48th Spring Meeting
Deutsche Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie
Mainz, March 13 – 15, 2007

		Abstract Numbers
Experimental Pharmacology		
1	Transport	1 – 16
2	Growth Factors	17 – 20
3	Molecular Receptor Pharmacology	21 – 62
4	Cholinergic Mechanisms	63 – 66
5	Adrenergic Mechanisms	67 – 74
6	Serotoninergic Mechanisms	75 – 78
7	Glutamatergic Mechanisms	79 – 81
8	Purinergic Mechanisms	82 – 87
9	Peptidergic Mechanisms, Opioids	88 – 91
10	Phospholipases, Eicosanoids	92 – 93
11	NO- and CO-mediated Mechanisms	94 – 116
12	Heterotrimeric G-Proteins	117 – 132
13	Small G-Proteins	133 – 151
14	Cyclic Nucleotides	152 – 156
15	Protein Kinases, Tyrosine Kinases	157 – 167
16	Calcium Channel Modulators, Calcium, Calmodulin	168 – 190
17	Potassium Channel Modulators	191 – 298
18	Renin-Angiotensin-System	199 – 209
19	Kinins	210
20	Endocrine Pharmacology	211 – 221
21	Immunopharmacology, Cytokines	222 – 242
22	Central Nervous System	243 – 270
23	Heart	271 – 300
24	Circulation	301 – 314
25	Respiratory Tract	315 – 319
26	Gastrointestinal Tract	320 – 322
27	Kidney	323 – 325
28	Pharmacokinetics and Drug Metabolism in Animals	326
29	Miscellaneous	327 – 341

		Abstract Numbers
Toxicology		
30	Drug Metabolizing Enzymes	342 – 351
31	Drug Metabolism / Toxicokinetics	352 – 360
32	Organ Toxicity	361– 373
33	Drug Toxicity	374 – 380
34	Toxic Metals	381 – 384
35	Toxicological Aspects of Natural Compounds	385 – 393
36	Tobacco Smoke	394 – 397
37	Apoptosis	398 – 408
38	DNA Modification	409 – 413
39	DNA Repair	414 – 430
40	Mutagenesis / Clastogenesis	431 – 434
41	Carcinogenesis	435 – 437
42	Cell Cycle Control / Cell Proliferation	438 – 442
43	Signal Transduction	443 – 458
44	Proteomics	459 – 462
45	Transport Mechanisms	463 – 470
46	Reactive Oxygen Species	471 – 479
47	In Vitro Test Systems	480 – 494
48	Screening Systems	495 – 498
49	Biomarkers / Biomonitoring	499 – 503
50	Genetic Polymorphism	504 – 507
51	Environmental Toxicology	508 – 517
52	Risk Assessment	518 – 522
Clinical Pharmacology		
53	Pharmacogenomics and Pharmacoepidemiology	523 – 541
54	Molecular Clinical Pharmacology and Disease Models	542 – 557
55	Clinical Studies	558 – 566
56	Pharmacokinetics	567 – 568
57	Other Areas of Clinical Pharmacology	569

AUTHOR INDEX
Figures refer to the abstract number

Abdel-Azim T. 316
Abdelrahman A. 84
Abel J. 363, 445, 447, 454, 455, 456
Aberle T. 170
Abraham G. 65
Abu-Taha I. 313
Ackermann F. 330
Adam R. 563
Adams V. 100
Afkham A. 197
Afonso J. 67
Agarwal N. 38
Aguilar-Bryan L. 194, 218
Aguilar-Pimentel A. 509
Ahles A. 26
Ahmad M.H. 427
Ahmadi S. 79
Ahmed K. 61
Ahnert-Hilger G. 338
Ahr H.J. 461
Ahrens W. 569
Aigner A. 131, 333, 334
Akhavan-Malyer R. 188
Akool E.-S. 33
Aktories K. 117, 127, 142, 146, 148, 151, 243, 462, 467
Al Hadithy A.F.Y. 532
Al-Anati L. 371
Al-Ansary D.M.M. 175
Albert F.W. 476
Albrecht C. 382, 430, 510
Albrecht U. 104, 181
Algaier I. 83
Alsdorf B.B.A. 84
Altenhöfer S. 112
Altenmüller D. 252
Althaus F.R. 422
Altis K. 562
Altmeyer M. 424
Alvanou A. 298
Amann T. 192
Amarenco P. 536
Amberg W. 75, 76
Ammer H. 89, 90, 91
Anagnostopoulou S. 312
Anders H.-J. 241
Andrade A.J.M. 383, 522
Angeli-Greaves M. 567
Angerer J. 502, 503
Angermann C.E. 546
Angioni C. 93, 548
Anke T. 239
Annett K. 423
Antic R. 500
Antonijevic I.A. 559
Antony J. 49
Antunes C. 420
Appel K.E. 353, 383, 436
Appenroth D. 66
Arlt V.M. 352
Armbruster N. 448
Arraj M. 314
Auer V. 213, 216
Augustin R. 2, 135, 136

Baasanjav C. 411
Baba H. 295
Bach M. 12
Bachmakov J. 561
Bachmann M. 222, 223
Bachschmid M. 477
Badenhoop K. 552
Bader M. 199, 203
Baer W.U. 250
Bäumer W. 234
Bakhiya N. 464
Bakiri L. 235
Bakker R.A. 53
Balboa N.E. 199
Balogh A. 539
Balz V. 56, 96, 309
Bankstahl J.P. 249
Barnewitz K. 348
Barrett P. 187
Bartels P. 187
Barth H. 457, 458, 468
Barth I. 7
Barth N. 399, 400
Barz D. 540
Bas M. 56, 114, 200, 309
Bassilana F. 55
Batista L.F.Z. 403, 404
Batke M. 464
Bauer A. 372
Bauer B. 248
Bauer D. 348
Bauer R. 98
Bauersachs J. 286, 302
Baum M. 413
Baumann-Wilschke I. 376
Baumgärtel-Allekotte D. 311
Baumgart P. 531
Baumstuemmler B. 469
Bayer M. 236, 238
Beck I. 62
Beck K.F. 106
Beckedorf A. 472
Becker A. 246
Bedini A. 232
Beer Stolz D. 480
Beetz N. 68, 69, 118
Behm C. 393, 394, 395
Behnke K. 187
Behr H. 279
Behrendt H. 367, 368, 509
Beisiegel U. 94
Belloni B. 368
Belyi Y. 462
Beneke S. 424
Benndorf R.A. 201
Benyó Z. 37
Berg C. 480
Berger W. 7
Berlot C.H. 23
Bernauer U. 349, 353
Bernden H. 363
Bernhard D. 163, 165
Bert B. 78
Bethmann K. 253
Beyerbach A. 75
Beyerle A. 333
Bhattacharya K. 382
Bhowmik S. 75
Biel M. 69, 118, 155, 262
Bien S. 553
Bier H. 200
Bierhals K. 454
Binder M. 490
Birnbaumer L. 274
Biskup E. 412
Blaich A. 261
Blana A. 284
Blaszkewicz M. 505
Blenn C. 422
Bliem C. 400
Blievernicht J.K. , 524, 534, 544, 568
Blömeke B. 364, 366
Blum A. 555
Blumbach K. 442
Blume R. 215
Blumenthal B. 142
Bobis Seidenschwanz I. 558, 560, 566
Bochud M. 535
Bock H.H. 80
Bock M. 292, 465
Bock U. 469, 470
Bockamp E. 372, 420
Bodie I. 179
Bödding M. 174
Böer U. 337, 341
Böger R.H. 94, 115, 201, 530
Boehm S. 63, 87, 88
Boekhoff I. 132, 330
Bönisch H. 10, 77, 256
Boere A. 367
Börner C. 232, 233
Bohnekamp B. 32
Boivin V. 546
Boknik P. 275, 288, 289, 297, 299
Bolay C. 438
Bollinger L. 97, 98
Bolt H.M. 394, 395, 434
Borchardt T. 79
Bornhorst A. 525, 541
Borth H. 132
Borza A. 395
Botham P. 495
Bothe H. 447
Brachetti T. 453
Brade V. 223
Braem C. 42
Braeuning A. 346, 449
Bramer R. 501, 502, 503
Bramlage P. 207
Brand E. 531, 535, 529
Brand M. 547, 557
Brand-Herrmann S.-M. 529, 531, 535, 536
Brandes R. 224
Brandt C. 253
Brandt D.T. 133, 134
Brandt M. 302
Brandt R. 339
Brandt U. 32
Brans R. 366
Braun M. 335
Brawek B. 251
Breddemann A. 563, 564
Brede M. 118
Breier-Wolski C. 566
Breit A. 54
Breithardt G. 284
Breuer D. 501, 502, 503
Breuer K. 509
Brink A. 409, 431
Brochhausen C. 20, 92
Brockes A. 361

Brockmöller J. 528
Broeckel U. 118
Broschard T.H. 498
Brouwers J.R.B.J. 532, 565
Bruckner T. 366
Brüning T. 501, 502, 503
Brüss M. 77
Bruggeman R. 532
Bruhnke S. 363
Brulport M. 372
Brune K. 240, 561
Bruns K. 113
Bryan J. 193, 194, 218
Bubik M.F. 305
Buchmann A. 346, 435, 436, 450
Buchner K. 241
Buchwalow I. 275
Büch T. 54, 131
Büchele B. 159, 267, 326
Büchter C. 493, 494
Bünemann M. 21, 22, 23, 25, 124, 125, 154, 179
Bürkle A. 408, 423, 424, 425, 426, 477, 484, 485
Buesen R. 492
Büsselberg D. 190, 265, 378, 379
Buitrago M. 286
Burghard S. 13
Burgold S. 126
Burian M. 562
Burk O. 544
Burkhardt S. 369
Burleigh K. 52
Burysek L. 230
Busquet F. 498
Buters J.T.M. 367, 368, 509

Caballero R. 196
Cabezas-Wallscheid N. 420
Cabrini D. 199
Calhau C. 205
Cambien F. 531, 536, 529
Carbajo J. 34
Cardoso C.C. 199
Carmeliet E. 284
Carmeliet P. 284
Cassee F.R. 510
Cassel J.C. 266
Castoldi M. 278
Cavalié A. 178
Cavassini M. 524
Cayla C. 199
Cerbai E. 72
Chahoud I. 383, 522
Chatzigianni E. 312
Chen J.G. 506
Cheremina O. 561
Chialda L. 240
Chovolou Y. 386, 387, 388, 405, 406, 407, 493, 494
Christ T. 292
Christel C. 167, 89
Christen S. 236, 238
Christen U. 236, 237, 238, 552
Christian F. 273
Christmann M. 401, 404, 416, 418, 419, 421
Christow C. 144
Chubanov V. 185
Ciecholewski S. 553
Cierny I. 215, 337
Cieslik J. 48
Claaßen S. 198
Closs E.I. 3, 8, 116, 302, 526
Coenraads P.J. 366
Coester C. 231
Cohausz O. 422
Coldewey M. 301
Cole T. 495
Colombo S. 524
Combadière C. 536
Combrink S. 77
Corban-Wilhem H. 548
Cortes-Cros M. 55
Cotovio J. 495
Csanády G.A. 360
Curren R. 495
Czapp M. 268
Czech E. 353
Czerwinski P. 18
Czeschik J.C. 265
Czubayko F. 334

da Costa G.G. 352
Daffner W. 343
Dai G. 281
Daiber A. 3, 113, 282, 301, 302, 547, 557
Damke D. 284
Damm G. 356, 357
Damrot J. 415
Dangel O. 108
Daniel C. 549, 550, 551
Dao T. 114
Daskala J. 312
Davis L.A. 491
Dawczynski J. 540
De Amici M. 50
de Groot J. 300
Debiak M. 485
Debrezion J. 547, 557
Decker M. 66
Decosterd L. 524
Degen G.H. 393, 434
Dehus O. 226
Dekant W. 442, 473, 497
Delpon E. 196
Delvizi E. 298
Demiraj F. 466
Dempe J.S. 389
Dendorfer A. 220, 221
Deng S. 99, 280
Deng S. 258
Dentz L. 530
Depping R. 220
Dettbarn G. 500
Dev K.K. 55
Dhein S. 71, 72
Dickel H. 366
Didié M. 279
Diefenbach J. 425
Diekert K. 7
Dietrich A. 171, 184
Dietrich C. 439
Dietrich H. 322, 476, 505
Dirsch V.M. 331
Dobrev D. 292, 293
Döhmer J. 356
Dördelmann C. 531, 536, 529
Dörrenhaus A. 344, 345
Doklea E. 511
Doller A. 111, 324
Dominiak P. 202, 203, 220, 221
Donath C. 410
Donovan C.M. 251
Dooley D.J. 251
Dopp E. 382
Dorn S.B. 434
Dorsch S. 25
Doser M. 519
Dragoi C. 222
Draheim R. 442
Drasdo D. 372
Draus E. 516
Dreger S. 140, 141, 151
Dreieicher E. 106
Drescher K.U. 76
Drews G. 194
Drobny H. 88
Du H. 506
Duckwitz W. 44
Düfer M. 194
Dünstel G. 227
Dürk H. 414
Dürst M. 539
Duffin R. 430
Dulich F. 443
Dumont L. 283
Dunford J. 328
Dyballa N. 285
Eberhardt W. 33, 111, 223, 324
Eberlein B. 368
Ebert B. 463
Eckhardt A. 365, 438
Eder E. 412
Eder P. 169
Egerer M. 467
Ehmke H. 122
Ehnert C. 264
Eiberger W. 428
Eichelbaum M. 568, 544, 534, 524
Eichele K. 327
Eichhorn B. 60
Eick J. 45
Eisele T. 43
Eisenbrand G. 413, 476
Eisinger D.A. 89
Eißmann F. 391
El-Armouche A. 288
El-Hawari Y. 351
El-Sakkar M.G. 316
Ellinger-Ziegelbauer H. 461
Ellis E.C. 480
Elsinghorst P.W. 48
Elsken C.S. 257
Eltze T. 426
Elz S. 35
Emmenegger Y. 104
Engel U. 121
Engelhardt S. 26, 278, 286, 287
Engst W. 464
Epe B. 428, 429
Erbach E. 360
Erdmann K. 242
Erkel G. 239
Erkes A. 501
Erler I. 175
Ertl G. 302
Eschbach M. 186
Eschenhagen T. 122, 279, 288
Eschrich D. 362, 483
Eshkind L. 420
Eskes C. 495,
Esser C. 440, 444
Estrada A.C. 159
Etzrodt J. 343
Eulberg D. 241

Fabian E. 356, 357

Fabian J. 204
Fabritz L. 275, 277, 284
Fackler O.T. 133
Fagh A. 376
Fahrer J. 408, 424
Farmer P.B. 352
Faussner A. 210
Faust D. 439
Fehr J. 132
Feil R. 104, 162, 65, 166, 167, 276, 308
Feil S. 104, 65, 166, 276, 308
Feldmann I. 460
Fend F. 164
Fentem J. 495
Fernandes C. 64
Fernandes M. 492
Ferrando-May E. 408, 484
Fery Y. 350
Feuerstein T.J. 251, 252, 266
Fieblinger D. 432
Filser J.G. 359, 360, 441
Fink F. 260
Fink H. 78, 207, 260
Fink L. 332
Finkenwirth C. 512
Fischer C. 259
Fischer C. 526
Fischer J.W. 281, 295, 300, 320
Fischer T. 278
Fleck C. 66
Fleckenstein-Grün G. 283
Flehmig J. 566
Flockerzi V. 170, 172, 173, 174, 175, 177, 178
Florea A.-M. 378, 379
Föllmann W. 393, 394, 395
Förster K. 502, 503
Förstermann U. 3, 17, 18, 95, 97, 98, 99, 105, 301, 302
Folwaczny M. 354, 380
Forster M.J. 257
Fortmüller L. 284
Foth H. 427, 486
Frank S. 160, 211, 212
Franken P. 104
Franklin C. 85
Frantz S. 286
Frechen T. 370
Frei E. 352
Freichel M. 170, 177
Frenzel C. 71
Frericks M. 444
Freudenberger T. 295
Freudenhammer C. 516
Freyberger A. 361
Fricker G. 6, 13
Friebe A. 102, 107, 108
Frieboes R.M. 559
Frieling T. 5
Fritsche E. 363, 364, 445, 447
Fritschy J.-M. 253
Fritz G. 149, 151, 415, 453
Frobel A.K. 564, 537
Fromm M.F. 527, 526, 542, 545
Frost R.J.A. 286, 287
Frotscher M. 80
Fuchs B. 171, 332
Fuchs H. 2
Füller F. 148
Fuellgraf H. 252
Fürst R. 305, 329
Furrer H. 524
Fusch C. 15
Gailus-Durner V. 2
Gallitz I. 454
Gamer A. 369, 370, 495
Gao W. 264
Garcia Moreno I. 375, 487
Garcia-Ladona F.J. 75, 76
Gawlik V. 2
Gazova A. 299
Gebauer G. 368
Gebhart G.F. 79
Geduhn J. 156
Geissler I. 130, 525, 538
Geisslinger G. 93, 161, 264, 548, 550, 551, 562
Genschow E. 492
Genth H. 140, 141, 149, 150, 151, 398
Genze F. 326
Gergs U. 289, 290, 291, 297
Gerhard R. 139, 149, 398
Gericke C. 383, 522
Gerlach M. 11, 377
Gerlofs-Nijland M.E. 510
Germann P.-G. 490
Gerstberger R. 64
Gerstner S. 358
Gerwien K. 516
Gessler T. 332
Gessner A. 240
Gestrich C. 418
Geurtsen W. 472
Geyer J. 64
Ghoneim M.T. 316
Giegold O. 554
Gier B. 194
Gierschik P. 123, 129, 137, 144, 145
Giesemann T. 467
Gille A. 37, 210
Gilsbach R. 68, 70, 179
Glaeser H. 542
Glahn F. 486
Glas J. 355, 380
Glatt H.R. 410, 411, 464
Glauert H.P. 436
Glockner C. 516
Glöckner R. 342
Glückmann M. 459
Gminski R. 520
Godoy J. 64
Göbel S. 113
Gödtel-Armbrust U. 99, 526
Göhler A. 517
Göke B. 321
Goergens A. 440
Göthert M. 76, 77
Göttle M. 156
Göttlicher M. 443
Goldstein M. 402
Golka K. 504, 505, 506, 567
Gombitang C. 493, 494
Gombitang C.
Goren I. 160, 211, 212
Goudeli A. 312
Goulimari P. 121
Gradhand U. 526
Grantcharova E. 29, 30
Grasl-Kraupp B. 474
Graziani A. 169, 182
Greaves E.D. 567
Grecksch G. 246
Grether-Beck S. 363
Grigat S. 12
Grimm M. 122
Grimminger F. 332
Grittner D. 191
Gröne H.J. 325
Grösch S. 93, 548
Gröticke I. 270
Gromadzinska J. 500
Groneberg D. 102, 107
Groschner K. 169, 182
Groß C. 278
Gross G. 75, 76
Gross S.A. 178
Grosse R. 121, 133, 134
Großer N. 242
Grote K. 383, 522
Grote-Wessels S. 275, 277
Grube M. 4, 15, 543
Gründemann D. 11, 12
Gründker N. 289
Grundt A. 206, 208
Grundt C. 206, 208
Grzelinski M. 131
Gsandtner I. 58
Gu N. 245
Gudermann T. 54, 131, 132, 171, 184, 185, 330
Günay H. 472
Günes D.A. 379
Günthard H.F. 524
Guenther R. 513, 514
Gütschow M. 48
Guiguemde A. 1
Guimarães S. 205
Gunder-Remy U. 353, 349, 461, 518
Guo K. 328
Guo W.C. 506
Gutermuth J. 509
Gutkind S. 303
Gutwein P. 111, 160, 325
Gutzmer R. 234

Haag R. 266
Haag S.D. 317, 318
Haarmann-Stemmann T. 447
Habermeier A. 3, 8, 116, 526
Haddad M. 530
Haenisch B. 256
Hänze J. 332
Hagedorn C. 531
Hagemeier C. 569
Hagenacker T. 265
Hahn A. 75
Hahn J.-U. 501, 502, 503
Hajek P. 65
Halabi-Cabezon I. 150
Halstenberg S. 20
Haltner-Ukomadu E. 469, 470
Halwachs S. 9
Hamann M. 255, 260
Hambrecht R. 100
Hamraz K. 115
Hannemann S. 133
Hannenberg H. 364
Hansen U. 516
Harati H. 266
Hard G.C. 442
Harjes M. 563
Harmann B. 81
Harorli T. 365
Harrison M. 118
Harst A. 306
Hartmann B. 79, 81
Hartmann L.M. 382
Hartung T. 226, 495

Hartz A.M. 248
Hasenfuss G. 280
Hasenkamp S. 535
Haslinger E. 474
Hassan-Oglu Z. 96
Hatlapatka K. 219
Haubold M. 70
Hauck-Weber N.C. 285
Hausmann R. 44
Hauser I. 325
Hausmann R. 85
Heck R. 420
Heeren J. 94
Heesen M. 366
Heidbreder M. 220, 221
Heidland A. 471, 475
Heil S. 112
Heilmann A. 201
Heilmann J. 57
Heim H.K. 306
Hein D.W. 366
Hein L. 68, 69, 70, 118, 155, 179
Hein P. 21, 23
Heine E. 432
Heine K. 458
Heinemeyer G. 519
Heinrich A. 341
Heinrich-Hirsch B. 349
Heiss E.H. 331
Hellmann J. 459
Henderson C.J. 352
Hengstler J. 372, 480
Henle F. 80
Henry M. 180
Herlyn H. 523
Hermann C. 226
Hermanns I. 465
Hermes M. 153, 372
Hermosilla R. 29, 30, 31, 32
Herrero San Juan M. 224, 225
Herroeder S. 120
Herz J. 80
Herzig S. 179, 187, 188, 274
Herzler M. 518
Heusch A. 564
Heusch G. 304
Hewitt P.G. 481, 482
Heydecke D. 330
Hickel R. 354, 355, 380, 521
Hildebrandt F. 184
Hilgenfeldt U. 210
Hill K. 168
Hillen H. 75
Hiller K.-A. 365, 438
Hink U. 113
Hintermann E. 236, 237, 238
Hinz B. 327, 561
Hippius M. 540
Hirsch-Ernst K.I. 348
Hirschberg A. 258
Hisgen V. 508
Hobler C. 383
Hochfeld J. 200, 309
Hochreiter A. 474
Höbel S. 333
Höcherl K. 209
Hoek H.W. 532
Höllt V. 39, 40, 41, 232, 233
Hölter S.M. 2, 104
Höltje H.D. 47
Höltje M. 338
Höschele D. 375, 487
Hoesl E. 276
Hoffmann C. 21, 23, 24, 26
Hoffmann E. 235, 382
Hoffmann F. 231, 305
Hoffmann K. 82, 84, 270, 373
Hoffmann S. 495
Hoffmann T.K. 200, 309
Hofinger A. 479
Hofmann A. 340
Hofmann F. 104, 140, 141, 150, 162, 163, 164, 165, 166, 167, 181, 189, 213, 216, 261, 262, 276, 308, 338, 339
Hofmann H.-D. 19
Hofmann S. 486
Hohlfeld T. 59, 272
Holdener M. 237
Hollt V. 39
Holtz S. 71
Holzgrabe U. 50
Hommann M. 343
Hommel A. 135, 136
Honscha W. 9, 466
Horke S. 95
Hornberger W. 75
Hortmann M. 301, 547, 557
Hosseini M. 280
Houtman P.M. 565
Hozjan V. 328
Hrabé de Angelis M. 2
Hrach J. 481
Hsien L. 564
Huber G. 370
Huber W.W. 474
Hübel U. 490
Hübenthal U. 445
Hübner N. 29, 30
Huelsenbeck J. 140, 141, 149, 150
Hüner H.-A. 357
Hullin R. 179, 188
Hunfeld K.P. 223
Huss-Marp J. 509
Hussl S. 87
Hutzler C. 359
Huwiler A. 324

Illes P. 297
Iori R. 411
Isermann M. 140
Isse T. 113, 282
Itter G. 197
Ittrich C. 436, 461

Jackisch R. 266
Jackisch-Riemann A. 560
Jacob J. 500
Jähnichen S. 53
Jahns R. 546
Jakob T. 509
Jakubowski N. 460
Jangsanthong W. 188
Jank T. 467
Janke D. 526
Jansen T.L.T.A. 565
Janssen A. 93, 548
Janzowski C. 476
Jaoulak H. 94
Jaschke A. 135, 136
Jedlitschky G. 4, 16, 543
Jerabeck S. 41
Jin S. 516
Jochim A. 163
Jöhren O. 202, 203, 220, 221
Jörns A. 214, 219
Jöst E. 417
Johannes C. 392
John A. 500
Johnson E.F. 237
Joksic G. 500
Jones P. 495
Joost H.-G. 2, 135, 136, 214
Jost S. 74
Just I. 139, 140, 141, 149, 150, 338, 339, 398
Just S. 278

Kaber G. 272
Kääb S. 293
Käfferlein H.U. 501, 502, 503
Kästner L. 191
Kahl E. 39
Kahl R. 386, 387, 388, 405, 406, 407, 493, 494
Kaibuchi K. 134
Kaina B. 401, 402, 403, 404, 414, 415, 416, 417, 418, 419, 420, 421, 453
Kaiser E. 468
Kaissling B. 323
Kalmes M. 364
Kalwa H. 54, 171, 184
Kamp H. 356
Kamp H.G. 357
Kampkötter A. 386, 387, 388, 405, 406, 407, 493, 494
Kandarova H. 495
Kappes F. 408
Kassner N. 556
Kathmann M. 73
Katona I. 38
Kavanagh K.L. 328, 351
Kawakubo Y. 366
Kawamoto T. 113, 282
Kayser A. 14, 525
Kehe K. 354, 355, 380, 465
Keipert C. 7
Kelber O. 5, 321
Kelety B. 7
Kellershohn K. 50
Kellert M. 496, 499
Kemp-Harper B.K. 101
Kessler B. 340
Kessler W. 359
Kettner-Buhrow D. 235
Khaljani E. 94
Khan I.F.Y. 179, 188
Khan M.D.H. 441
Khodadoust M. 408
Kiedron O. 404
Kienhöfer J. 477
Kietzmann M. 234, 335, 336
Kinkel R. 554
Kirchhartz N. 200, 309
Kirchhefer U. 294
Kirchheiner J. 533
Kirchhof P. 277, 284
Kirkpatrick C.J. 20, 92, 465
Kirsch M. 19
Kissel T. 334
Kitzing T. 133
Klaffke H.S. 353
Klaus G. 92
Klein C. 274
Klein D. 441
Klein K. 526, 527, 534, 544, 568
Kleine-Katthöfer P. 531
Kleinert H. 105, 112
Kleppisch T. 167, 189, 262
Kleschyov A.L. 116

Kleuser B. 42, 109, 110
Klimas J. 299
Klimm S. 208
Kling A. 75, 76
Klöss T. 530
Kloor D. 153
Klotz K.N. 25
Kloz U. 425, 426
Klugbauer N. 147, 186, 259
Kluge R. 135, 136, 214
Klussmann E. 273
Klussmann S. 241
Kmecova J. 299
Knaapen A.M. 430, 510
Knaus A. 69, 155
Knaut M. 292
Knepel W. 215, 337, 341
Knerr S. 473
Kneuer C. 9, 466
Knieling H. 121, 133
Knipper B. 531
Knopf H.J. 567
Knust Z. 142
Kocgirli O. 56
Koch T. 39, 40
Koch W.J. 274
Kochan G. 328
Köberle B. 414
Köck K. 4, 15, 543
Koehle C. 346, 449, 450
Köhler C.U. 347, 460, 504
König B. 156
König J. 527, 542
König P. 102
Königsdörffer E. 540
Koesling D. 102, 107, 108, 152, 246
Kojda G. 56, 96, 100, 103, 114, 200, 309
Koltermann A. 157
Kom G.D. 94
Konstantinides S. 397
Kooter I.M. 510
Kopp-Schneider A. 461
Korhonen H.-K. 128
Korhonen R. 105
Korostylev A. 258
Kosenburger K. 88
Kotlyarov A. 149
Kouvelas D. 298
Kovacs F. 250
Kracht M. 235
Krätzner R. 215
Kraft A. 469
Kraiczy P. 223
Kramer P.-J. 459
Kranaster R. 424
Kraus J. 232, 233
Krause E. 31, 436
Krause G. 28
Kreile A.K. 125
Krenek P. 299
Krenn M. 169
Krens L. 471
Kretschmer N. 74
Kreusler B. 558
Kreyling W. 367
Kriebs U. 177
Krippeit-Drews P. 194
Kristiansen A.-M. 245
Kröger J. 203
Kröger M. 459, 461
Kroemer H.K. 4, 15, 16, 525, 543, 553
Krötlinger F. 361
Krüger A. 525, 541
Krüger D. 5
Krutmann J. 363, 510
Krystof V. 158
Kubatzky K. 19
Kucerova D. 299
Kudlacek O. 58
Kühlein H. 264
Künstle G. 227, 228
Küper J. 191
Kuhlmann J. 395
Kuhn E. 129
Kuhn U. D. 343, 540
Kulawik A. 385, 386, 387, 388
Kulkarni O. 241
Kulle B. 280
Kumpf S. 100, 103, 309
Kuner R. 38, 79, 81, 258
Kuner T. 38
Kunz I. 190
Kunzmann A. 423
Kurejova M. 186
Kurowski C. 111
Kurtz A. 209
Kurtz S. 531
Kurz C.M. 73
Kutschenko A. 215
Kuznetsova T. 535
Kyselovic J. 299

Laake J. 464
Laakmann S. 284
Labreuche J. 536
Lacinova L. 186
Lackner K.J. 99, 113
Läer S. 537, 563, 564
Lahaniatis M. 519
Lakner U. 431
Lambert S. 172, 173
Lamers D. 454
Lampen A. 463
Lamy E. 390, 391
Landsiedel R. 356, 357, 369, 370, 508
Lang H. 474
Lang S. 127
Lang T. 526
Lange K. 113
Lange U.E.W. 75
Langmesser S. 104
Langner I. 569
Langwieser N. 181, 189
Lanzer M. 1
Lass C. 348
Laumonnier Y. 229, 230
Lautz K. 29, 30, 31, 32
Lazar A. 11, 12
Lebrun S. 397
Lecher B. 470
Lee H.H. 247
Lee J.-H. 187
Leemhuis J. 19, 80, 243, 259
Lehmann J. 66, 113, 301
Lehmann L. 392, 451, 488
Leibold E. 370
Leineweber K. 304
Leiss V. 213, 216
Lektarau Y. 393
Lemmer B. 206, 208, 313, 314
Lemoine H. 191
Lenz A.-G. 333
Lesch K.P. 11
Leurs R. 53
Levkau B. 295
Leyhausen G. 433, 472
Leyhausen G.
Li H. 17, 18, 97, 98, 99, 301
Li Q. 229
Li X. 535
Liang Y.J. 40
Liao Y. 245
Liebl J. 158
Liebsch M. 495
Liem W.-K. 296
Limtanyakul J. 365
Lin G.F. 506
Linder S. 162
Lindner M. 373
Linke A. 211
Linker K. 105, 112
Linsel N. 47
Linz D. 197
Lis A. 173
Lison A. 566
Litchfield D. 45
Liu D. 423
Loch S. 173
Löffler M. 251, 266
Löhlein S. 504
Loeppky R.N. 413
Löscher W. 249, 253, 270, 373
Lösel R. 36
Lohse M.J. 21, 22, 23, 24, 26, 69, 118, 124, 125, 154, 271, 286, 287, 546
Lommerse J.P.M. 434
Looman N.M.G. 532
Lorenz D. 273
Lorenz R.L. 392
Lorenzen T. 540
Lou Y. 385
Lu D.R. 506
Lu H.C. 506
Lubitz S. 368
Luch A. 396
Ludwig A. 261, 262, 276
Ludwig A. 563
Ludwig J. 198
Lüllmann-Rauch R. 295
Lüpertz R. 405
Lüscher B. 45
Luk J.T.Y. 101
Lukacik P. 328
Lukasova M. 303
Lukowski R. 162, 164, 165, 213, 308
Lunkenbein S. 490
Luo C. 81
Lupp A. 343, 540
Luppa P. 164
Lutz S. 34, 138, 143
Lutz W.K. 409, 499

Ma-Hock L. 369, 370, 508
Maas R. 115, 530
Mackie K. 38
Maechler H. 169
Maeurer C. 161
Magnussen M.P. 515
Mahr J. 435
Mahringer A. 6
Maier T.J. 93, 548
Mair S. 509
Majchrzak M. 266
Malanga M. 422
Malek A. 334
Mally A. 442, 473
Mangerich A. 425
Mannebach S. 172, 173
Manns M.P. 237

Mantovani M. 252
Manz A. 460
Marczynski B. 501, 502, 503
Maringer M. 470
Marion S. 134
Markovitz D. 408
Markwardt F. 45
Marquardt W. 354, 355, 380, 521
Marquez-Klaka B. 86
Martin H.-J. 351, 556
Martin M. 272
Martiné U. 8
Marumo M. 182
Marx A. 424
Marx-Stoelting P. 435, 449, 450
Marzoll A. 281
Maser E. 351, 516, 517, 555, 556
Matar N. 180
Matroos G.E. 532
Matt L. 262
Matthes J. 179, 187, 188, 274
Matthiesen S. 317, 318
Matus M. 277
Matzdorf C. 209
Mauti O. 258
Mayer B.A. 305
Mayer F. 509
Mayer M. 452
Mayer P. 306
McCullough A.K. 429
Mederos y Schnitzler M. 185
Mehralivand S. 526
Meier S. 226
Meineke I. 528
Meissner M. 444
Melchior A. 295
Melichar K. 72
Menck C.F.M. 403
Meng H. 506
Mensing T. 501, 502, 503
Mergia E. 246
Merk H.F. 366
Merkel O. 334
Merkle S. 286
Mersch-Sundermann V. 390, 391, 520
Mersmann K. 295
Messerschmidt J. 460
Messow D. 340
Metzger S. 285
Metzler M. 358, 389
Meurrens K. 397
Meyer A.H. 75
Meyer D. 132, 330
Meyer D.K. 19, 80, 243, 259
Meyer F. 490
Meyer H.J. 151
Meyer-Kirchrath J. 59
Mezler M. 75
Michalakis S. 262
Michalopoulos G.K. 480
Michels G. 123, 129
Michels G. 247
Miller D.S. 248
Millrose M. 374
Minn J. 322
Misner G. 204
Moepps B. 123, 145, 129
Moers A. 128
Mohr F.W. 71, 72
Mohr K. 47, 48, 49, 50
Mohr-Andrä M. 47
Moilanen E. 105
Monjón Tortosa E.I. 332
Monyer H. 38
Moosmang S. 167, 181, 189
Morawietz H. 60
Moreno-Villanueva M. 408, 477, 484
Moser A. 252
Moser M. 135, 136
Moss S.J. 247
Moura D. 205
Moura E. 67
Moura J.E. 68, 118
Muckenthaler M. 278
Mückter H. 511
Mühl H. 222, 223
Mühlbauer B. 558, 560, 566, 569
Mühlenstädt C. 339
Müller C. 392
Müller C.E. 84, 217
Müller D. 342, 343, 482
Müller E. 160
Müller F.U. 275, 277, 294, 296, 310
Müller G. 60
Müller H. 202, 203
Müller J. 282, 412, 547, 557
Müller R. 259, 324
Müller S.O. 350, 459, 481, 489, 498
Müllershausen F. 55
Münzel P.A. 448
Münzel T. 3, 113, 116, 282, 301, 302, 547, 557
Müßig E. 485
Mugelli A. 72
Murck H. 559
Muth M. 50
Muthig V. 70
Muttray A. 512

Nagel G. 420, 421
Nagel R. 498
Nagy N. 320
Nanoff C. 58
Nattel S. 293
Nau H. 490, 492
Naumann S.C. 401, 404
Nawrath H. 116
Neschen S. 214
Nestler M. 214
Netz A. 75
Neumann J. 289, 290, 291, 294, 297
Neumeyer A. 364
Ng S. 328
Nguyen J. 332
Nicaud V. 529, 531
Nicke A. 86
Nickl K. 57
Niederberger E. 264
Niedorf F. 336
Niehues T. 563
Nielsen P. 94
Niemeyer B.A. 175
Niesen F. 351
Niesler B. 77
Nikolaev V.O. 546
Nobrega J.N. 254
Nohl H. 478, 479
Noll C. 337
Nottrott S. 398
Nürnberg B. 274
Nüsing R.M. 92
Nunez L. 196
Nussler A. 544
Nussler N. 544

Oberemm A. 461
Oberwinker J. 172, 173
Obrdlik P. 7
Ochs S. 367
Ochse M. 75
Ochsenhirt V. 99
Oelze M. 113, 282, 301, 302, 547, 557
Oesch F. 439, 446, 452, 507
Oesch-Bartlomowicz B. 446, 507
Oesterle D. 441
Oetjen E. 215
Oevermann L. 16
Oey D. 391
Offermanns S. 37, 61, 119, 120, 128, 258, 303
Ohngemach S. 420
Okpanyi S.N. 321
Oksche A. 32
Olausson J. 177
Oppermann M. 96, 103, 114, 309
Oppermann U. 328, 351
Origer J. 416
Orth J. 117, 127, 146
Osinde M. 55
Osswald H. 153, 323
Ostrouska I. 58
Oswald S. 543
Ott T. 435, 450
Ottersen P. 245
Oxynos A. 354, 355, 380, 521

Paal K. 436
Pagani S. 533
Pahl A. 240, 319
Palavinskas R. 353
Palitti F. 500
Papameletiou D. 519
Papamitsou T. 298
Parolaro D. 38
Parzefall W. 474
Passreiter C. 386, 387, 388
Paul C. 167
Paul M. 531, 536, 529
Paulsen S. 517
Pautz A. 105, 112
Pechan P. 158
Pehl U. 7
Pekcec A. 248
Penachioni J.Y. 258
Penning T.M. 556
Perez Sastre A. 307
Perez-Reyes E. 187
Perneczky A. 421
Pertz H.H. 109
Pesch B. 501, 502, 503
Petersenn H. 130
Petrik C. 295
Petzinger E. 64, 371
Pfeifer A. 340
Pfeiffer E. 358, 389
Pfeiffer M. 41
Pfeil R. 518
Pfeilschifter J. 33, 106, 111, 160, 211, 212, 222, 223, 224, 324, 325, 552, 554
Pfeilschifter J.M. 225
Pfreimer M. 145
Philipp S.E. 172, 173
Phillips D.H. 352
Pieh J. 459
Piekorz R. 274
Pielarski T. 493, 494
Pierre S.C. 161
Pietzsch T. 366
Pigeot I. 569

Pilka E. 328, 351
Pinheiro H. 205
Pinkenburg O. 131, 183
Pitterle K. 267, 533
Plant T.D. 183
Platzek T. 432, 519
Platzer M. 523
Pleskova M. 106
Plöttner S. 394, 395
Podda M. 562
Pohl C. 465
Poirier O. 536
Popovic M. 230
Popp O. 425
Postma A.V. 294
Poteser M. 169, 182
Potschka H. 248, 249, 268
Potstada W. 161
Potteck H. 42
Pourzitaki C. 298
Preuß I. 117, 127
Prilla S. 49
Proksch P. 385
Püntmann I. 558, 560, 569
Puljic R. 319
Purschke W. 241
Pust S. 457
Qiu H. 523

Quante T. 235
Quast U. 192, 193
Quintanilla-Fend L. 441
Qiu H. 528

Raasch W. 202, 203
Rabausch B. 300
Rached E. 442
Racké K. 317, 318
Radeke H.H. 224, 225, 324, 549, 550, 551, 552, 554
Radicke S. 196
Radke K. 508
Rafalzik S. 64
Rahmer H. 433, 472
Ramer R. 327
Ramos-Lopez E. 224, 552
Rancourt D.E. 491
Rath T. 476
Rauch B.H. 311
Rauch M. 518
Raulf-Heimsoth M. 501, 502, 503
Rauwald J.W. 72
Ravens U. 60, 196, 292, 293
Raymond R. 254
Reichel V. 13
Reichl F.-X. 354, 355, 380, 521
Reimer J. 553
Reinders M.K. 565
Reinelt K. 511
Reisinger K. 362, 483
Renner F.J. 90
Renner T. 11
Reszka E. 446, 507
Retlich M. 137, 144
Rettenmeier A.W. 382
Rettinger J. 86
Reusch H.P. 307
Reuther S. 15
Rex A. 260
Richart T. 535
Richter A. 254, 255, 260, 263
Richter C. 224, 225
Richter F. 255
Richter I. 409
Richter-Reichhelm H.-B. 461
Rieg T. 323
Riehle R. 153
Rignall B. 449
Rimmbach C. 14, 130, 525, 541
Rio P. 364
Ritter C.A. 4, 16, 553
Ritter M. 72
Rittmann P. 451
Ritz V. 348, 518
Robles C. 495
Rochais F. 26
Röhm S. 537
Rösener N. 374
Röser C. 68
Rogge C. 279
Rohländer S. 342
Rohr-Udilova N. 479
Rohrig R. 385, 386, 387, 388, 493
Rojas-Gomez D.M. 71
Rojkova A. 328
Romanos M. 11, 377
Rood A. 191
Roos P.H. 344, 345, 347, 460, 504
Roos W.P. 401, 402, 403, 404, 417
Rorsman P. 213
Rosenau T. 479
Rosenfeld M.E. 308
Rosenthal W. 27, 28, 29, 30, 31, 32, 273
Rossbach B. 512
Rosskopf D. 14, 130, 525, 538, 541
Rotger M. 524
Roth G. 505
Roth M. 332
Rothemund S. 289
Rothmeier A.S. 437
Rothmund C. 448
Rotmann A. 1, 8, 116
Rottbauer W. 278
Rouguet R. 495
Rubino T. 38
Rüdell G. 348
Rüßmann C. 529
Rütten H. 197
Ruhl S. 385, 386, 387, 388
Ruitenberg M. 7
Runden-Pran E. 245
Runge A. 530
Runge D. 480
Runnebaum I. 539
Rupp J. 116
Russ U. 192, 193
Russwurm M. 152
Rustenbeck I. 214, 218, 219
Ruth P. 245, 323
Rutz C. 27
Ruwiedel K. 363, 445

Sadik C.D. 223
Sahadevan A. 133
Salameh A. 71
Sanchez C. 1
Sander S.E. 254, 263
Sandrock K. 147
Sartorius T. 245
Sartory N.A. 549, 550, 551
Sassmann A. 119
Sauermann A.M. 451, 488
Saur O. 257
Sausbier M. 245, 323
Sausbier U. 323
Saussele T. 524, 534, 544, 568
Schachner D. 474
Schäfer C. 363
Schäfer K. 397
Schäfer L. 325
Schaefer M. 126, 168, 183, 307
Schäfer-Korting M. 42
Schäfers M. 265
Schaeffeler E. 534
Scheel J. 483
Scheepers A. 2
Scheitz J. 16
Schemann M. 5
Schermuly R.T. 332
Scherneck S. 214
Schicker K. 63, 88
Schiedel A.C. 84
Schiefelbein D. 212
Schieke S.M. 363
Schiffmann S. 93, 548
Schilder Y.D.C. 331
Schins R.P.F. 430, 455, 510
Schlack W. 285
Schladt L. 361
Schlaegel A. 540
Schlatter J. 499
Schlechter K. 492
Schleef R. 397
Schleifenbaum J. 124
Schlicker E. 73, 76
Schlösser R. 539
Schlossmann J. 163, 164, 165
Schlüter G. 280
Schmalz G. 365, 438
Schmalzing G. 43, 44, 45, 85
Schmechel A. 122
Schmehl S. 325
Schmehl T. 332
Schmid U. 471, 475
Schmidberger H. 417
Schmidt A. 280
Schmidt G. 142, 147, 148, 151, 243
Schmidt H.H.H.W. 101
Schmidt K.G. 563, 564
Schmidt M. 184
Schmidt R. 93, 548, 551, 562
Schmidt S. 2, 136, 489
Schmidt T. 159
Schmidtko A. 264, 562
Schmitt J.P. 271
Schmitt S. 414, 420
Schmitteckert E. 179, 278, 286
Schmitz H.-J. 322
Schmitz M. 423
Schmitz O.J. 352
Schmitz W. 275, 277, 289, 294, 296, 297, 310
Schmitz-Salue C. 348
Schmolz K. 214
Schmutz I. 181
Schneider D.T. 563
Schneider E. 51, 57
Schneider H. 518
Schneider K. 519
Schneider T. 180
Schneiders U.M. 399
Schnell D. 52
Schober W. 367, 368, 509
Schoemaker H. 75, 76
Schömig E. 11, 12
Schön M.P. 286
Schöneberg T. 46, 176
Schönfelder J. 531, 536
Schoentaube J. 139, 398

Scholich K. 161
Scholze C. 40
Schooß C. 59
Schormann W. 372
Schott C. 79
Schramme A. 325
Schreck I. 439, 452
Schreiber S. 435, 449, 450
Schreiner M. 411
Schrenk D. 322, 350, 473
Schrobang J. 47
Schröder H. 242
Schröder H.C. 512
Schröder K. 362, 483
Schroeder P. 363
Schröder S. 201
Schrör K. 59, 272, 311
Schroeter M. 397
Schubert H. 20
Schuchlautz H. 45
Schülein R. 27, 28
Schüppel M. 110
Schürger S. 242
Schürks M. 538
Schürmann A. 2, 135, 136, 214
Schütz G. 310
Schuhholz J. 123
Schuhmacher S. 282, 547, 557
Schulte J.S. 71
Schulte S. 508
Schulte-Hermann R. 474
Schultz G. 126
Schulz A. 46
Schulz B. 409
Schulz E. 282
Schulz F. 149
Schulz H. 333
Schulz K. 27
Schulz N. 290
Schulz S. 41, 242
Schulz T. 396
Schulze F. 115
Schulze-Bahr E. 294
Schupp N. 471, 475
Schuppan S. 547, 557
Schwab M. 524, 526, 534, 544, 568
Schwahn C. 525
Schwan C. 80, 243
Schwaninger M. 206
Schwanstecher C. 195
Schwanstecher M. 192, 195
Schwartz A. 179
Schwartz S.M. 308
Schwarz M. 346, 435, 436, 448, 449, 450
Schwedhelm E. 94, 115, 530
Schweikl H. 365, 438
Schwieger I. 30, 31
Schwinzer R. 340
Schwoerer A. 122
Schyschka L. 399, 400
Seeburg P.H. 79
Seefeld A. 51
Seeger W. 332
Seeling A. 113, 301
Seeringer A. 533
Sehrt D. 528
Seidel A. 396, 410, 463, 464, 500
Seidel T. 505
Seidl M.D. 296, 310
Seifert R. 35, 51, 52, 57, 156
Seiler A. 492
Seiss M. 354, 355, 380, 521
Seithel A. 527
Selve N. 241
Semtner M. 183
Sendzik J. 376
Sens B. 370
Serrão M.P. 67
Serwe A. 239
Settmacher U. 343
Seuberth A.E. 91
Seuwen K. 55
Shafqat N. 328
Shan M. 266
Sheeta A.A. 316
Shen J.H. 506
Shih M. 257
Sibaev A. 321
Sichelschmidt O. 272
Siebelmann M. 204
Sieber M. 496, 497
Siedschlag V. 266
Siegmund W. 543
Sieke C. 518
Sigl K. 163
Silber R.E. 291, 297
Simm A. 291, 297
Simmet T. 159, 229, 230, 267, 326
Simon A. 3, 8, 116
Simon K. 496
Simon S. 489
Singh R. 352
Sitte H.H. 87
Skladnikiewicz T. 396
Slawik B. 492
Sluka K. 79
Solbach T.F. 545
Solecki R. 518
Sommer C. 421
Sommer K. 466
Spahl W. 521
Spanier G. 99
Spickenheuer A. 501, 502, 503
Spielmann H. 492, 495
Splettstoesser F. 378, 379
Spormann T. 476
Sprengel R. 79
Staessen J.A. 535
Stahl J. 336
Stahlmann R. 374, 376
Stalleicken D. 301
Standl T. 530
Stangel M. 373
Staniek K. 478
Stark H. 257
Stasch J.P. 101
Steensen C. 7
Steger U. 442
Stehfest E. 486
Steiger A. 559
Stein J.M. 549, 550, 551
Stein R. 215
Steinfelder H.J. 433
Steinhoff L. 113, 547, 557
Steinkamp-Fenske K. 97, 98
Stewart R.E. 532
Stiborova M. 352
Stoeckli E.T. 258
Stöger T. 333
Störk S. 546
Stoll M. 118
Stolze K. 479
Stopper H. 377, 409, 431, 471, 475
Storch U. 184
Storm J.F. 245
Storr M. 321
Strack S. 513, 514
Strähle U. 514
Strand D. 95, 526
Straßer A. 35
Strasser R.H. 292
Strathmann J. 436
Stratmann H. 146
Straub I. 173
Strauss V. 369
Ströfer M. 486
Strom S.C. 480
Strotmann R. 176
Stübs G. 226
Stumm R. 40, 41
Stuppner H. 400
Suckow-Schnitker A.K. 386, 387, 388
Sullivan J.P. 75, 76
Sultan K. 94
Suvorava T. 96, 100, 103, 114, 309
Swiercz J.M. 38, 258
Sydlik U. 454, 455, 456
Sydor S. 406
Syrovets T. 159, 229, 230, 267
Szabo B. 244, 250
Szasak M. 273

Tafti M. 104
Talsness C.E. 522
Tamagnone L. 258
Tamargo J. 196
Tang T. 520
Tang W.J. 156
Tannert A. 126
Tappe-Theodor A. 38, 81
Taudien S. 523
Tegeder I. 79
Tegude H. 524
Teichert M. 214
Tekook M. 284
Telenti A. 524
Telgmann R. 529, 531
Tenbrock K. 296
Teschner C. 191
Tesseromatis C. 312
Tessmann G. 525, 538
Thielen S. 413
Thiemann M. 352
Thijs L. 535
Thoms W. 530
Thor D. 46
Thude H. 540
Thum T. 302
Tigges J. 445
Tigka E. 312
Timm J. 569
Timpel C. 493
Tiret L. 529
Töllner K. 248
Tönnies M. 349
Töpfer M. 217
Toliat M. 526
Toma O. 285
Toman A. 195
Tomicic-Christmann M.T. 416, 419
Torky A. 427, 486
Torti F.M. 474
Torti S.V. 474
Torzewski M. 99
Toscano C. 568
Tot E. 537
Touboul P.-J. 536
Tränkle C. 48, 49

Traidl-Hoffmann C. 162, 509
Trapp C. 429
Trentmann G. 517
Triebel S. 356
Tröger K. 539
Truss M. 567
Tschirbs S. 344, 345, 504
Tschöpe C. 295
Tsoukali E. 298
Tsvilovskyy V. 170
Tüllmann C. 200, 309
Tunaru S. 61
Tuschl G. 481
Twarock S. 320

Ündeger Ü. 393
Uhde I. 195
Ullrich A. 480
Ullrich V. 477
Ulmer A.J. 226
Unfried K. 430, 454, 455, 456
Ungemach F.R. 65, 466
Unger T. 295
Urban-Klein B. 333
Urbanski M.J. 244

Vallon V. 323
Valtcheva N. 166
van Berlo D. 430, 510
van der Hoeven F. 425
van der Horst G.T.J. 446
van Harten P.N. 532
van Os J. 532
van Ravenzwaay B. 356, 357, 369, 370
van Roon E.N. 565
van Schooten F.J. 510
Vang G. 515
Vaquero M. 196
Venkatachalam A. 460
Verlohner A. 357
Verspohl E.J. 204, 217
Vettel C. 138, 143
Viegas O. 205
Vieira Coelho M.A. 67, 68, 118
Vierling W. 74
Vignali S. 213, 216
Vilardaga J.-P. 24
Visan A. 492
Vodrazka P. 258
Völkel W. 496
Völker U. 553
Völzke H. 525, 541
Vogel H. 214
Vogelbein S. 28
Vogelsang M. 304
Vogt A. 138
Voigt J.-P. 78, 207
Voigt N. 292
Volk J. 433, 472
Volkery D. 284
Volknandt W. 7
Volland J. 490
Vollandt R. 539
Vollmar A.M. 157, 158, 231, 305, 329, 399, 400, 437
von Eiff B. 459
von Hayn K. 154
von Herrath M.G. 237
von Kries J.-P. 273
von Kügelgen I. 82, 83, 84
von Landenberg F. 498
von Vietinghoff S. 179
Vormfelde S.V. 528
Voss P. 430
Vulcu S.D. 62

Wäring J. 185
Wätjen W. 385, 386, 387, 388, 405, 406, 407, 493, 494
Wagner E.F. 235
Wagner G. 539
Wagner J. 392, 451, 488
Wagner S. 496, 499
Wagner T.F.J. 172, 173
Wahl M. 513, 514
Wakabayashi I. 182
Wakili R. 293
Walitza S. 11, 377
Walker J. 247
Wallbach J. 321
Wallerath T. 397
Walliser C. 137, 144
Walstab J. 77
Walther D.J. 78
Walther S.C. 381
Walther T. 199
Walther U.I. 381
Wang Y. 385
Wang Z. 385
Warnke A. 11, 377
Warnken M. 317
Wasowicz W. 446, 507
Watzer B. 20
Webb J. 374, 376
Weber K. 442
Weber M. 36
Weber S. 165
Wegener J.W. 163, 165
Wehling M. 36
Wehrmeister D. 294
Weichenmeier I. 367
Weidinger M. 262
Weiergräber M. 180
Weiland T. 228
Weiller M. 227
Weimer M. 461
Weindl K. 104
Weiner H. 113
Weinmeister P. 162, 164, 165, 166, 167
Weirich J. 283
Weiser D. 5
Weiss C. 439, 452, 513, 514
Weissenberg A. 455, 456
Weißgerber P. 177
Weissmann N. 171, 332
Weistenhöfer W. 504
Welge P. 347, 501, 502, 503
Welling A. 213, 216
Wendel A. 226, 227, 228
Wendorff S. 234
Wenge B. 10
Wenzel P. 113, 282, 301, 302, 547, 557
Wenzel U.O. 201
Werfel T. 234
Werner A. 335
Werner B. 377
Werner F. 289
Wernet W. 75
Werthmann R.C. 154
Wessels A. 430, 510
Westerkamp J. 535
Westermann D. 295
Westphal D. 518
Wettschureck N. 119, 120, 128, 303
Wettwer E. 196, 292
Wewetzer M. C. 11
Weyerbrock A. 251
Whistler J. 38
Wichert Grande S. 383, 522
Wicke K. 76
Widmayer P. 132
Wiegand C. 362
Wieland T. 34, 138, 143
Wienbergen A. 218, 219
Wiench K. 369, 508
Wiertz M. 375, 487
Wiesner B. 30, 31
Wiewrodt D. 421
Wilde A.A. 294
Wilffert B. 532, 565
Wilgenbus P. 95
Wilhelm B. 132, 330
Wilhelm C.S. 199
Will F. 322, 476
Will J. 224, 225, 554
Wille H. 558, 560
Willecke K. 435
Willenborg M. 218
Willmer C. 542
Winkler M. 193
Winkler P. 143
Winter G. 231
Winter J. 248
Wirth A. 303
Wirth K.J. 197
Wishart W.L. 55
Wissenbach U. 174, 175, 178
Witte I. 95
Wittköpper K. 288
Wittmann H.-J. 35
Wobst I. 93
Wojnowski L. 99, 280, 523, 526, 556
Wolf C.R. 352
Wolf E. 340
Wolff K. 54
Wolff S. 445
Wollenberger L. 518
Wolski P. 349
Woltersdorf R. 45
Wood R. 414
Wormke M. 443
Worzfeld T. 258
Wotjak C. 189
Wsol W. 351
Wu D.F. 39, 40
Wu X. 328
Wurst W. 2, 104
Wuttke M. 114

Xiong G. 516, 517
Xu H. 17, 18, 97, 98, 99
Xu J. 302

Yang L. 514
Yao Y. 97, 98
Yates A.E. 169
Yeh Y.-H. 293
Young M.F. 295
Yuece B. 321

Zabel U. 23, 24
Zacharowski K. 295
Zahler S. 157, 158, 231, 305, 329, 437
Zahn C. 135, 136
Zahn N. 549, 550, 551
Zander S. 516
Zang Q. 213
Zanger U.M. 524, 526, 527, 534, 544, 568

Zatonski H. 446, 507
Zehbe R. 20
Zeilhöfer H.U. 79
Zelaszczyk D. 76
Zeller F. 5
Zezula J. 58
Zhang H. 535
Zhang T.B. 506
Zhang X.J. 506
Zholos A.V. 170
Zidek N. 482
Ziemann C. 348, 433, 472
Zillies J. 231
Zimmermann A. 505
Zimmermann K. 109
Zimmermann N. 275
Zimmermann W.H. 279
Zitranski N. 330
Zok S. 508
Zolk O. 545
Zong X. 69, 118, 155
Zou M.-H. 302
Zuang V. 495
Zubakova R. 210
Zünkler B.J. 198
Zürn A. 24
Zugmaier W. 326
zur Nieden N.I. 491
Zurawski R. 493, 494

1

CHARACTERIZATION OF THE *PLASMODIUM FALCIPARUM* CALCIUM/PROTON ANTIPORTER PfCHA

A. Rotmann, A. Guiguemde, C. Sanchez, and M. Lanzer

During intraerythrocitic development, malaria parasites tightly regulate their intracellular Ca^{2+} and a sudden increase in cytosolic Ca^{2+} plays a crucial role in the parasite developmental processes. Little is known about the calcium homeostasis regulation mechanisms in *Plasmodium falciparum*. Therefore, we aimed to characterize the putative *P. falciparum* Ca^{2+}/H^{+} antiporter (PfCHA) expressed in *Xenopus laevis* oocytes. In uptake experiments with radiolabeled Ca^{2+} we observed an increased accumulation of $^{45}Ca^{2+}$ over time in oocytes injected with the PfCHA-cRNA compared to water injected controls. This uptake was inhibited by the Ca^{2+}/Na^{+} exchanger inhibitor KB-R7943, by the L-type Ca^{2+}-channel blocker verapamil, and by the Ca^{2+} competitor La^{2+}. Using red phenol dyed ringer solution, we observed an acidification of the incubation medium surrounding PfCHA expressing oocytes, suggesting that PfCHA extrudes protons from the cells. In addition, PfCHA-injected oocytes demonstrated a more depolarized membrane potential, indicating an electrogenic exchange of Ca^{2+} against H^{+}. This was confirmed by two-electrode voltage-clamp experiments in which PfCHA expressing oocytes showed an increased inward current compared to the controls. This increased conductance was blocked by verapamil and La^{2+}. Taking this data together we conclude: PfCHA is a Ca^{2+}/ H^{+} antiporter and may play a crucial role in the Ca^{2+} homeostasis of *P. falciparum*.

Universitätsklinikum Heidelberg, Hygiene-Institut, Abteilung Parasitologie, 69120 Heidelberg, Germany

2

FUNCTIONAL REGULATION OF OATP2B1 (SLCO2B1) BY PROTEIN KINASES

K. Köck[1], M. Grube[1], G. Jedlitschky[1], C. A. Ritter[2] and H. K. Kroemer[1]

OATP2B1 is a member of the organic anion transporting polypeptide family (OATP/SLCO) and shows a wide tissue distribution including intestinum, liver and placenta, where it might be involved in the uptake of endogenous or xenobiotic compounds, like organic anions, steroid conjugates and pharmacological important compounds like statins. Regarding the regulation of transport function of OATP uptake transporters mainly transcriptional or protein expression alterations have been studied so far. However, cells possess a plethora of other mechanisms to adapt their transport activity, just to mention the phosphorylation of proteins by several protein kinases. In this study we therefore investigate the effect of posttranslational modifications on transport function of OATP2B1. Initially, we could identify several potential protein kinase A (PK A) and C (PK C) phosphorylation sites within the OATP2B1 using *in silico* analyses based on phosphorylation site prediction programs (NetPhos1, GPS, DisPhos). Using an OATP2B1-overexpressing MDCK cell line, we further measured the transport function of OATP2B1 in the presence of several PK A or PK C activating or inhibiting compounds. While PK A induction by Forskolin, 8-Br-cAMP and DB-cAMP had no effect on OATP2B1-mediated [^{3}H]DHEAS and [^{3}H]Estrone-3-sulfate (E3S) uptake, preincubation with the PK C activator phorbol-12-myristate-13-acetate (PMA) (0.01µM-10µM) significantly inhibited the [^{3}H]DHEAS and [^{3}H]E3S uptake in a time and concentration dependent manner, with a maximum inhibition of around 40 % for 10 µM PMA. This effect could be reversed by coincubation with the PK C inhibitor Bisindolylmaleimide (BIM) I (1 µM), which alone had no effect on OATP2B1 function. Mechanistically, immunofluorescent staining of PMA treated cells revealed an internalisation of OATP2B1. In conclusion, our data suggest a rapid regulation of OATP2B1-mediated transport by PK C, which may be relevant for uptake and transepithelial transport of endogenous or exogenous OATP2B1 substrates, especially in organs like placenta, intestinum or liver.

[1]Dept. of Pharmacology, Ernst Moritz Arndt University, Greifswald, Germany
[2]Dept. of Pharmacy, Ernst Moritz Arndt University, Greifswald, Germany

3

Deletion of GLUT8 in mice increases anxiety-related behavior and stress responsivness

S. Schmidt*, R. Augustin*, S. M Hölter#, A. Scheepers*, V. Gawlik*, V. Gailus-Durner#, H. Fuchs#, W. Wurst#, M. Hrabé de Angelis#, H.-G. Joost* and A. Schürmann*

Facilitative transport of hexoses into mammalian cells is catalysed by members of the GLUT family. GLUT8, one of 14 members of the GLUT family, is a high-affinity glucose transporter (Km ~2mM) which also transports fructose and galactose. GLUT8 mRNA is expressed at highest levels in testis, at intermediate levels in brain, and at low levels in heart, skeletal muscle, adipose tissue, and liver. GLUT8 contains an N-terminal dileucine sorting signal that retains the transporter in an intracellular compartment. So far, stimuli leading to translocation of the transporter to the plasma membrane have not been found. Therefore, an intracellular function for GLUT8 is considered. In order to analyze the physiological role of GLUT8, a conventional *Slc2a8* knockout mouse was generated. *Slc2a8*$^{-/-}$ mice are viable and fertile and do not show significant differences in body weight development under standard-diet conditions. However, in the modified hole board behavioral test *Slc2a8*$^{-/-}$ mice differ distinctly in terms of anxiety-related behavior from their wild-type littermates. As compared to control mice, *Slc2a8*$^{-/-}$ mice showed a longer latency to enter unprotected areas, suggesting increased anxiety in these animals. In addition, *Slc2a8*$^{-/-}$ mice showed significantly elevated hyperactivity in the box but less rearings on the board of the modified hole board. Thus, the behavioural characterization of *Slc2a8*$^{-/-}$ mice is likely to represent increased stress responsiveness at the behavioural level. In order to understand this phenotype we performed *in situ* hybridization experiments of total brains of wild-type and knockout mice. GLUT8 specific signals were detected in hippocampus, thalamus and cortex of wild-type mice. We suggest that the neuronal phenotype of *Slc2a8*$^{-/-}$ mice is the result of dysfunctions in vegetative and cognitive processes which might be due to a defect in the neuronal glucose utilization.

*Department of Pharmacology, German Institute of Human Nutrition, 14558 Potsdam-Rehbruecke, Germany, #German Mouse Clinic, GSF National Research Center for Environment and Health, Neuherberg, Germany

4

FUNCTIONAL REGULATION OF OATP2B1 (SLCO2B1) BY PROTEIN KINASES

K. Köck[1], M. Grube[1], G. Jedlitschky[1], C. A. Ritter[2] and H. K. Kroemer[1]

OATP2B1 is a member of the organic anion transporting polypeptide family (OATP/SLCO) and shows a wide tissue distribution including intestinum, liver and placenta, where it might be involved in the uptake of endogenous or xenobiotic compounds, like organic anions, steroid conjugates and pharmacological important compounds like statins. Regarding the regulation of transport function of OATP uptake transporters mainly transcriptional or protein expression alterations have been studied so far. However, cells possess a plethora of other mechanisms to adapt their transport activity, just to mention the phosphorylation of proteins by several protein kinases. In this study we therefore investigate the effect of posttranslational modifications on transport function of OATP2B1. Initially, we could identify several potential protein kinase A (PK A) and C (PK C) phosphorylation sites within the OATP2B1 using *in silico* analyses based on phosphorylation site prediction programs (NetPhos1, GPS, DisPhos). Using an OATP2B1-overexpressing MDCK cell line, we further measured the transport function of OATP2B1 in the presence of several PK A or PK C activating or inhibiting compounds. While PK A induction by Forskolin, 8-Br-cAMP and DB-cAMP had no effect on OATP2B1-mediated [^{3}H]DHEAS and [^{3}H]Estrone-3-sulfate (E3S) uptake, preincubation with the PK C activator phorbol-12-myristate-13-acetate (PMA) (0.01µM-10µM) significantly inhibited the [^{3}H]DHEAS and [^{3}H]E3S uptake in a time and concentration dependent manner, with a maximum inhibition of around 40 % for 10 µM PMA. This effect could be reversed by coincubation with the PK C inhibitor Bisindolylmaleimide (BIM) I (1 µM), which alone had no effect on OATP2B1 function. Mechanistically, immunofluorescent staining of PMA treated cells revealed an internalisation of OATP2B1. In conclusion, our data suggest a rapid regulation of OATP2B1-mediated transport by PK C, which may be relevant for uptake and transepithelial transport of endogenous or exogenous OATP2B1 substrates, especially in organs like placenta, intestinum or liver.

[1]Dept. of Pharmacology, Ernst Moritz Arndt University, Greifswald, Germany
[2]Dept. of Pharmacy, Ernst Moritz Arndt University, Greifswald, Germany

5

MECHANISMS OF ACTION OF THE PRO-SECRETORY EFFECTS OF STW 5 IN HUMAN INTESTINE

D. Krüger[a], F. Zeller[b], O. Kelber[c], D. Weiser[c], T. Frieling[d], M. Schemann[a]

Phytotherapy is a successful alternative approach to treat functional gut disorders including irritable bowel syndrome. The action of the hydroethananolic drug STW 5 (Iberogast) consisting of nine herbal extracts (bitter candy tuft, chamomile flower, peppermint leaves, caraway fruit, liquorice root, lemon balm leaves, angelica root, greater celandine herbs, and milk thistle fruit) on secretory activity in isolated human intestine was tested with the Ussing chamber technique. Serosal application of STW 5 (256µg/ml-1024µg/ml) concentration dependently increased the short circuit current in human mucosa-submucosa preparations. Mucosal application had no effect. The response was similar in small and large intestine and the data were therefore pooled. The increase was 9.7±2.9µA/cm^2 for 256µg/ml, 22±7.9µA/cm^2 for 512µg/ml and 29±8.1µA/cm^2 for 1024µg/ml ($p<0.05$ at all concentrations). The STW 5 evoked secretory effect was bumetanide (100µM) sensitive and therefore due to increased chloride secretion. This was confirmed by the inhibitory action of glibenclamide (400µM), the selective CFTR inhibitor CFTR 172inh (10µM) and the cAMP blocker MDL12330A (20µM). Reduction of the secretory response by SITS (1mM) suggests additional involvement of Ca- activated Cl-channels. STW 5 had also a pro-secretory effect in the human colonic cell line T84 supporting a direct effect on the epithelium. However, nerve blockade by tetrodotoxin (1µM) significantly reduced the STW 5 evoked secretion indicating an additional neural involvement. Our results indicate that STW 5 has a powerful pro-secretory effect in the human intestine in vitro. It stimulates chloride secretion at the level of the epithelial cell and the enteric nervous system. Generally, patients with reduced secretion may profit from this drug. These mechanisms of action may be a basis for the use of STW 5 (Iberogast®) as a treatment option for IBS patients, in particular those with constipation predominant IBS.

[a]Human Biology, TU Munich, Freising, Germany, [b]Surgery, Clinic Freising, Freising, [c]Steigerwald Arzneimittelwerk GmbH, Scientific Department, Darmstadt, Germany, [d]Internal Medicine, Clinic Krefeld, Krefeld, Germany

6

A NOVEL FLUORESCENCE BASED IN VITRO ASSAY FOR THE DETECTION OF DRUG INTERACTIONS WITH BREAST CANCER RESISTANCE PROTEIN (BCRP)

A. Mahringer, G. Fricker

The Breast Cancer Resistance Protein (BCRP) is a member of the ABC-transporter family which is involved in the efflux of a broad spectrum of structurally unrelated substances causing multidrug resistance. Apart from its expression in mammary glands, placenta tissue, kidney, liver and stem cells the efflux transporter is also expressed at the blood-brain barrier, where BCRP provides a neuroprotective function but is also considered to limit entry of drugs into the brain. Therefore, newly developed CNS drug candidates have to be tested for potential interactions with BCRP in preclinical phases to exclude possible effects on drug distribution. Purpose of the study was the development of a fluorescence based assay that characterizes the extent of interactions between BCRP and innovative drugs. The assay compares two self-fluorescent, potent BCRP substrates - mitoxantrone and BODIPY-Prazosin - and uses quantitative fluorescent analysis in porcine brain capillary endothelial cells (PBCECs) as well as in the human breast cancer cell line MDA-MB231. The assay principle is based on the measurement of intracellularly accumulated fluorescent dye in absence or presence of drug substances interacting with BCRP. Assay parameters like the optimum substrate concentration, substrate specificity and incubation times have been established. Additionally, the permeability coefficients for mitoxantrone and BODIPY-Prazosin in PBCECs and MDA MB231 cells have been investigated and the interaction of a series of known compounds with BCRP has been studied using FACS-analysis. This assay

provides an efficient tool to identify modulators of BCRP in drug discovery, which is also applicable to high throughput systems.
IPMB, INF 366, Ruprecht-Karls University Heidelberg, 69120-Heidelberg, Germany

7

ANALYSIS OF TRANSPORTER FUNCTION IN NATIVE SYNAPTIC VESICLES USING A NOVEL ELECTROPHYSIOLOGICAL TECHNOLOGY

U. Pehl[1], P. Obrdlik[1], K. Diekert[1], I. Barth[1], Chr. Keipert[1], C. Steensen[1], W. Berger[1], M. Ruitenberg[1], W. Volknandt[2], B. Kelety[1].

Pharmacological studies on intracellular compartments, in particular of native synaptic vesicles are difficult to perform due to their limited accessibility. On the other hand, integration of intracellular transport proteins into the plasma membrane utilizing heterologous expression systems often results in non-specific effects on the pharmacological and biophysical transporter properties potentially leading to a misinterpretation of the data. The SURFE^{2}R technology permits the electrophysiological analysis of transporters as well as other electrogenic proteins on solid supported membranes (SSMs). Vesicles and membrane fragments are adsorbed onto a gold-coated sensor surface. The resulting biosensor is mounted into the fluidic system of the setup. Protein-specific charge movements resulting from rapid solution exchanges can be detected as electrical currents in a highly sensitive manner. Here, rat brain synaptic vesicles were isolated and functionally examined utilizing the SURFE^{2}R technology to demonstrate its suitability for pharmacological characterization of intracellular transporters. First of all, we examined the synaptic v-type ATPase that generates a proton electrochemical gradient across the vesicular membrane required for the uptake of neurotransmitters into the organelles by specific transport systems. Activation of the v-ATPase through ATP concentration jumps induced electrical currents that could be inhibited by the specific v-ATPase inhibitor bafilomycin A1. These currents were Mg^{2+}- and ATP-dependent with an apparent $K_{0.5}$ for ATP of approximately 50 µM. Assay development and experiments allowing the functional identification and pharmacological characterization of further vesicular transporters and ion channels are ongoing. Preliminary recordings from sensors coated with synaptic vesicles indicate that electrical responses can be elicited by application of different neurotransmitters. To our knowledge, this study represents the first electrophysiological investigation of proteins from native synaptic vesicles. In summary, our data demonstrate that the SURFE^{2}R technology is a powerful tool to study native transport systems not only in plasma membranes but in intracellular compartments like synaptic vesicles as well.

[1]IonGate Biosciences, Frankfurt; [2]Institute of Cell Biology and Neuroscience, University of Frankfurt.

8

ACTIVATION OF CLASSICAL PROTEIN KINASE C (PKC) DECREASES TRANSPORT VIA SYSTEM y^+L

U. Martiné, A. Rotmann, A. Habermeier, A. Simon, and E. I. Closs

Systems y^+ and y^+L are widely expressed transport systems for cationic amino acids (CAA). Their activities are mediated by CAA transporters (CATs) and system y^+L AA transporters (y^+LATs), respectively. Both systems transport CAA in a Na^+-independent manner. They can however be distinguished by their interaction with large neutral AA that only inhibit y^+LATs (competitively), but not CATs. We have previously shown that PKC activation results in reduced CAT activity (Rotmann et al., 2004, J. Biol. Chem.; Rotmann et al., 2006, Biochem. J). However, others found that PKC increases arginine transport in various mammalian cell types, suggesting opposite PKC effects on different CAA transporters. We thus investigated the effect of PKC activation on system y^+L. The PKC activating phorbol ester phorbol-12-myristate-13-acetate (PMA, 100 nM) reduced system y^+L-mediated (leucine-sensitive) arginine transport in all but one cell lines tested, independent of the y^+L isoform expressed (y^+LAT1 or -2). The extent and onset of inhibition varied between cell lines, however, a PMA-induced increase in arginine transport was never observed. A time course of PMA inhibition in ECV304 cells showed a delay in the maximal inhibition of system y^+L in comparison with system y^+ (2h versus 30min). The IC_{50} values for PMA were 9.2±1.6 and 3.0±1.4, for system y^+ and y^+L, respectively. Inhibition of both systems could be prevented by Gö6976, a specific inhibitor of conventional PKCs. Thymelea toxin that activates preferentially classical PKC had a similar inhibitory effect as PMA. In contrast PIP, an activator of atypical PKC, and the inactive phorbol ester 4αPDD had no effect. To investigate the PMA effect on each isoform, y^+LAT1 and 2 were expressed individually in Xenopus laevis oocytes, together with the glycoprotein 4F2. A pronounced inhibition, that could be prevented by the PKC inhibitor BIMI, was consistently found for both transporters. 4αPDD had again no effect. Taken together our data demonstrate that both system y+L transporters are downregulated by classical PKC.

Johannes Gutenberg University Mainz, Department of Pharmacology, 55101 Mainz, Germany

9

DOWNREGULATION OF RFC1 MEDIATED METHOTREXATE UPTAKE BY PB-TYPE CYP450 INDUCERS INVOLVES PKC

S. Halwachs, C. Kneuer and W. Honscha

Drug interactions are an ongoing concern in treatment of cancer. Recent clinical studies suggest that longterm anticonvulsant therapy with Phenobarbital (PB) and Carbamazepine (CBZ) may reduce the efficacy of cancer chemotherapy, including high dose methotrexate (MTX) treatment. Seizures are a common complication in cancer patients that require additional treatment with antiepileptic drugs. Both agents represent cytochrome (CYP) P450 inducers of the PB-type, which increase CYP gene expression via the Constitutive Androstane Receptor (CAR, NR1I3) pathway. The antifolate MTX is widely used for the treatment of malignancies, e.g. ALL and lymphomas. Our *in vivo* and *in vitro* studies support downregulation of the sodium-dependent reduced folate carrier (Rfc1; Slc19a1) in hepatocytes by PB, CBZ, chlorpromazine, clotrimazole as well as the CAR agonist TCPOBOP and indicated a post-transcriptional mechanism. As RFC/Rfc provides the major route for cellular uptake of antifolate drugs such as MTX into liver, kidneys and other tissues, we have therefore systematically investigated its regulation in an *in vitro* model for rat liver.

Methods: HPCT-1E3 hepatocytoma cells were pre-treated with various modulators or controls and Rfc1 activity was measured as the time- and sodium-dependent uptake of [^{3}H] MTX. Effects on Rfc1 mRNA and protein levels were investigated by quantitative RT-PCR and by quantitative immunocytochemistry.

Results: Decreased MTX uptake following pre-treatment (48h) with therapeutic levels of PB-type CYP450 inducers and TCPOBOP was not associated with reduced mRNA and protein levels. Interestingly, short-term treatment (2h) with the proteinkinase C (PKC) inducer PMA (0.6µg/ml) and protein phosphatase 2A inhibitor okadaic acid (0.08µg/ml) also caused a reduction of Rfc1 mediated MTX uptake. The down-regulation of Rfc1 activity by PMA as well as PB and TCPOBOP was almost completely reversed by the PKC inhibitor Bisindolylmaleimide (0.02µg/ml).

Conclusion: This study documents for the first time, that clinically relevant concentrations of PB-type CYP450 inducers cause a significant PKC-dependent reduction in the Rfc1 mediated MTX uptake on the posttranscriptional level.

Institute of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, University of Leipzig, 04103 Leipzig, Germany

10

INTERFERENCE OF THE NORADRENERGIC NEUROTOXIN DSP4 WITH NEURONAL AND NON-NEURONAL MONOAMINE TRANSPORTERS

B. Wenge and H. Bönisch

The haloalkylamines, xylamine and DSP4 (N[-2-chloroetyl]-N-ethyl-2-bromobenzylamine) are proposed to be selective substrates for the noradrenaline transporter (NAT) and to be toxic for noradrenergic neurons. Their systemic application is known to cause a significant reduction of endogenous norepinephrine and [^{3}H]desipramine binding in the frontal cortex of rats. DSP4 is used for the denervation of locus coeruleus projections. Previously, we showed that xylamine and DSP4 also interferes with the human neuronal transporters for dopamine (hDAT), although with a 20-fold lower potency compared to that at the hNAT. The aim of the present study was to investigate 1. which region of the hNAT may be responsible for the recognition of DSP4, 2. whether DSP4 interacts with human non-neuronal monoamine (organic cation) transporters (hOCT1, hOCT2 and hOCT3), 3. whether the mode of action is irreversible, and 4. whether the selective neurotoxic effects is dependent on transporters with high affinity for DSP4. Using hNAT or hDAT (or hNAT/hDAT chimeras) expressing HEK293 cells we now show that the transmembrane domains 6-8 of the hNAT seem to be involved in the inhibition of [^{3}H]MPP^+ by DSP4. We also show that DSP4 inhibits hOCT1, hOCT2 and hOCT3 (K_i about 2 µM) but in a reversible manner (in contrast to hNAT and hDAT). Finally, we demonstrate that those cells which express a transporter with high affinity for DSP4 (e.g. the hNAT) show a much higher sensitivity to cytotoxic DSP4 effects. However, this cytotoxic effect (measured by means of an ATP-based viability assay) was not yet observed after 90 min but after a delay of 24 hours. In conclusion: Our results indicate that DSP4 (and related compounds) at low concentrations cause irreversible inactivation only of the NAT.

Institute of Pharmacology & Toxicology, University of Bonn, 53113 Bonn, Germany

11

GENETIC ANALYSIS OF THE EXTRANEURONAL MONOAMINE TRANSPORTER IN CHILDREN AND ADOLESCENTS WITH ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

A. Lazar[1], M. Romanos[2], S. Walitza[2], T. Renner[2], K.P. Lesch[3], M. Ch. Wewetzer[2], A. Warnke[2], M. Gerlach[2], D. Gründemann[1], E. Schömig[1]

Attention-deficit/hyperactivity disorder (ADHD) is the most common behavioral disorder in childhood with a considerable genetic component. The underlying pathophysiology is complex and the implication of several transmitter systems has been discussed. Because of the beneficial effects of the catecholamine-selective reuptake inhibitor methylphenidate in the treatment of ADHD genes of the noradrenergic pathway are logical candidates for a contribution to the etiology. The extraneuronal monoamine transporter (EMT) is responsible for the non-neuronal inactivation of noradrenergic signaling in the central nervous system and represents a candidate gene for a variety of neuro-psychiatric disorders. The aim of the present study was to determine whether genetic variants of EMT are associated with ADHD in children and adolescents. We conducted a case-control-study of 70 Caucasian children and adolescents with ADHD according to DSM-IV and 100 healthy adults by direct sequencing of all 11 exons, exon-intron-boundaries and the putative core promoter region of EMT. Single polymorphism and haplotype analysis was performed using Genecounting 2.2 software. As a result, known polymorphisms (-29A>G, 360T>C, 1233G>A, IVS9 –60delC, -50A>G, 1734C>T) were not associated with ADHD in the patients analyzed. Furthermore, no novel mutations were detected. On the other hand, there were significant differences in the distribution of some common haplotypes. Consequently, common polymorphisms in the EMT gene were not associated with ADHD in the present sample. The pathological relevance of formally associated haplotypes require confirmatory investigations.

[1]Department of Pharmacology, University of Cologne, Cologne; [2]Departments of Child and Adolescent Psychiatry, Julius-Maximilians-University, Würzburg;
[3]Molecular and Clinical Psychobiology Department of Psychiatry and Psychotherapy, Julius-Maximilians-University, Würzburg

12

FUNCTIONAL ANALYSIS OF NATURAL MUTANTS OF THE HUMAN ERGOTHIONEINE TRANSPORTER BY USE OF TRANSPORTER TANDEMS

M. Bach, S. Grigat, A. Lazar, E. Schömig, and D. Gründemann

Recently, we have discovered the ergothioneine (ET) transporter ETT (gene symbol *SLC22A4*). The precise physiological role of ET is still unclear; most researchers consider it an intracellular antioxidant. ETT from human (ETTh) has high affinity for ET (K_m = 21 µmol/l) and catalyzes cotransport of ET with Na^+. The carrier is strongly expressed in erythrocyte progenitor cells ($CD71^+$) and monocytes ($CD14^+$). Much interest in human ETT has been generated by case-control studies that suggest an association of polymorphisms in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases. The aim of the present study was to characterize the function of 2 naturally occuring mutants of ETTh, *i.e.* Gly462Glu and Leu503Phe, with the physiological substrate ET. Since substitution of single amino acids can profoundly alter protein expression level or cellular sorting, it is necessary for a meaningful comparison of intrinsic transport activity to take the number of active transporters in the plasma membrane into account. Here we constructed transporter tandems to achieve reliable normalization of active transporter. The cDNA of the human organic cation transporter type 2 (OCT2h), which efficiently transports 1-methyl-4-phenylpyridinium (MPP^+), was fused in frame to the C-terminus of ETTh. 293 cells were stably transfected with wild-tpye and mutant cDNAs inserted into vector pEBTetD. Initial rates of uptake of ET and of MPP^+ (1 min, 10 µmol/l) were determined by LC-MS/MS. In control experiments with the original transporters, it was verified that ETTh does not transport MPP^+ and that OCT2h does not transport ET. The ET clearance / MPP^+ clearance ratio was 0.38 for the wild-type ETTh tandem, and 0.77 for the Leu503Phe mutant tandem. With the Gly462Glu tandem, the MPP^+ clearance was similar to wild-type tandem, but there was no uptake of ET at all. In conclusion, the Gly462Glu variant is a natural null mutant, while the intrinsic activity of Leu503Phe is two-fold higher than wild-type.

Department of Pharmacology, University of Cologne, Gleueler Straße 24, 50931 Cologne, Germany

13

PGP AND BCRP EXPRESSION CHANGES IN CHOROID PLEXUS AND BLOOD BRAIN BARRIER CAPILLARIES IN DIABETIC RATS

S. Burghard[1], G. Fricker[1] and V. Reichel[1]

The blood brain barrier, formed by capillary endothelial cells, and the choroid plexus are major barriers which separate the brain from flowing blood. A number of transport proteins are expressed in both barriers. These transport proteins are involved in efflux of metabolites and potentially harmful substances out of the brain and uptake of nutrients and other substances from blood. In the present study we investigated transport processes mediated by two major efflux transporters, P-glycoprotein (Pgp) and Breast Cancer Resistance Protein (Bcrp) in control and diabetic rats. Both proteins are ATP-dependent active transporters with important roles at the blood brain barrier. Pgp is the 170kDa gene product of multidrug-resistance (MDR1) gene. Its absence in the blood brain barrier is known to cause increased brain penetration of a number of drugs. Bcrp is a 655 amino acid polypeptide that functions as a homodimer. Like Pgp, it is located to cell membranes in a variety of tissues. Overexpression of Bcrp in capillary endothelial leads to resistance against a number of drugs while downregulation of Bcrp causes increased accumulation of some substances in the brain. We compared Pgp and Bcrp mRNA levels in freshly isolated brain capillaries and choroid plexuses in wildtype (Sprague Dawley) and diabetic rats using RT-PCR. The diabetic rats suffer from at least 12 days lasting diabetes induced by streptozotocin (STZ), which models type 1 diabetes. The comparison showed that Pgp expression level in brain capillaries was enhanced in diabetic rats. Concerning Bcrp changes in expression level could not be detected using brain capillaries. However, mRNA expression levels of both transporters were not clearly modified in choroid plexus samples of wildtype and diabetic rats. Semi-quantitative analysis are carried out to verify these results. For analysis of Pgp and Bcrp protein expression level, western blot studies will be accomplished, similarly functional comparisons using fluorescent substrates and confocal microscopy will be carried out.

[1] Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, Heidel-berg, Germany

14

EPIGENETIC REGULATION OF MDR1 TRANSCRIPT EXPRESSION BY DIFFERENTIAL PROMOTER METHYLATION

A. Kayser, C. Rimmbach, D. Rosskopf

Efflux transporters for xenobiotics (e.g. MDR1, MRP2, MRP4, MRP5) have an important role in drug disposition. Overexpression of these pumps has been implicated in the generation of drug resistance, particularly in cancer cells. Epigenetic regulation by differential methylation of CpG islands on the DNA has become an interesting mechanism, how cancer cells can modulate gene expression. Here, we analyzed the methylation status of already identified and novel CpG motifs in the MDR1 gene. DNA from different cell lines (lymphoblasts, fibroblasts, CaCo, HeLa, HL60,…) was prepared. Treatment with bisulfite is an accepted procedure for the differentiation between non-methylated and methylated C's, for bisulfite treatment leads to a conversion of unmethylated C to T. Bisulfite-treated DNA was subsequently amplified with strand-specific primers under consideration of the C to T conversion. The extent of methylation at a distinct DNA CpG locus was quantified by the pyrosequencing procedure upon comparison of T or C sequencing signals at a given locus. Controls showed that the bisulfite treatment caused the C to T conversion with an efficacy of > 90%. Among different cell lines we observed considerable differences in the extent of methylation of 5 CpG sites, ranging from -121 to -85 upstream of the start codon. While lymphoblasts and fibroblasts were completely methylated at this locus, HeLa cells and other tumour-derived cell lines showed a partial methylation in the range of 40 to 80% only. Together, we present evidence that promoters of export pumps are subject to differential methylation at CpG motifs. We observed first suggestive evidence that the promoter methylation of MDR1 in common tumor cell lines differs from the methylation of primary cells.

Institut für Pharmakologie – Ernst-Moritz-Arndt Universität Greifswald

15

TRANSPORT PROTEINS IN THE PLACENTA: REGULATION OF RNA-EXPRESSION OVER GESTATION TIME

S. Reuther[1], M. Grube[1], K. Köck[1], C. Fusch[2], H. K. Kroemer[1]

The human placenta as the fetal-maternal interface has both protective and nurturing functions for the fetal organism. Both features are associated with transport processes, which are performed by transport proteins of the solute carrier (SLC)- and ABC-family. However, the placental transport function has to adapt to the requirements of the developing fetus; therefore, transporter expression is changing during pregnancy. For this reason we examined mRNA expression of several SLC- and ABC-transporter in 80 placentas of different gestation age (22 term (> 37. week of gestation), 26 preterm (30. - 37.) and 22 early preterm (< 30.) placentas). After RNA isolation real-time PCR was performed for the organic anion-transporting polypeptides 2B1 and 4A1 (OATP2B1 and 4A1), the organic anion transporter 4 (OAT4), the sodium-dependent organic anion transporter (SOAT) and the organic cation transporter novel type II (OCTN2) as well as for the breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp). In addition to these transport proteins, we measured several housekeeping genes (18SrRNA, ß-Actin, GAPDH and TBP (TATA box-binding protein) for normalization. Thereby, we were able to show significant regulation of P-gp, OATP2B1, BCRP and OAT4 for the different gestation stages and we observed a positive correlation between OATP2B1 and BCRP ($R^2 = 0.59$; $p < 0.05$) and Pgp and OAT4 ($R^2 = 0.6$; $p < 0.05$). However, we also found substantial differences between the housekeeping genes. For example, we detected a significant variation in expression of 18S rRNA or ß-Actin over pregnancy, while TBP remained relatively stable. In summary, we were able to show gestation dependent effects in placental transporter expression, which may be due to different needs of the foetus during pregnancy. Moreover, we observed an important impact in the choice of housekeeping genes with TBP being the most appropriate one for our expression analyses.

[1] Dept. of Pharmacology, Peter Holtz Research Center of Pharmacology and Experimental Therapeutics; Ernst-Moritz-Arndt University, Greifswald, Germany
[2] Dept. of Neonatology, Ernst-Moritz-Arndt University, Greifswald, Germany

16

EXPRESSION AND FUNCTION OF MRP4 AND MRP5 DURING HAEMATOPOEITIC DIFFERENTIATION

J.F. Scheitz, L. Oevermann, G. Jedlitschky , H.K. Kroemer and C.A. Ritter

Background: The multi-drug-resistance proteins (MRP) 4 and 5 are known to mediate the transport of nucleotides, nucleoside analogues and cyclic nucleotides. Recent studies of our laboratory indicate that their expression is specifically regulated during differentiation of cord-blood (CB) derived $CD34^+$ stem cells, which suggests a role of these transporters during haematopoietic development.

Methods: In order to investigate this regulation in more detail, we differentiated human leukemia cell lines widely used as in-vitro models for myeloid development. To imitate the myelomonocytic differentiation HL-60 and U-937 cells were cultivated with vitamin D_3, 8-Cl-cAMP, butyrat, DMSO or PMA. To mimic the megakaryocytic differentiation K-562 and M-O7e cells were treated with 8-Cl-cAMP, PMA or TPO, respectively. State of differentiation was determined by FACS staining using the lineage specific surface markers CD11b, CD14, CD15, CD71 (transferrin receptor) for myelomonocytic culture as well as CD61a (Glycoprotein IIIa) and CD235 (GlycophorinA) for megakaryocytic culture. Expression of MRP4 and 5 was investigated using Taqman® Real-time PCR and immunoblotting and transporter activity was assessed by accumulation assays.

Results: When U-937 and HL-60 cells were treated with the above-mentioned substances we identified an increase of CD11b, CD14 and CD15, typical phenotypic features of myelomonocytic cells, and a decrease of CD71. MRP4 and 5 protein expression was found to be decreased under these conditions, which is consistent with our findings concerning CB differentiation. Differentiation of K-562 cells stated by an increase of CD61a and a decrease of CD235a was accompanied by a significant decrease of MRP4 and MRP5 expression on mRNA and protein level. Furthermore, reduction of protein expression was accompanied by a significant 2-fold accumulation when K562 cells, differentiated with PMA for 72 h, were incubated with radiolabeled (^{3}H)-Bis(POM)-PMEA, a substrate of MRP4 and MRP5, as compared to untreated K562 cells.

Conclusion: These results show that expression of MRP4 and MRP5 is specifically regulated during haematopoeitic differentiation suggesting physiological functions in this process.

Department of Pharmacology, Research Center of Pharmacology and Experimental Therapeutics and Institute of Pharmacy, University of Greifswald

17

PKC ACTIVATION ENHANCES ENDOTHELIAL VEGF EXPRESSION AND PROMOTES ANGIOGENESIS

H. Xu, U. Förstermann, and H. Li

Protein kinase C (PKC) represents a family of enzymes that play crucial roles in intracellular signal transduction and have been implicated in many cellular responses important in health and disease. Here we report that PKC is also an important regulator of endothelial cell function. In a matrigel angiogenesis assay, treatment of human umbilical vein endothelial cells (HUVEC) with the PKC activator phorbol-12-myristate-13-acetate (PMA) resulted in enhanced tube formation. To analyze the underlying mechanisms, we studied the expression of vascular endothelial growth factor (VEGF) in human endothelial cells. Treatment of HUVEC and HUVEC-derived EA.hy 926 cells with PMA resulted in a marked upregulation of VEGF mRNA expression. This effect was reproducible with a second PKC activator, phorbol-12,13-dibutyrate (PDBu), but not with the inactive phorbol ester analog 4alpha-phorbol-12,13-didecanoate. These results indicate that the effects of PMA and PDBu were PKC-dependent. Accordingly, the enhancement of VEGF mRNA expression by PMA was preventable with PKC inhibitors. EA.hy 926 cells express a total of eight PKC isoforms. Treatment with PMA led to an activation of PKC alpha, delta and epsilon. Preliminary results from siRNA experiments showed that knockdown of PKC alpha could prevent PMA-induced VEGF expression, demonstrating a regulatory role of this PKC isoform. VEGF mRNA showed a half-life of about 6 hours and PMA treatment did not change VEGF mRNA stability. A causal link between PMA-induced VEGF upregulation and endothelial cell function (angiogenesis) is currently under investigation.

Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany

18

REGULATION OF BDNF EXPRESSION IN PERIPHERAL ENDOTHELIAL CELLS THROUGH PKC ACTIVATION AND TNF-ALPHA

P. Czerwinski, H. Xu, U. Förstermann, and H. Li

Brain derived neurotrophic factor (BDNF) is a neurotrophin best characterized for its survival and differentiative effects on neurons. However, recent studies demonstrated that BDNF and its receptors are also expressed in the peripheral vasculature (particularly in intramyocardial vessels), where it stimulates angiogenesis and promotes survival of endothelial cells. Because myocardial ischemia and reperfusion is associated with PKC activation and enhanced release of tumor necrosis factor-alpha (TNF-alpha), we hypothesized that part of the effects of PKC and TNF alpha may be mediated by a reduction of BDNF production in endothelial cells. Treatment of human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells with the PKC activator phorbol-12-myristate-13-acetate (PMA) led to a concentration- and time-dependent downregulation of BDNF mRNA expression. This effect was reproducible with a second PKC activator, phorbol-12,13-dibutyrate (PDBu), but not with the inactive phorbol ester analog 4alpha-phorbol-12,13-didecanoate. These results indicate that the effects of PMA and PDBu were PKC-dependent. Accordingly, the reduction of BDNF mRNA expression by PMA was preventable with the PKC inhibitor Gö 6983. Also TNF alpha reduced BDNF expression in HUVEC and EA.hy 926 cells concentration- and time-dependently. This effect, however, was a PKC-independent event because it was not prevented by PKC inhibitors. In a matrigel angiogenesis assay, treatment of HUVEC with exogenous BDNF resulted in enhanced tube formation, whereas TNF reduced angiogenesis of HUVEC. Thus, the reduced BDNF expression and angiogenesis ability of endothelial cells may be involved in the detrimental effects mediated by PKC and TNF alpha.

Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany.

19

PHARMACOLOGICAL INHIBITION OF JAK/STAT SIGNALING INDUCES PROCESS FORMATION IN NEURAL CELLS

D.K. Meyer, J. Leemhuis, M. Kirsch, K. Kubatzky and H.-D. Hofmann

In early studies, CNTF was described as a neurotrophic protein which promotes survival and differentiation of immature neurons. More recent studies indicate that CNTF prevents differentiation and promotes self renewal in neural stem cells. Effects of CNTF are mediated by a tripartite cytokine receptor complex containing gp130 and LIFRß as signal transducing subunits. Ligand binding activates receptor-associated protein kinases (JAKs) resulting in the phosphorylation of STAT1/3 which influence gene expression by directly binding to DNA. In the present study, we have investigated how inhibition of JAK/STAT signaling affects the morphology of cultured rat neocortical astroglial cells, rat hippocampus neurons and SH-SY5Y human neuroblastoma cells. In SH-SY5Y, the JAK protein kinase inhibitor 2-(1,1-dimethylethyl) - 9 - fluoro - 3,6 – dihyd- ro-7H-benz-[h]-imidaz [4,5-f]isoquinolin-7-one (JAK-I) (Thompson et al. (2002) Bioorgan. & Med. Chem. Let. 12:1219-1223) initiated within 2 h morphological changes, when applied at concentrations of 0.1 - 1 µmol/L. The polygonal cells started to extend long processes and the cell body became spindle shaped. With prolonged exposure to JAK-I, the cells acquired a neuron-like morphology. Also cultured astroglial cells developed long extensions, whereas cultured hippocampus neurons extended more neurites with multiple branches. Blocking STAT3 effects with cucurbitacin-1 (Blaskovich et al. (2003) Cancer Res. 63:1270-1279) induced very similar morphological changes in SH-SY5Y cells, when applied at concentrations of 0.1 – 1 µmol/L. In contrast, treatment of SH-SY5Y and neocortical astroglial cells with CNTF (50 ng/ml) promoted an epithelial morphology, whereas CNTF-treated hippocampus neurons apparently did not differ from controls. We consider these data as first evidence that activation of JAK protein kinase can suppress differentiation in neural cells. The signal pathways involved are currently investigated.

Institute of Anatomy & Cell Biology and Institute of Pharmacology, Albert-Ludwigs-University, 79104 Freiburg, Germany.

The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

20

PLGA-MICROSPHERES AS A SYSTEM FOR PROLONGED PROSTAGLANDIN E_2 RELEASE – FIRST RESULTS OF KINETIC ANALYSIS

C. Brochhausen[1], R. Zehbe[2], B. Watzer[3], S. Halstenberg[1], H. Schubert[2], C.J. Kirkpatrick[1]

Background: Prostaglandin E_2 (PGE_2) is an arachidonic acid metabolite involved in physiological homoeostasis and numerous pathophysiological conditions, such as stress response and inflammation. Futhermore, it has already been demonstrated that prostaglandins have stimulating effects not only on angiogenesis *in situ* and *in vitro* but also on chondrocyte proliferation *in vitro*. Thus, PGE_2 represents an interesting target molecule as a therepeutic agent to promote angiogenesis and as a signalling molecule for cell expansion *ex vivo* in the scope of tissue engineering strategies. However, the half life of PGE_2 is very low, with a value of approximately 10 minutes under physiological conditions. We tested if the release of prostaglandin E_2 could be prolonged by its integration in a degradable polyglycolide-based microsphere. Materials and Methods: PGE_2-modified microspheres were produced by dissolving PLGA (85:15 - lactide-glycolide ratio) in $CHCl_3$ and adding a solution of PGE_2 in a fluorinated alcohol. This solution was added drop wise to stirring 0.5 % polyvinyl alcohol as emulsifier. The solution was left stirring overnight to allow for complete evaporation of the used solvents. Twelve identical microsphere charges were produced; to each charge 1 ml of serum free medium was added. The supernatant was removed at different time points (0, 0.5, 1, 2, 4, 6, 13, 24, 48, 72, 154 h). PGE_2 release rates were measured by gas chromatography and the microspheres were further characterized morphologically using SEM. Results: Within the first 6 hours a linear release of PGE_2 could be observed with a plateau at 24 hours until 48 hours, followed by a discrete decrease within the following 5 days. Discussion: PGLA-microspheres allowed a prolonged release of PGE_2. The decrease and exact kinetics of decrease need further investigations. In conclusion, the findings open up interesting aspects for the use of PGE_2 as an innovative signalling molecule for cell expansion in tissue engineering strategies.

REPAIRlab, Institute of Pathology, Johannes Gutenberg-University, Mainz
Institute of Biotechnology, Technical University Berlin
Department of Paediatrics, Philipps-University, Marburg

21

DIFFERENT SIGNAL AMPLIFICATION IN G_S AND G_I COUPLED PATHWAYS

P. Hein, C. Hoffmann, M.J. Lohse, M. Bünemann

Signal transduction on G protein-coupled receptors allows for amplification of the signal. Specifically, few activated receptors can lead to a full effector response, a phenomenon which is termed spare receptors or receptor reserve. We used fluorescence resonance energy transfer (FRET)-based assays for receptor / G protein interaction and G protein activation together with assays for effector activation to directly compare amplification processes in G_s and G_i coupled systems in single cells. When stimulating HEK293 cells transfected with appropiately tagged A_{2A} adenosine receptors, G_s protein subunits or an EPAC-based cAMP sensor, we measured receptor / G_s interaction, G_s activation and whole-cell cAMP accumulation. For a G_i coupled system, cells were transfected with α_{2A} adrenergic receptors, G_i proteins or GIRK channels, and GIRK channel activation was used to asses effector activation. Again, receptor / G_i interaction, G_i activation and GIRK channel activation were measured. Comparing these results, we observed a substantial higher shift in EC_{50} curves for the G_i compared to the G_s coupled system. We conclude that signal transduction processes involving different G protein systems may be differently organized with regard to their ability to amplify the signal.

University Würzburg, Department of Pharmacology, Würzburg, Germany

22

VOLTAGE DEPENDENT ACTIVATION OF G PROTEIN COUPLED RECEPTORS DETECTED BY FRET MICROSCOPY

Moritz Bünemann[1], Martin J. Lohse[1]

Many G protein coupled receptors such as α_{2A} adrenergic receptors are expressed in excitable cells and regulate important physiological parameters such as electrical excitability and transmitter release. These membrane receptors are exposed to changes in membrane potential during action potentials. Monitoring effector responses of certain GPCRs voltage dependent regulation of receptor activity has been demonstrated. We attempted to measure effects of voltage on receptor activity directly by means of a recently developed FRET probes for α_{2A}-adrenergic receptors (α_{2A}-AR). The combination of whole cell patch clamp recording and single cell FRET-detection revealed that at negative membrane potentials norepinephrine (NE) was more potent in activating α_{2A}-AR in comparison to positive potentials. Voltage exerted effects on the receptor activity only in the presence of agonist. At intermediate concentrations of NE (10 µM) the receptor switched on and off in dependence of the membrane potential. The concentration response curve of NE-evoked α_{2A}-AR activation was shifted 2-3 fold by application of positive potential without affecting maximal receptor activation. In addition, analysis of the kinetics uncovered a correlation between agonist concentration and speed of voltage induced receptor activation. These results are in line with the hypothesis that binding of the charged agonist within the electrical field of the membrane represent a major mechanism for voltage dependence of GPCR activation. This concept is further supported by the notion that A_{2A}-adenosine receptors which bind non charged agonists did not show any voltage dependence as detected by means of a similar FRET approach. Taken together, our results demonstrates that G protein coupled receptors are regulated by the membrane potential and support the hypothesis that the underlying mechanism of voltage dependence is the influence of the electrical field on the binding of charged ligands to their receptors.

[1] Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Strasse 9, 97078 Würzburg.

23

KINETIC ANALYSIS OF THE Gq-COUPELD RECETOR SIGNALING IN LIVING CELLS

C. Hoffmann[1], P. Hein[1], U. Zabel[1], C.H. Berlot[2], M.J. Lohse[1] and M. Bünemann[1]

The activation of G protein-coupled receptors (GPCRs) is the initial step in the signaling cascade which is triggered by binding of an agonist to a GPCR. Here we report the kinetics of the initial steps in the signaling cascade of a Gq-coupled receptor. We employed the M_3-subtype of the muscarinic acetylcholine receptor (M_3-AChR) as a model receptor to study Gq-signaling. Using a fluorescence resonance energy transfer (FRET) approach based on the FlAsH-tetracysteine tag technology in combination with CFP we generated a M_3-receptor construct that reports conformational changes during receptor activation by changes in the FRET-signal. Acetylcholine or carbachol both induced rapid receptor activation with similar kinetics on a millisecond timescale. However, receptor deactivation was significantly faster with carbachol than acetylcholine. Receptor – G-Protein interaction was measured by FRET between a M_3-AChR-YFP construct and an N-terminally tagged CFP-γ_2 subunit of the heterotrimeric G-protein. The receptor – G-Protein coupling induced by carbachol was at a similar time scale as receptor activation. G-Protein activation was measured using YFP- tagged Gαq and an N-terminally tagged CFP-γ_2 subunit of the heterotrimeric G-protein. Activation of the Gq-Protein was significantly slower than receptor activation and undistinguishable for acetylcholine or carbachol.

[1] Universität Würzburg, Institut für Pharmakologie und Toxikologie, Versbacher Strasse 9, 97078 Würzburg.
[2] Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania, 17822-2623, USA

24

OPTICAL RECORDING OF DIFFERENTIAL CONFORMATIONAL CHANGES WITHIN THE 3RD INTRACELLULAR LOOP OF THE α2A-ADRENERGIC RECEPTOR FOR FULL AND PARTIAL AGONISTS IN LIVING CELLS

A. Zürn[1], U. Zabel[1], J.-P. Vilardaga[2], M.J. Lohse[1] and C. Hoffmann[1]

Stimulation of G protein-coupled receptors by an agonist leads to a conformational change and hence to a transition of the receptor into an active conformation, which can subsequently couple to a G-protein. Conformational changes have been well established to occur within the transmembrane domain (TM) III and VI and could have ramifications into the 3rd intracellular loop, which is one of the coupling regions for G-proteins. Evidence is accumulating that agonists of different efficacy could induced different changes in receptor conformations. Here we report, for the first time in living cells, differences in conformational changes in specific locations of the third intracellular loop when a receptor is stimulated with full or partial agonists. Using a

Fluorescence resonance energy transfer (FRET) based approach employing the FlAsH-tetracystein tag technology in combination with CFP (1), we were able to site-specifically label the α_{2A}- adrenergic receptor in different positions within the 3rd intracellular loop. The positions were chosen to be underneath TM VI, underneath TM V or in the middle of the 3rd intracellular loop, while CFP was fused to the C-terminus. All constructs were characterized with respect to ligand binding and no significant changes were observed when compared to the wild-type receptor. All receptors constructs were expressed at the cell surface and showed agonist induced changes in the FRET-ratio when stimulated with the full agonist norepinephrine. Significant differences were observed for a set of ligand with different efficacy for G-protein stimulation. The full agonists, dopamin and clonidine, both induced changes in the FRET signal for all three receptor constructs tested, but with significantly smaller signal amplitude than norepinephrine. The partial agonists, norphenylephrine and octopamine, only induced changes in the FRET-signal for the construct underneath TM VI, while no change was observed for the other two receptor constructs. From these data we can speculate, that full agonists, with respect to G-protein signaling, can induce conformational changes within the entire 3rd intracellular loop, while partial agonists do only induce conformational changes close to TM VI.

[1] Universität Würzburg, Institut für Pharmakologie und Toxikologie, Versbacher Strasse 9, 97078 Würzburg.
[2] Endocrine Unit and Division of Nephrology, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

(1) Hoffmann, C., Gaietta, G., Bunemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) *Nat Methods* **2**, 171-176

25

β-ADRENERGIC RECEPTOR DIMERIZATION/OLIGOMERIZATION REVISITED BY FRAP MICROSCOPY.

S. Dorsch, K.N. Klotz, M. Bünemann

Dimerization or oligomerization of G protein-coupled receptors has been intensely investigated most prominent by means of resonance energy transfer measurements. BRET measurements suggested that β_1- and β_2-adrenergic receptors constitutively form dimers, either homo- or heterodimers. As suggested by a recent study on the reliability of BRET measurements for detection of receptor dimerization other independent methods for detecting and quantifying of protein-protein interaction in living cells are needed. We therefore attempted to adjust a novel FRAP-assay to analyze receptor-receptor interaction. This method is based on determination of the lateral mobility of proteins in the plasma membrane. Upon restriction of the mobility of one interaction partner, changes of the mobility of the potential interaction partner can be quantified and used as readout for protein-protein interactions (Digby et al., PNAS, 103, 17789). In our modified two-colour FRAP approach β_1- or β_2-adrenergic receptors tagged with CFP on the C-terminus and receptors fused with YFP at the extracellular N-terminus of the receptor were coexpressed. In order to restrict mobility of only the N-terminally tagged receptors we preincubated cells with antiserum against green-fluorescent-protein (GFP) before subjecting to dual color FRAP protocols. Using this approach, we found marked differences between β_1- and β_2-adrenergic receptors. The majority of β_1-CFP receptors exhibited an unchanged mobility whereas almost all β_2-CFP receptors were severely restricted in their mobility, suggesting that β_2-adrenergic receptors indeed oligomerize in intact cells.

Universität Würzburg, Institut für Pharmakologie und Toxikologie, Versbacher Str. 9, 97078 Würzburg

26

DETERMINATION OF β_1- AND β_2-ADRENERGIC RECEPTOR CONFORMATIONAL CHANGES IN LIVING CELLS IN REAL TIME

F. Rochais[1], A. Ahles[1], C. Hoffmann[2], M. J. Lohse[2] & S. Engelhardt[1]

β_1- and β_2-adrenergic receptors (βARs) are coexpressed on several different cell types of the human body. While both receptor subtypes can exert similar effects with respect to several intracellular target proteins, they differ with respect to others. In this study, we directly determined and compared the activation characteristics of both receptor subtypes. To do so, we generated mutants of the human β_1AR (β_1AR-sensor) and of the β_2AR (β_2AR-sensor) which contain the cyan- and the yellow-emitting variants of the green fluorescent protein (CER and YFP). Using a fluorescence resonance energy transfer (FRET) approach, the βAR-sensors permit the direct monitoring of conformational changes of both receptors subtypes upon ligand binding.

Transfection of both receptor sensors in HEK293 cells showed proper expression at the cell membrane. Activation of the FRET receptors by norepinephrine lead to a symmetrical increase in CER emission and decrease in YFP emission and consequently to a decrease of the FRET ratio reaching 4% and 7% for the β_1AR-sensor and the β_2AR-sensor, respectively. Concentration response curves of β_1AR- and β_2AR-sensor activation for isoproterenol gave EC_{50} values which are in good agreement with radioligand binding studies performed with the native receptors. We are currently comparing activation and deactivation kinetics of both receptor subtypes in living cells in real time. In addition, we have generated FRET-sensors for the most frequent receptor polymorphisms, which allows us to study directly the impact of receptor polymorphisms on the response of β_1- and β_2ARs to agonists and antagonists.

[1]Rudolf Virchow Center, DFG-Research Center for Experimental Biomedicine, University of Wuerzburg
[2]Institute of Pharmacology and Toxicology, University of Wuerzburg

27

THE PSEUDO SIGNAL PEPTIDE OF THE CORTICOTROPIN-RELEASING FACTOR RECEPTOR TYPE 2A PREVENTS BETA-ARRESTIN RECRUITMENT

K. Schulz[1], C. Rutz[1], W. Rosenthal[1,2], and R. Schülein[1]

Intracellular trafficking of G protein-coupled receptors (GPCRs) starts with the insertion of the proteins into the membrane of the endoplasmic reticulum. The ER insertion of GPCRs and other membrane proteins is mediated by two different types of signal sequences. The majority of the proteins contain signal sequences forming part of the mature protein (usually transmembrane domain 1 as a so called internal "signal anchor sequence"). A smaller group possesses additional N-terminal signal peptides that are normally cleaved off during the ER insertion process. The corticotropin-releasing factor receptors (CRF_1 and $CRF_{2(a)}$ receptors) contain N-terminal signal peptides. However, in the case of the $CRF_{2(a)}$ receptor, we could show that the signal peptide is surprisingly unable to mediate ER insertion. Moreover, it is uncleaved and forms part of the mature receptor. This "pseudo signal peptide" forms a novel functional domain within the GPCR family (Rutz et al., J. Biol. Chem. 281, 24910-24921; 2006). We have now assessed the significance of the pseudo signal peptide for $CRF_{2(a)}$ receptor function using a signal peptide mutant. Trafficking studies revealed that the domain facilitates receptor trafficking through the early secretory pathway. Moreover, internalization studies demonstrated that this sequence prevents recruitment of the beta-arrestin 2 molecule following agonist stimulation. The wild-type $CRF_{2(a)}$ receptor carrying the pseudo signal peptide thus internalizes *via* an yet undefined, beta-arrestin-independent mechanism.

[1]Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin. [2]Institut für Pharmakologie, Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Thielallee 67-73, 14195 Berlin.

28

RETENTION MECHANISMS OF MUTANT VASOPRESSIN V2 RECEPTORS IN THE EARLY SECRETORY PATHWAY

S. Vogelbein[1], G. Krause[1], W. Rosenthal[1,2], and R. Schülein[1]

Transport of G protein-coupled receptors along the secretory pathway starts with the insertion of the proteins into the membrane of the endoplasmic reticulum (ER). During ER insertion, proteins are folded and correct folding is monitored by a quality control system (QCS). Correctly folded proteins are allowed to enter the vesicular transport via the ER/Golgi intermediate compartment (ERGIC) and the Golgi apparatus to the plasma membrane; misfolded forms are retained. Taking the human vasopressin V_2 receptor (V_2R) as a model, we have previously shown that misfolded V_2Rs fall into two classes. Class A mutants are retained exclusively in the ER. Class B mutants reach the ERGIC and are rerouted to the ER (Hermosilla et al., Traffic 5, 993-1005; 2004). In principle, the retention mechanism may be determined by the severity of the mutation or by the location of the mutation within the receptor molecule. We have now characterized the properties of mutant V_2Rs determining their retention mechanism (class A or class B). Using a molecular modeling and a site-directed mutagenesis strategy, we have constructed a series of V_2R mutations covering the whole receptor molecule. The mutant receptors were expressed as GFP fusion proteins in transiently transfected HEK 293 cells and located within the individual compartments of the early secretory pathway by confocal laser scanning microscopy and glycosylation state analyses. Receptors carrying mutations in the extra or intracellular domains were able to leave the ER and reach the ERGIC. In contrast, an exclusive ER retention was observed for receptors with mutations in transmembrane domains 1, 3 and 7. These results are consistent with the view that the location of the mutation within the receptor molecule determines the retention mechanism.

[1]Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin. [2]Institut für Pharmakologie, Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Thielallee 67-73, 14195 Berlin.

29

NEPHROGENIC DIABETES INSIPIDUS-CAUSING V2 VASOPRESSIN RECEPTOR MUTANTS INDUCE ENDOPLASMIC RETICULUM OVERLOAD RESPONSE

E. Grantcharova[1,2], K. Lautz[1,2], N. Hübner[3], W. Rosenthal[1,2], R. Hermosilla [1,2]

Numerous human diseases are caused by the retention of proteins by the quality control system within the cell, such as diabetes mellitus, retinitis pigmentosa, cystic fibrosis, Alzheimer's disease, α_1-antitrypsin deficiency and congenital nephrogenic diabetes insipidus (NDI). NDI is caused by mutations of the *AVPR2* gene leading to misfolded V2 vasopressin receptors (V2R) that are retained in different intracellular compartments. Retention of misfolded proteins within the cell leads not only to non-functional proteins, but also to cell stress responses like the unfolded protein response (UPR) and the endoplasmic reticulum overload response (EOR). However, the molecular mechanisms, which lead to the selective activation of the UPR and/or EOR by NDI-causing V2R mutants, are not well understood. The EOR-signalling pathway involves an activation of NF-κB and an elevation of pro-inflammatory cytokine expression. Gene expression analysis (>47,000 genes, Affymetrix technology) of several V2R mutant stable cell clones showed a significant upregulation of genes involved in UPR and additionally I-κB/NF-κB regulating genes, among others. Therefore, we further analysed NF-κB activation of several intracellulary retained NDI-causing V2R mutants, e.g.: L62P, InsQ292, R337X and T204N. In stably expressing HEK293 cells we could show that only InsQ292 and R337X mutants were able to induce a cell stress response characterized by a phosphorylation of NF-κB and its translocation into the nucleus. Furthermore, we could demonstrate *via* Western Blot analysis that also Akt is involved in this signalling pathway. These results suggest that EOR is additionally switched on to the UPR only by some NDI-causing mutants. The therapeutical implications of this pathway for NDI treatment are discussed.

This work is supported by the DFG grant HE 4486/1-1/2

[1]Institut für Pharmakologie, CBF, Charité-Universitätsmedizin Berlin, 14195 Berlin.
[2]Leibniz-institut für Molekulare Pharmakologie (FMP), 13125 Berlin.
[3]Max-Delbrück-Centrum für Molekulare Medizin (MDC), 13125 Berlin.

30

INTRACELLULARLY RETAINED V2 VASOPRESSIN RECEPTOR MUTANTS ACTIVATE DIFFERENT UPR SIGNALLING PATHWAYS.

K. Lautz[1,2], , E. Grantcharova[1,2], I. Schwieger[1,2], W. Rosenthal[1,2], B. Wiesner[2], N. Hübner[3], R. Hermosilla[1,2]

The human V2 vasopressin receptor (V2R) is predominantly expressed in the basolateral membrane of principal cells of the renal collecting ducts. Mutations in the *AVPR2* gene cause X-linked nephrogenic diabetes insipidus. Most of these disease-causing mutants are recognized as misfolded proteins by the quality control system of

the cell and are retained in the ER, the ER/Golgi Intermediate Compartment (ERGIC) or the Golgi apparatus. The retention of misfolded proteins in the cell leads to an activation of specific stress response signalling pathways, e. g. the unfolded protein response (UPR). Three different sensors of the UPR activate specific pathways, resulting in an upregulation of chaperones and, upon prolonged stress, apoptosis. These sensors are the PKR-like ER kinase (PERK), the Activating transcription factor 6 and the Inositol requiring protein 1 (IRE-1). We studied in stable expressing HEK293 cell lines the different UPR pathways triggered by the retention of GFP-tagged V2R mutants. We could show an induction of UPR by detecting an upregulation of several chaperones, like calnexin, GRP78, GRP94 and PDI, but not all of the mutants show the same expression pattern of these chaperones. To check which UPR signalling pathway is activated we analysed the induction of PERK and IRE-1, but only the PERK signalling pathway is activated in all of the mutants. In contrast to this an activation of IRE-1 could only be detected in two of the mutants. Furthermore, we studied gene expression of several V2R mutant expressing cell clones and probed up to 47,000 transcripts. We found amongst others an upregulation of genes involved in the regulation of apoptosis. Our results show that there is not just one kind of UPR and it has to be distinguished between different UPR signalling pathways.

This work is supported by the DFG grant HE 4486/1-1/2.

[1] Institut für Pharmakologie, CBF, Charité-Universitätsmedizin Berlin, 14195 Berlin.
[2] Leibniz-Institut für Molekulare Pharmakologie (FMP), 13125 Berlin.
[3] Max-Delbrück-Centrum für Molekulare Medizin (MDC), 13125 Berlin.

31

P97/VALOSIN-CONTAINING PROTEIN LEADS V2 RECEPTORS TO ER-ASSOCIATED DEGRADATION.

I. Schwieger[1,2], K. Lautz[1,2], E. Krause[2], B. Wiesner[2], W. Rosenthal[1,2], R. Hermosilla[1,2]

Two principal mechanisms of the quality control system in the cell are the unfolded protein response (UPR) that contributes to the refolding of aberrant proteins in the ER and the ER-associated degradation (ERAD), which mediates the degradation of misfolded proteins. Mutations in the human V2 vasopressin receptor (V2R) gene lead to X-linked nephrogenic diabetes insipidus. Most of them result in transport-defective V2Rs that are retained in different compartments of the early secretory pathway. L62P is retained in the ER exclusively. Y205C and V226E reach the ER-Golgi intermediate compartment (ERGIC) and G201D is transported to the Golgi apparatus. Here we show that the V2R and all investigated mutants are polyubiquitinylated in the presence of the proteasomal inhibitor MG 132. Treatment with MG 132 also stabilizes the immature receptor types of all mutants at the ER membrane. They interact with p97/valosin-containing protein (VCP), an ATPase, which acts as a chaperone to unfold proteins and extract them out of the ER membrane. Furthermore, the mutants can be co-immunoprecipitated with the 19S regulatory ATPase subunit 7 of the 26S proteasome. The 19S regulatory complex recognizes polyubiquitinylated proteins and unfold them for degradation. Our results indicate an entire degradation *via* the ERAD for mutants L62P and V226E, retained in the ER and ERGIC, respectively. Quality control in the ERGIC seems to regulate the retrograde transport to the ER. Mature receptors of the V2R and the mutants Y205C and G201D with a transport-competent folding state are able to escape this quality control process and show a co-localization with lysosomes. The ability to escape this control determines their way of degradation: immature forms are degraded *via* the ERAD and mature forms undergo lysosomal degradation.

The work is supported by the DFG grant HE 4486/1-1/2.

[1]Institut für Pharmakologie, CBF, Charité Berlin, 14195 Berlin
[2]Leibniz-Institut für Molekulare Pharmakologie, 13125 Berlin

32

CHARACTERISATION OF TWO NDI-CAUSING V2 RECEPTOR MUTANTS: ARRESTIN-DEPENDENT AND –INDEPENDET INTERNALISATION

B. Bohnekamp[1,2], K. Lautz[1,2], U. Brandt[1,2], W. Rosenthal[1,2], A. Oksche[3], R. Hermosilla[1,2]

The human V2 vasopressin receptor (V2R) belongs to the family of G protein-coupled receptors and is expressed in principal collecting duct cells. Mutations in the *AVPR2* gene cause X-linked nephrogenic diabetes insipidus (NDI). The two NDI-causing mutants V227A and F287L were tagged with the green fluorescence protein (GFP) at their C-terminus for microscopic analysis. Both mutants were expressed in HEK293 cells and could be localized at the cell surface. Plasma membrane localization was confirmed by cell surface biotinylation assays. Cell surface binding experiments were performed and mutant V277A had a five fold higher K_D-value (K_D= 22.2 ± 4.1 nM) then the wild type receptor, but the B_{Max} value (683 ± 56 fmol/mg) was similar. The K_D-value for mutant F287L.GFP was also increased (K_D = 92.3 ± 12.1 nM) and underestimated, since the curve did not reach a plateau at 100 nM AVP (B_{Max} value of 108.45 ± 10.0 fmol/mg). The results of the adenylyl-cyclase-assays (V277A.GFP, EC_{50} = 368 ± 94.2 nM; F287L.GFP, EC_{50} = 388 ± 45.3 nM) showed that EC_{50}-values are 150 fold over the wild type values. Both mutants activate the ERK1/2-signalling pathway, but less efficiently then the wild type. Next we studied the agonist-induced internalisation of both NDI-causing mutants in living cells and demonstrate that upon AVP-stimulation (1 µM) both mutants were internalised after 20 min. These results were confirmed by cell surface biotinylation assays, showing a reduction of about 50% of the receptor expression levels at the plasma membrane. Furthermore, we could show that the wild type and both mutants do recruit β-arrestin 2 to the plasma membrane after AVP-stimulation. Interestingly, mutant F287L internalises without β-arrestin 2; mutant V277A internalise with β-arrestin 2, as the wild type receptor does. Our results suggest that certain receptor conformations (F287L) lead to a β-arrestin 2-independent internalisation process of the V2R.

[1] Institut für Pharmakologie, CBF, Charité-Universitätsmedizin Berlin, 14195 Berlin
[2] Leibniz-Institut für Molekulare Pharmakologie (FMP), 13125 Berlin
[3] Mundipharma GmbH, 65549 Limburg/Lahn

33

PROFIBROTIC MECHANISMS OF CALCINEURIN INHIBITORS CSA AND FK506IN GLOMERULAR MESANGIAL CELLS

E.-S. Akool, J. Pfeilschifter, and W. Eberhardt

The calcineurin inhibitors cyclosporine (CsA) and tacrolimus (FK506) are structurally dissimilar immunosuppressive drugs that by inhibiting the cellular phosphatase calcineurin (CN) suppress T-cell activation. CN-inhibitors are among the most efficient immunosuppressive drugs and therefore are widely used in transplantation and for the treatment of many inflammatory diseases including psoriasis, and rheumatoid arthritis. However, the clinical use of both drugs is strongly limited by acute and chronic nephrotoxicity which is mainly characterised by glomerulosclerosis and tubulointerstitial fibrosis. TGF-β and downstream smad signaling pathways have been found to be the most important pathways involved in renal fibrosis via induction of profibrotic genes such as the tissue inhibitors of MMPs (TIMPs), plasmogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF). Previously, we could demonstrate that CsA specifically interfere with the transcriptional induction of cytokine-induced MMP-9. In the present work we demonstrate that the CN-inhibitors CsA and FK506 similar to TGF-β induce a rapid smad activation as indicated by smad accumulation in the nucleus. The induction is paralleled with induced DNA-binding of different smads as shown by electrophoretic mobility shift assay (EMSA) and supershift analysis. Functionally, CsA and FK506 both cause a significant increase in a SBE-driven reporter gene. Using pharmacological inhibitors and silencing RNA (si RNA) technique we found that smad phosphorylation by CN-inhibitors not only depend on TGF-β receptor but also on p38 MAPK activity. Functionally, CsA and FK506 caused amplification in the constitutive CTGF and PAI-1 expression in rat mesangial cells. Our data imply that the CN-inhibitors CsA and FK506 via p38-MAPK and smad-dependent pathways can cause a rapid induction of CTGF and PAI-1 gene expression which may play a causative role in CN-inhibitor-induced fibrosis.

pharmazentrum frankfurt/ ZAFES, Klinkum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

34

ANGIOTENSIN II TYPE 1 AND TYPE 2 RECEPTORS DIFFERENTIALLY REGULATE SPROUTING ANGIOGENESIS IN RAT VASCULAR ENDOTHELIAL CELLS

J. Carbajo, S. Lutz, T. Wieland

The vascular endothelial growth factor (VEGF) and angiotensin II (Ang II) are important regulators of angiogenesis. We recently demonstrated that Ang II-mediated signalling interferes with the VEGF-stimulated signal cascades, leading to enhanced migration of endothelial cells. The contribution in this cross regulation of the different Angiotensin II receptors AT_1R and AT_2R is not understood until now. We therefore used rat fat pad endothelial cells (RFPEC), a spontaneous immortalized cell line derived from dermal microvascular endothelial cells, to further study the influence of Ang II on VEGF-induced responses. In sprouting assays, a three-dimensional in vitro angiogenesis assay, VEGF induced an increase in sprout length of maximally 30±10% in a concentration dependent manner (0-100 ng/ml). In order to test the effect of Ang II on the basal sprout formation, cells were exposed to increasing concentrations of Ang II ($0\text{-}10^{-6}$ M). A similar increase of about 30±10% of the basal sprout length was observed at concentrations > 10^{-7} M. Co-stimulation of RFPEC with 50 ng/ml of VEGF and 10^{-8} M Ang II caused a synergistic increase of about 50±10% in sprouting. Surprisingly, in the presence of the AT_1R antagonist Telmisartan (10^{-7} M), 10^{-8} M Ang II blunted the VEGF-induced increase to 15±10% in sprout length. A complete inhibition of VEGF-induced sprouting was obtained by co-application of the specific AT_2R agonist CGP-42112A (10^{-8} M). The inhibitory effect of CGP-42112A was reversed by the specific AT_2R antagonist PD123,319 (10^{-7} M). VEGF-induced sprouting requires the activation of the monomeric GTPase RhoA. Therefore, we quantified activation of RhoA in RFPEC by the G-Lisa assay. VEGF induced an about 3-fold activation of RhoA which was completely suppressed by CGP-42112A. We conclude, that AT_1R and AT_2R differentially regulate angiogenic sprouting in RFPEC. Whereas AT_1R stimulation exerts pro-angiogenic effects with a similar magnitude than VEGF, AT_2R inhibit sprouting by interfering with VEGF-incuced RhoA activation.

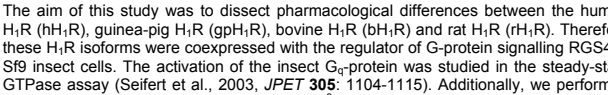
Institut für Experimentelle und Klinische Pharmakologie und Toxikologie,
Universität Heidelberg, Maybachstr.14, D-68169 Mannheim, Germany.

35

THE HISTAMINE H_1-RECEPTOR: MOLECULAR ANALYSIS OF FOUR H_1-RECEPTOR SPECIES ISOFORMS

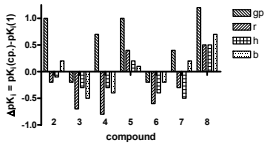
A. Straßer[1], H.-J. Wittmann[2], S. Elz[1], R. Seifert[3]

Histamine H_1-receptor (H_1R) agonists are important tools to analyse differences in species isoforms of the H_1R at a molecular level. Histaprodifen (**1**) and its derivatives, like suprahistaprodifen (**2**) (R_1=H, R_2=H), are very potent and selective H_1R agonists.



The aim of this study was to dissect pharmacological differences between the human H_1R (hH_1R), guinea-pig H_1R (gpH_1R), bovine H_1R (bH_1R) and rat H_1R (rH_1R). Therefore these H_1R isoforms were coexpressed with the regulator of G-protein signalling RGS4 in Sf9 insect cells. The activation of the insect G_q-protein was studied in the steady-state GTPase assay (Seifert et al., 2003, *JPET* **305**: 1104-1115). Additionally, we performed competition and kinetic binding assays with [^{3}H]mepyramine. With these three types of pharmacological assays we found differences between gpH_1R on the one hand and hH_1R, bH_1R and rH_1R on the other hand, as can be seen for the competition binding assay in the next figure.

	compound	
1	Histaprodifen	
2	H	H
3	CH_3 (S)	H
4	CH_3 (R)	H
5	H	Phenyl
6	CH_3 (S)	Phenyl
7	CH_3 (R)	Phenyl
8	Dimeric Histaprodifen	



The project is supported by the Deutsche Forschungsgemeinschaft (GRK 760)
[1] Department of Pharmaceutical and Medicinal Chemistry I, University of Regensburg
[2] EDV-Referent of the Faculty Chemistry and Pharmacy, University of Regensburg
[3] Department of Pharmacology and Toxicology, University of Regensburg

36

A SCREENING APPROACH TO IDENTIFY PROTEINS INTERACTING WITH MINERALOCORTICOID RECEPTOR AND LIGAND DEPENDENT CHANGES THEREOF

M. Weber, M. Wehling and R.Lösel

In an effort to search for proteins interacting with the mineralocorticoid receptor, several constructs with different affinity tags at both N and C terminus were designed to overcome the limitations of inherently low expression and antibodies unsatisfactory for IP. All constructs were functional yielding aldosterone dependent activation of a MMTV reporter gene construct, but only one tag gave satisfactory yields. After cell lysis and immunoprecipitation, interacting proteins were separated by 1D and 2D electrophoresis, digested by trypsin and identified by MALDI MS (peptide mass fingerprinting). As expected, MR isolated from the cytosol of cells in the absence of steroids yielded several interacting heat shock protein species (Hsp90 and Hsp70 proteins, GroEL family members) that were reduced in the presence of aldosterone at various time points. Additional proteins and cofactors of known or potential significance have been identified. Surprisingly, a number of metabolic enzymes, including dehydrogenases, were found to interact with both the N- terminal and the C-terminal fused constructs, thus suggesting a specific phenomenon. Even more surprising, some enzymes seem to interact depending on the kind of steroid bound to MR (aldosterone, spironolactone, cortisol, none), thus discriminating between different steroids. As MR normally is present at low concentration, i.e. considerably lower than most metabolic enzymes, it is very unlikely to modulate their function or activity. On the other hand, the question arises whether the interaction with those enzymes could serves as some kind of coupling of receptor function to cellular metabolism, or whether the interaction has no or little function.

Abteilung für klinische Pharmakologie, Institut für experimentelle und klinische Pharmakologie, Medizinische Fakultät Mannheim der Universität Heidelberg, Mannheim.

37

MECHANISM OF NICOTINIC ACID-INDUCED FLUSHING IN MICE

A. Gille, Z. Benyó and S. Offermanns

The use of nicotinic acid as an anti-dyslipidemic drug has been affected by a flushing phenomena, which decreases the patient-compliance. The flush is mediated by the by the nicotinic acid receptor as demonstrated by the absence of this phenomenon in GPR109Adeficient mice. Flushing has been shown to be accompanied by a cutaneous formation of vasodilatory prostanoids. Transplantation of wildtype bone-marrow onto irradiated nicotinic acid receptor deficient mice was able to rescue the flushing response. However the cellular mechanism of the flushing response is still unclear. We depleted mice of macrophages ($CD11b^+$), dendritic cells ($CD11c^+$) or Langerhans cells ($langerin^+$) using selective expression of the diphteria toxin receptor. Depletion of Langerhans cells but not of macrophages or dendritic cells abrogated the flushing response in mice. Langerhans cells, which respond to nicotinic acid with an increase of intracellular calcium express prostaglandin D_2 and E_2 synthases. These data indicate that Langerhans cells but not macrophages or dendritic cells are necessary for the nicotinic acid induced flushing response. Indeed, Langerhans cells and keratinozytes have been shown to express GPR109A. Further studies are nessecary to determine whether Langerhans cells are sufficient to mediate the nicotinic acid induced flush. Our data indicate that Langerhans cells besides their immunological role are involved in the regulation of dermal blood flow.

Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg

38

A MOLECULAR BASIS OF ANALGESIC TOLERANCE TO CANNABINOIDS

A. Tappe-Theodor[1], N. Agarwal[1], I. Katona[2,] T. Rubino[3], J. Swiercz[1], K. Mackie[4], H. Monyer[5], D. Parolaro[3], J. Whistler[6], T. Kuner[7], R. Kuner[1]

Clinical usage of cannabinoids in chronic pain states is limited by their central side effects and the pharmacodynamic tolerance which sets in after repeated dosage. Analgesic tolerance to cannabinoids in vivo could be caused by agonist-induced down-regulation and intracellular trafficking of cannabinoid receptors, but little is known about the molecular mechanisms involved. We show here that the type 1 cannabinoid receptor (CB1) interacts physically with G-protein-associated sorting protein1 (GASP1), a protein which sorts receptors in lysosomal compartments destined for degradation. CB1-GASP1 interaction was observed to be required for agonist-induced down-regulation of CB1 in spinal neurons ex vivo as well as in vivo. Importantly, uncoupling CB1 from GASP1 in mice in vivo abrogated tolerance towards cannabinoid-induced analgesia. These results suggest that GASP1 is a key regulator of the fate of CB1 following agonist exposure in the nervous system and critically determines analgesic tolerance to cannabinoids.

[1]Pharmacology Institute, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany; [2]Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony utca 43, 1083 Budapest, Hungary; [3]DBSF, Pharmacology Section and Neuroscience Center, University of Insubria, Busto Arsizio, Italy; [4]Department of Anesthesiology, University of Washington School of Medicine, Seattle, WA 98195-6540, USA; [5]Department of Clinical Neurobiology, Interdisciplinary Center for Neuroscience, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany; [6]Ernest Gallo Clinic and Research Center, University of California, San Francisco, CA 94608, USA; [7]Department of Anatomy & Cell Biology, University of Heidelberg, Im Neuenheimer Feld 307, 69120 Heidelberg, Germany

39

ROLE OF PHOSPHOLIPASE D2 IN MU-OPIOID RECEPTOR-STIMULATED SYNTHESIS OF REACTIVE OXYGEN SPECIES (ROS)

T. Koch, D.F. Wu, E. Kahl, and V. Höllt

We have recently shown that the mu-opioid receptor (MOR1) is associated with the phospholipase D2 (PLD2), a phospholipid-specific phosphodiesterase located in the plasma membrane. We further demonstrated that in HEK293 cells coexpressing MOR1 and PLD2, treatment with DAMGO led to an increase in PLD2 activity and an induction of receptor endocytosis, whereas morphine, which does not induce opioid receptor endocytosis failed to activate PLD2. We report here that mu-opioid receptor-mediated activation of PLD2 stimulates production of reactive oxygen molecules, primarily H_2O_2, via NADH/NADPH oxidase. Oxidative stress was measured with the fluorescent probe dichlorodihydrofluorescein diacetate (DCFDA) and the role of PLD2 was assessed by the PLD inhibitors ethanol and D-erythro-sphingosine (sphinganine). To determine whether NADH/NADPH oxidase contributes to production of reactive oxygen species, mu-agonist stimulated cells were pretreated with the specific flavoprotein inhibitor diphenylene iodinium (DPI). We found that receptor-internalizing agonists (like DAMGO and ß-endorphine) strongly induce ROS-synthesis via PLD-dependent signaling pathways, whereas agonists that do not induce mu-opioid receptor endocytosis and PLD2-activation (like morphine and buprenorphine) failed to activate ROS-synthesis in transfected HEK293 cells.

Department of Pharmacology and Toxicology, Otto-von-Guericke University, 39120 Magdeburg

40

MEMBRANE GLYCOPROTEIN M6A IS A REGULATORY PROTEIN OF μ-OPIOID RECEPTOR INTERNALIZATION AND INTRACELLULAR TRAFFICKING

D.F. Wu[1], Y.J. Liang[1], R. Stumm[1], C. Scholze[2], V. Höllt[1] and T. Koch[1]

The μ-opioid receptor (MOPr) mediates the analgesic effects of opioids but is also important for initiating adaptive changes in the nervous system causing opioid tolerance, dependence and addiction. In the present study, MOPr was identified to be associated with the neuronal membrane glycoprotein M6a, a member of the proteolipid protein (PLP) family. After coexpression in HEK293 cells, M6a cointernalizes and then corecycles with MOPr to cell surface via recycling endosomes, and augments opioid receptor trafficking. As a consequence, M6a-enhanced MOPr trafficking results in decreased receptor desensitization and reduced opioid tolerance. Overexpression of a M6a negative mutant prevents μ-opioid receptor endocytosis demonstrating an essential role of M6a in MOPr trafficking. The finding that M6a and MOPr are widely coexpressed in rat brain together with primary neuronal culture data implicates a physiological role of M6a for MOPr trafficking and signaling.

[1]Department of Pharmacology and Toxicology, Otto-von-Guericke University, 39120 Magdeburg, Germany
[2]Institute of Experimental Internal Medicine, 39120 Magdeburg, Germany

41

PHOSPHORYLATION OF SERINE 363 OF THE DELTA OPIOID RECEPTOR (DOP) DETECTED BY A PHOSPHOSPECIFIC ANTIBODY

M.Pfeiffer, S.Schulz, R.Stumm, S.Jerabeck and V.Höllt

Using an antibody that selectively recognizes the Ser^{363}-phosphorylated form of mouse DOP we examined the agonist-dependent phosphorylation of the receptor in stably transfected HEK293 cells or transiently transfected primary neuronal cells. Ser^{363}-phosphorylation of receptors were not seen in untreated cells. However when DOP-expressing cells were incubated with agonists and subjected to Western blot analysis Ser^{363}-phosphorylated receptors were detectable within 2 min. Using confocal microscopy a similarly rapid Ser^{363} phosphorylation of the receptor after exposure to the peptide agonist [D-$Pen^{2,5}$]enkephalin (DPDPE) or to the alkaloid agonist etorphine was observed. Within 2 min Ser^{363}-phosphorylated receptors were first detectable at the plasma membrane. Following endocytosis the majority of DOPs were confined to perinuclear clusters of vesicles after 30 min exposure to either substance. After removal of etorphine, Ser^{363}-phosphorylated receptors were rapidly dephosphorylated and recycled to the plasma membrane within 60 min. In contrast, in cells exposed to DPDPE Ser^{363}-phosphorylated receptors persist in vesicle-like structures within the cytoplasm for prolonged periods (up to 120 min) before undergoing dephosphorylation and recycling back to the membrane. It is assumed that opioid agonists can direct internalization of DOP in distinct vesicles which differ in the rate of DOP dephosphorylation and that dephosphorylation is required for recycling. Although phosphorylation of DOP has been shown to be a crucical step for DOP endocytosis, we surprinsingly found that buprenorphine, a mixed opioid receptor agonist-antagonist, induced Ser^{363} phosphorylation but no internalization of DOP.

Dept. of Pharmacology and Toxicology, University Magdeburg, Germany

42

PHOSPORYLATION OF THE IMMUNOMODULATOR FTY720 PROTECTS FIBROBLASTS FROM APOPTOSIS VIA THE $S1P_3$ RECEPTOR SUBTYPE

H. Potteck., C. Braem., M. Schäfer Korting, B. Kleuser

The immunmodulator FTY720 has been indicated to induce apoptosis in a variety of cells. Here we show that phosphorylation of FTY720 to its metabolite FTY720-phosphate (FTY720P) possesses antiapoptotic effects in human fibroblasts. Apoptosis was induced by TNFα and actinomycine D and was inhibited by preincubation with FTY720P in a dose dependant manner. This action is specific for the phosphorylated compound as FTY720 did not protects fibroblasts from apoptosis. FTY720, after phosphorylation, is a structural analogue of sphingosine 1-

phosphate (S1P) and therefore acts as a high-affinity agonist at four of the five G-protein-coupled S1P-receptors. It was of interest, whether the FTY720P-induced protection of human fibroblasts is mediated via S1P-receptors. It has been suggested that the $S1P_3$ is the crucial receptor subtype involved in the antiapoptotic property of S1P. Indeed, measurement of apoptosis in fibroblasts isolated from $S1P_3$-deficient mice almost completely abrogated the ability of FTY720P to protect cells from apoptosis. This indicates that FTY720P mediates its cell protective effects via this receptor subtype. To further examine the intracellular molecular mechanism, we identified phosphatidylinositol-3-kinase (PI3K) in the protective ability of FTY720P. Cell protective actions of FTY720P were abolished by inhibition of PI3K. In analogy, stimulation of fibroblasts from wildtype mice with FTY720P resulted in an activation of PI3K, whereas this pathway was not activated after treatment with FTY720P in cells isolated from S1P3-deficent mice.
This data indicate that FTY720P exerts its ability to protect fibroblasts from undergoing apoptosis via the $S1P_3$ receptor subtype followed by an activation of the PI3K pathway.
Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, D-14195 Berlin

43

DETERMINATION OF THE ASSEMBLY STATE OF $P2X_1$ RECEPTOR HETEROLOGOUSLY EXPRESSED IN PICHIA PASTORIS

T. Eisele and G. Schmalzing

P2X receptors are ligand-gated cation channels that open an intrinsic cation-selective channel in response to their physiological ligand, extracellular ATP. P2X receptors are expressed by a large variety of cells throughout the body and play crucial roles in many cellular and physiological processes. Biochemical experiments have shown that functional P2X receptor subtypes assemble as homotrimers or heterotrimers from a repertoire of seven subunit isoforms designated $P2X_1$ to $P2X_7$ (Aschrafi et al. 2004). Further structural information has been inferred so far from site-directed mutagenesis experiments. High resolution structures from X-ray diffraction or NMR are not available.In this study, we addressed the question whether the methylotrophic yeast *Pichia pastoris* might represent a suitable host for the production of large amounts of $P2X_1$ receptors. To this end, we generated a *P. pastoris* cell line stably expressing the hexahistidine-tagged rat $P2X_1$ subunit under the control of the methanol-inducible alcohol oxidase 1 promotor. The $P2X_1$ protein was purified from detergent extracts of these cells by one-step Ni^{2+} NTA affinity chromatography and resolved by polyacrylamide gel electrophoresis (PAGE), followed by immunodetection with a $P2X_1$ subunit-specific antibody. n-Dodecyl ß-D-maltoside showed the best solubilization properties among a number of detergent examined. The *P. pastoris*-expressed $P2X_1$ subunit was found to migrate as polypeptides of 47-52 kDa carrying 1-3 high-mannose type N-linked glycans. To determine their oligomeric state, $P2X_1$ subunits were resolved by blue native PAGE, which has the capacity to correctly display the quaternary state of receptor channels and transporters. The *P. pastoris*-expressed $P2X_1$ receptor showed a very low electrophoretic mobility indicative of aggregates. In contrast, the $P2X_1$ receptor purified from *X. laevis* oocytes migrated as a defined homotrimer under otherwise identical conditions. Taken together the results show that the $P2X_1$ subunit is synthesized as an N-glycosylated polypeptide of the expected mass in *P. pastoris*. However, in contrast to other expression systems such as *X. laevis* oocytes or HEK293, *P. pastoris* does not support the ready formation of properly defined homotrimeric $P2X_1$ receptors.
Molecular Pharmacology, RWTH Aachen University, 52074 Aachen.

44

ROLE OF TRANSMEMBRANE DOMAINS 1 AND 2 IN HOMO-TRIMERIZATION OF HUMAN $P2X_5$ SUBUNITS

W. Duckwitz, R. Hausmann, and G. Schmalzing

P2X receptors are ATP-gated cation channels that mediate fast signaling in a large variety of cells. Seven subunits have been cloned, designated $P2X_1$-$P2X_7$, which share a common topology with cytosolic N-and C-terminal domains and two transmembrane regions (TM1 and TM2), connected by a large N-glycosylated extracellular loop. Functional P2X receptors have been demonstrated to display a characteristic trimeric architecture of three identical or homologous subunits (Nicke et al. EMBO J 1998; Aschrafi et al. J Mol Biol 2004), which are thought to arrange circularly to form a central cation-conducting pore. By systematic scanning mutagenesis of the $P2X_5$ subunit, we have further shown recently that TM2 plays an indispensable scaffolding role in homotrimerization without providing much specific subunit recognition information (Duckwitz et al. JBC 2006). The sole exception was the only charged TM2 residue, ^{355}D, which contributed in an unclear manner to trimer formation efficiency. Here we studied the role of TM2-embedded ^{355}D in subunit homotrimerization in greater detail. To unequivocally demonstrate that of all TM2 residues solely ^{355}D contains specific assembly information, the TM2 domain (residues 337-356) was replaced by a total of 20 consecutive leucine residues. While the polyleucine $P2X_5$ mutant homotrimerized only weakly, efficient homotrimerization was restored when ^{355}D was introduced into the polyleucine stretch. To examine whether TM2-embedded ^{355}D supports homotrimerization by driving self-association of transmembrane helices through the formation of interhelical hydrogen bonds, we mutated ^{355}D to a series of other amino acids. Replacing ^{355}D by a hydrophobic residue (V, L or F) impaired homotrimerization, while replacing ^{355}D by residues capable of forming intramembrane hydrogen bonds (E, R, N or W) supported efficient homotrimerization. Replacing TM1-embedded polar residues ^{33}Y, ^{34}R or ^{37}Q by L did not impair homotrimerization efficiency. This suggests that hydrogen bond formation occurs only between TM2 domains, and not between TM1 and TM2. We conclude from these results that ^{355}D contributes to homotrimerization by forming homomeric hydrogen bonds between TM2 domains.
Department of Molecular Pharmacology, RWTH Aachen University , 52074 Aachen

45

GENERATION OF AN INDUCIBLE STABLE CELL LINE EXPRESSING A TANDEM AFFINITY PURIFICATION-TAGGED HUMAN $P2X_7$ RECEPTOR

J. Eick[1], H. Schuchlautz[2], R. Woltersdorf[3], F. Markwardt[3], D. W. Litchfield[4], B. Lüscher[2] and G. Schmalzing[1]

$P2X_7$ receptors function as trimeric ATP-gated cation channels in epithelial and antigen-presenting cells, where they exert immunomodulatory functions. A special feature of $P2X_7$ receptors is that prolonged ATP stimulation promotes the formation of pores, which allow the entry of organic cations of up to 900 Da into the cell, ultimately leading to necrosis or apoptosis. The mechanism how the pores are formed is under debate. Data have been presented showing that $P2X_7$ receptors undergo 'pore dilatation' upon sustained stimulation, that is, a progressive increase of the diameter of the initially small cation-selective pore to accommodate larger and larger molecules. In contrast, single $P2X_7$ channel measurements clearly indicate that the $P2X_7$ channel diameter stays constant during sustained activation, suggesting that the macroscopically observable permeability increase must involve accessory proteins (Riedel et al. Biophys J 2007).
Here we attempted to capture proteins interacting with the $P2X_7$ receptor under close-to-physiological conditions for subsequent identification by mass spectrometry. To this end, we generated a tetracycline-inducible HEK293 cell line, which stably expresses a fusion protein consisting of the TAP tag (protein A linked by a TEV protease cleavage site to the calmodulin binding peptide) fused in frame to the N-terminus of the complete human $P2X_7$ subunit. Resolution of non-denatured digitonin extracts of HEK293 cells expressing the TAP-tagged $P2X_7$ receptor (TAP-$P2X_7$R) on a blue native PAGE gel, followed by immunodetection, showed that the TAP-$P2X_7$R existed entirely as a correctly assembled trimeric protein. The fusion protein and bound accessory proteins were recovered from n-dodecyl ß-D-maltoside extracts of ~5 x 10^8 HEK293 cells by two sequential purification steps. SDS-PAGE combined with Coomassie staining visualized >10 co-purified polypeptides in addition to the prominent TAP-$P2X_7$R band. The identity and the potential functional relevance of the associated proteins will be discussed.
[1]Molecular Pharmacology and [2]Biochemistry and Molecular Biology, RWTH Aachen University, 52074 Aachen; [3]Julius-Bernstein-Institute for Physiology, Martin Luther University, 06097 Halle/Saale; [4]Department of Biochemistry, University of Western Ontario, London, Canada.

46

RECEPTOR/G PROTEIN INTERPLAY CONTRIBUTES TO THE PHARMACOLOGIC QUALITY OF LIGANDS AT THE M_3 MUSCARINIC ACETYLCHOLINE RECEPTOR

D. Thor, A. Schulz, and T. Schöneberg

Upon agonist binding at G protein-coupled receptors (GPCR) conformational changes involving the transmembrane helices lead to G-protein activation. However, helix movement is also observed following binding of inverse agonists. Further, minor mutational changes in GPCR have been shown to convert the pharmacological ligand quality of inverse agonists into agonists. These facts implicate a fine-tuned receptor/G protein interplay which distinguishes between different ligand-induced structural changes but the molecular basis of gating such conformational stages is unclear. We took advantage of a genetically modified yeast strain which allows the efficient screening of large libraries of mutant M_3 muscarinic acetylcholine receptors (M_3R) to identify and study mutants which can be activated by atropine, an inverse agonist on wild type M_3R. Screening of more than 10 million clones led to the identification of 13 atropine-sensitive M_3R mutants. All atropine-sensitizing mutations were located in transition of the intracellular loop 3 and helix 6. Interestingly, the pharmacological switch was not only seen for atropine but for all other M_3R blockers investigated. It is therefore likely that the M_3R and probably other GPCR contain gate keeping structures which restrict activating conformational changes to the proper agonist(s). Mutations can unlock such constraints allowing ligands, regardless of their chemical structure, to act as agonists. However, the atropine sensitivity of all mutants was restricted to the yeast expression system and could not be transferred to mammalian expression systems despite retained high affinity ligand binding. Therefore, we interpret our findings that proper receptor/G protein interplay significantly contributes to the pharmacological quality of a ligand.
University of Leipzig, Medical Faculty, Institute of Biochemistry, Molecular Biochemistry, Johannisallee 30, 04103 Leipzig, Germany

47

REPLACING THE ALLOSTERIC CORE EPITOPE ^{422}TRYPTOPHAN BY ALANINE AT MUSCARINIC M_2 RECEPTORS DOES NOT AFFECT BINDING OF THE ATYPICAL ALLOSTERIC MODULATOR DUO3

Linsel, N.[1], Mohr-Andrä, M.[1], Schrobang, J.[2], Höltje, H.-D.[2], Mohr, K.[1]

The atypical muscarinic allosteric modulator Duo3 (4,4´-bis[(2,6-dichloro-benzyloxy-imino)-methyl]-1,1´-propane-1,3-diylbis-pyridinium-dibromide) is distinguished from common allosteric agents by particular characteristics such as steep concentration effect curves. Docking simulations in a bovine rhodopsin based 3-dimensional model of the M_2 receptor in which the orthosteric site is blocked by the antagonist [^{3}H]N-methylscopolamin ([^{3}H]NMS) suggested that Duo3 does not enter the core binding region of the allosteric site [1]. $M_2{}^{422}$tryptophan represents an epitope that is conserved among the muscarinic receptor subtypes and that is part of the critical cluster of amino acids forming the M_2 receptor's allosteric core region [2]. We checked whether the allosteric action of Duo3 depends on $M_2{}^{422}$Trp. Binding experiments using [^{3}H]NMS as an orthosteric agent were conducted to determine the affinity of the test compound for [^{3}H]NMS-bound receptors. Experiments were performed with mutant receptors where $M_2{}^{422}$Trp was replaced by alanine. The human wild type M_2 receptors and the $M_2{}^{422}$Trp→Ala mutant receptors were expressed in COS7 cells.
The affinity of the allosteric ligand is reflected by the modulator concentration that inhibits [^{3}H]NMS dissociation half-maximally:

Duo3	M_2 wild type	$M_2{}^{422}$Trp→Ala
$pEC_{0.5diss}$	7.17 ± 0.03	6.99 ± 0.03
Hill slope n_H	-1.50 ± 0.17	-1.59 ± 0.12

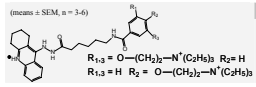
These results show a loss of affinity at mutant receptors by only 0.18 decades.
In accordance with the 3D receptor model we conclude that binding affinity of Duo3 is not affected by replacing $M_2{}^{422}$tryptophan by alanine.
[1] Tränkle et al. (2005) *Mol Pharmacol* 68: 1597-1610
[2] Prilla et al. (2006) *Mol Pharmacol* 70:181-193
[1]Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Str. 3, Germany;
[2]Institute of Pharmaceutical and Medicinal Chemistry, University of Düsseldorf, Universitätsstr. 1, Germany

48

ALLOSTERIC MODULATION IN MUSCARINIC M_2 RECEPTORS: LINKAGE OF TWO ALLOSTERIC INHIBITOR RELATED MOIETIES YIELDS AN ALLOSTERIC ENHANCER

Cieslik J., *Elsinghorst P.W., *Gütschow M., Mohr K., Tränkle C.

The atypical allosteric action of tacrine in muscarinic M_2-receptors can be switched to a typical behaviour by dimerisation of the molecule; the allosteric potency of the dimer was increased whereas the cooperativity with [^{3}H]N-methylscopolamine ([^{3}H]NMS) remained strongly negative (1). Here, we tested the hybrid compounds Gü1178 and Gü1182 (cf. Table1) in which the atypical tacrine is attached to moieties related to the typical modulator gallamine. Binding experiments applying the conventional ligand [^{3}H]NMS were carried out in



modulator	$\log K_X$	$\log\alpha$	$-\log EC_{50,diss}$
gallamine	8.15±0.33	-1.38±0.11	6.94±0.09
tacrine	6.29±0.02	-1.72±0.09	5.20±0.03
Gü1178	8.21±0.17	-0.29±0.06	9.07±0.09
Gü1182	8.71±0.51	0.27±0.08	8.88±0.06

porcine heart homogenates (4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4, 23 °C). The hybrids showed log equilibrium binding affinity constants K_X in free M_2 receptors similar to gallamine (cf. Table 1). In contrast to the strong negative cooperative interaction of either gallamine or tacrine with [^{3}H]NMS equilibrium binding, Gü1178 showed a weak negative and Gü1182 even a positive cooperativity as reflected by larger log cooperativity factors α. The –log equilibrium dissociation constants $EC_{50,diss}$ of the hybrids indicating half maximal binding to NMS-occupied M_2-receptors were both larger than those of tacrine or gallamine, respectively. The curves slopes characterizing the allosteric effects on [^{3}H]NMS equilibrium binding and dissociation were not different from unity suggesting a typical behaviour of the hybrids. In conclusion, linking moieties originating from an atypical and a typical modulator yielded new hybrid modulators of high allosteric potency, typical behaviour and weakly negative or even positive cooperativity with a conventional radioligand. The findings support the concept that the allosteric binding area in M_2-receptors may accommodate two small molecules simultaneously.

(1) Tränkle et al. (2005) Mol Pharmacol 68:1597-1610

Pharmacology & Toxicology and *Pharmaceutical Chemistry, Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Str. 3, 53121 Bonn, Germany.

49

MUSCARINIC RECEPTORS: THE ALLOSTERIC/ORTHOSTERIC EPITOPE M_2^{422}TRYPTOPHAN MEDIATES STRONG NEGATIVE COOPERATIVITY BETWEEN AN ALLOSTERIC ANTAGONIST AND ACETYLCHOLINE

Prilla S., Antony J., Tränkle C., and Mohr K.

The muscarinic allosteric site contains a core region with the conserved tryptophan M_2^{422}W as a key component [1]. Here we show that M_2^{422}W is also important for the orthosteric agonist acetylcholine (ACh) whereas the antagonist N-methylscopolamine (NMS) does not utilize this residue. Allosteric modulators often strongly reduce agonist but not antagonist (NMS) binding [2]. We hypothesized that the pronounced negative cooperativity might result from an allosteric/orthosteric competition for this tryptophan. We studied the interaction between ACh and the allosteric antagonist naphmethonium (Naphm) [3] in experiments with [^{3}H]NMS as a probe using membranes from CHO cells expressing either M_2wt or the receptor mutant M_2^{422}W→A (10mM HEPES, 10mM $MgCl_2$, 100mM NaCl, 10µM GDP, pH 7.4 at 30°C). ACh binding curves were recorded with or without a fixed concentration (10xK_D) of Naphm. Curve shifts were used to estimate the minus log factor of cooperativity (pα).

	Apparent affinity (pK_{app})			Apparent cooperativity (pα)	
	ACh	NMS	Naphm	NMS / Naphm	ACh / Naphm
M_2 wt	6.36 ± 0.11	9.44 (9.44; 9.43)	7.69 ± 0.14	0.43 ± 0.08	< -2
M_2^{422}W→A	5.12 ± 0.17	9.44 (9.47; 9.40)	5.91 ± 0.18	-0.15 ± 0.01	-0.96 ± 0.16
Δ	1.24	0	1.78	0.58	> 1

pK_{app} (means ± S.E., n = 3-6) reflect apparent affinities of the indicated ligands for the respective receptor. Replacement by alanine (A) of M_2^{422}W led to a pronounced reduction of negative cooperativity between Naphm and ACh. In contrast, cooperativity between Naphm and NMS was affected only to a minor degree and in the opposite direction from positive to negative. We conclude that M_2^{422}W is a "cooperativity switch" especially for the interaction of allosteric antagonists and orthosteric agonists. Allosteric enhancers of ACh binding should not depend on M_2^{422}W as a binding epitope.

[1] Prilla et al. (2006) Mol Pharmacol 70:181-196; [2] Grossmüller et al. (2005) Naunyn Schmiedebergs Arch Pharmacol 372:267-276; [3] Muth et al. (2005) J Med Chem 48:2212-2217

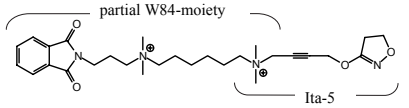
Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, 53121 Bonn

50

COOPERATIVE INTERACTION BETWEEN ATROPINE AND A MUSCARINIC M_2 RECEPTOR SELECTIVE ALLOSTERIC/ORTHOSTERIC HYBRID AGONIST

Kellershohn K.[1], Muth M.[2], Holzgrabe U.[2]; De Amici M.[3] and Mohr K.[1]

Besides the orthosteric acetylcholine binding site, muscarinic receptors exhibit an allosteric binding site which is located at the entrance of the ligand binding pocket. Usually compounds that bind to the allosteric site exhibit a higher subtype selectivity, because this site is less conserved than the orthosteric ligand binding site. Ita-5 is an oxotremorine-M related highly potent nonselective muscarinic agonist, whereas W84 is an M_2 selective allosteric antagonist. Covalent linkage of the essential portion of W84 with Ita-5 yields a hybrid compound with M_2 selective agonist properties.



In order to probe the hypothetical bitopical allosteric/orthosteric binding mode of the hybrid compound, we investigated its interaction with the orthosteric antagonist atropine. Hybrid-induced M_2 receptor-mediated G protein activation was measured using a [^{35}S]GTPγS binding assay in membranes of CHO cells stably expressing human M_2 receptors. Atropine shifted the hybrid agonist curve to the right in a saturable fashion and reduced the hybrid's efficacy concentration-dependently. These findings indicate a negative cooperativity between the hybrid and atropine with regard to both, receptor binding and receptor activation. It is concluded that atropine hinders hybrid binding to the orthosteric site and thereby receptor activation by the hybrid, whereas hybrid binding to the allosteric site remains possible thus allowing an allosteric action on atropine binding.

[1]Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, 53121 Bonn, Germany, [2]Pharmaceutical Chemistry, Institute of Pharmacy and Food Chemistry, University of Würzburg, 97074 Würzburg, Germany, [3]Institute of Medicinal and Toxicological Chemistry, University of Milano, 20131 Milano, Italy

51

DIFFERENTIAL EFFECTS OF RGS-4 AND RGS-19 ON HISTAMINE $H_4R/G\alpha_{i2}$ COUPLING

Schneider, E., Seefeld, A., Seifert, R.

The human histamine H_4 receptor (H_4R) is a G-protein-coupled receptor that interacts preferentially with G_i-proteins, resulting in adenylyl cyclase inhibition and phospholipase C activation. The (patho)physiological role of the H_4R is far from being understood and has yet to be elucidated using receptor-specific ligands as pharmacological tools. Several publications suggest that the H_4R is involved in the regulation of immunological processes including eosinophil migration and inflammation. For the functional characterization of new H_4R ligands we use the steady-state GTPase assay with membranes from baculovirus-infected Sf9 insect cells that co-express the H_4R with components of the intracellular signaling machinery ($G\alpha_{i2}$ and $G\beta_1\gamma_2$). In this system, the H_4R exhibits high constitutive activity, resulting in a small agonist signal (maximum stimulation just 25-30% above basal). In an effort to improve the signal intensity, we additionally expressed RGS-4 and RGS-19, two regulators of G-protein signaling (RGS proteins), which stimulate the GTPase activity of the $G\alpha_{i2}$ subunit. The RGS-proteins were co-expressed with the hH_4R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$. Moreover, we fused the N-terminus of RGS4 or RGS-19 to the C-terminus of the hH_4R and co-expressed the resulting fusion proteins with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. The largest agonist stimulation relative to the baseline was found for the hH_4R-RGS-19 fusion protein co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. The pharmacological properties of the hH_4R-RGS-19 fusion protein were comparable to those of the non-fused receptor. Interestingly, compared to RGS-4, RGS-19 increased only the histamine-stimulated GTPase activity, whereas RGS-4 enhanced both the basal and the agonist-induced signal. Differences in the effect of RGS4 and RGS-19 were also found in kinetic studies, determining K_m and V_{max} of the Gα GTPase activity. Our data suggest that the investigated RGS proteins do not only interact with the Gα subunit, but also with the H_4R protein in a manner dependent on the activation state of the receptor.

Department of Pharmacology and Toxicology, University of Regensburg, D-93040 Regensburg

52

MOLECULAR ANALYSIS OF HUMAN HISTAMINE H_3 RECEPTOR USING THE BACULOVIRUS/SF9 CELL SYSTEM

D. Schnell, K. Burleigh, R. Seifert

Our challenge was the fact, that there has been no uniform test system to compare all histamine receptor subtypes. Baculovirus/Sf9 cell systems have been developed to characterize the H_1-, H_2- and, most recently, the H_4 receptor. We generated a baculovirus containing the open reading frame of the human histamine H_3 receptor (hH_3R) cDNA. The receptor sequence was tagged with a N-terminal FLAG epitope, to allow immunological detection of the corresponding protein via the M1-antibody, and a C-terminal hexahistidine tag, e. g. for further purification steps or as a linker in fusion proteins between the receptor and coupling G-proteins. We produced membranes of Sf9 cells expressing the hH_3R in combination with $G\alpha_{i2}$ or $G\alpha_o$ and $G\beta_1\gamma_2$. Additionally, we co-expressed the receptor and G-protein subunits with regulators of G-protein signalling (RGS proteins GAIP and RGS-4) that stimulate the GTPase activity of $G\alpha_{i2}$ and $G\alpha_o$. Protein expression was confirmed by immunoblot, using specific antibodies for the FLAG epitope, $G\alpha_{i2}$ or $G\alpha_o$, and RGS-4 or GAIP. Receptor density was determined independently of radioligand binding, using the M1-antibody and FLAG-tagged $h\beta_2$-adrenoceptor as standard. The expression levels of all proteins were similar when comparing different membrane preparations. In the steady-state GTPase assay, the hH_3R showed constitutive activity and a poor signal-to-noise ratio for agonists (just ~20% stimulation). After co-expression of RGS proteins, the signal intensity increased substantially (~100% stimulation) with highest values for RGS-4. We also found different levels of agonist-independent receptor activity depending on which Gα-subunit was co-expressed. We characterized several potent and selective standard H_3 receptor ligands and our results are consistent with the known hH_3R pharmacology. In conclusion, we established a novel and sensitive system to study hH_3R and to examine H_3 receptor ligand effects at a very proximal point of the signal transduction cascade. We are now able to reclassify ligands for all histamine receptors in terms of potency, efficacy and selectivity under similar conditions, using radioligand binding, steady-state GTPase and [^{35}S]GTPγS binding assays.

(Supported by Deutsche Forschungsgemeinschaft - GRK 760 and Deutscher Akademischer Austauschdienst)

53

FUNCTIONAL SELECTIVITY FOR LISURIDE AT HISTAMINE H_1 RECEPTORS

S. Jähnichen[1], R.A. Bakker[1,2], R. Leurs[1]

Stimulation of the G-protein coupled H_1 receptor by histamine results in an activation of different signal transduction pathways which include an increase of intracellular inositol trisphosphate (IP_3) and cyclic AMP (cAMP). In the present study, we performed second messenger and reporter gene assays in HEK293T cells to investigate the phenomenon of ligand-directed stimulus trafficking at H_1 receptors. First, we screened H_1 receptor ligands belonging to different structural classes for their ability to differently activate signaling pathways. The investigated histamines, phenylhistamines, and histaprodifens behaved as full or partial agonists in both, the nuclear factor-κB (NFκB) and cAMP response element (CRE)-mediated activation of reporter genes. Agonist potencies obtained for these ligands were about 15 times higher for the activation of NFκB compared to the activation of CRE whereas no differences in the rank order of agonist efficacies and potencies was observed. However, the ergoline lisuride potently activated NFκB (pEC_{50} 8.8, E_{max} 87% relative to 100 µM histamine) while this compound only weakly activated

CRE (E_{max} < 10%). The lisuride-induced activation of NFκB was competitively antagonized by mepyramine (pA_2 8.9). Selective activation of the NFκB pathway was also observed for other ergolines including terguride (pEC_{50} 7.6, E_{max} 34%) and ergonovine (pEC_{50} 6.7, E_{max} 57%). Furthermore, we investigated G-proteins and second messengers involved in the selective activation of the NFκB pathway by lisuride. Lisuride increased the cumulation of IP_3 (pEC_{50} 7.9, E_{max} 55%). In contrast to histamine, lisuride failed to elevate the level of intracellular cAMP. Overexpression of G_q and G_{11}-proteins amplified the lisuride signal in both, the IP_3 and NFκB assay, whereas shRNA-mediated silencing of G_q and G_{11}-proteins strongly diminished the lisuride-induced cumulation of IP_3 and the activation of NFκB. In conclusion, lisuride is a potent H_1 receptor agonist that shows functional selectivity for the $G_{q/11}$-dependent IP_3/NFκB pathway over the cAMP/CRE pathway.

[1]Leiden/Amsterdam Center for Drug Research, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081HV Amsterdam, NL; [2]current address: Boehringer Ingelheim Pharma GmbH und Co. KG, Abt. Stoffwechselforschung, Birkendorferstr. 65, 88397 Biberach.

54

AGOUTI-RELATED PROTEIN EXHIBITS AGONISTIC PROPERTIES ON THE ARRESTIN-MEDIATED ENDOCYTOSIS PATHWAY OF MELANOCORTINRECEPTORS

A. Breit, K. Wolff, T. Büch, H. Kalwar and T. Gudermann

Agouti-related protein (Agrp), one of the two naturally occurring inverse agonists known to inhibit G protein-coupled receptor activity, regulates food-intake and energy expenditure by decreasing basal and blocking agonist-promoted melanocortin receptor (MCR) signalling. Here we report that, in addition to its inverse agonistic activities, Agrp exhibits agonistic properties on the endocytosis pathway of melanocortin receptors. Sustained exposure of HEK-293 cells to Agrp induced endocytosis of the MC3R or the MC4R. The extent and kinetics of Agrp-promoted MCR endocytosis were similar to the endocytosis induced by melanocortins. Using the bioluminescence resonance energy transfer technique, we further show that after binding of Agrp, both MCR interact with β-arrestin-2. In line with this observation, co-expression of β-arrestin-2 enhanced Agrp-induced MCR endocytosis in Cos-7 cells, indicating that arrestins initiate receptor endocytosis promoted by the inverse agonist. This so far unappreciated regulatory mechanism was likewise detectable in a cell line derived from murine hypothalamic neurons endogenously expressing MC4R, pointing to the physiological relevance of Agrp-promoted receptor endocytosis. Removal of Agrp induced recycling of the MC4R protein to the plasma membrane, indicating that Agrp-mediated receptor endocytosis is a reversible process. In conclusion, we demonstrate that Agrp does not solely act by directly blocking MCR signalling, but also by temporarily reducing the amount of MCR molecules accessible to melanocortins at the cell surface. This "long-term antagonism" of Agrp reveals a new aspect of MCR signalling in particular and refines the concept of GPCR antagonism in general.

Institut of Pharmacology and Toxicology, Philipps-University Marburg, Germany Karl-von-Frisch-Str.1, 35043 Marburg

55

FTY720 SIGNALING THROUGH S1P RECEPTORS IN CEREBELLAR ASTROCYTES

F. Müllershausen[1], M. Cortes-Cros[3], M. Osinde[2], F. Bassilana[1], WL. Wishart[3], K. Seuwen[1] and KK. Dev[2] .

Sphingosine-1-phosphate (S1P) receptors are targets of the immunomodulator FTY720 which is currently in phase III clinical trials for the treatment of multiple sclerosis. In addition to the expression in peripheral tissues, S1P receptors are also widely expressed in the CNS where they may contribute to the therapeutic efficacy of FTY720. FTY720 becomes phosphorylated in vivo and FTY720-p is an agonist on S1P receptor subtypes 1, 3, 4 and 5. The aim of our study was to identify cells in the CNS that respond to FTY720-p and to identify the subtypes of S1P receptors responsible for mediating agonist action. Using a combined approach of live intracellular calcium imaging and immunocytochemistry, we identify astrocytes as cells responding to FTY720-p in mixed cortical cultures from rat brain. Cross-desensitization studies demonstrate that S1P and FTY720-p signal through the same receptor subtypes. In pure rat astrocyte cultures, we find mRNA for S1P1 = S1P3 >> S1P2, whereas S1P4 and S1P5 were not detectable. Accordingly, we demonstrate that FTY720-p triggers signaling to adenylyl cyclase and ERK via S1P1 receptors and Gi proteins, whereas inositol phosphate formation is mediated predominantly by S1P3 receptors and Gq proteins. We suggest that activation of S1P receptors on astrocytes may contribute to the therapeutic efficacy of FTY720 in multiple sclerosis.

[1]G Protein-Coupled Receptors, [2]Neuroscience, [3]Genome & Proteome Sciences, Novartis Institutes for Biomedical Research, Basel, Switzerland

56

EXPRESSION OF THE BRADYKININ B2-RECEPTOR IN HUMAN HEAD AND NECK SQUAMOUS CARCINOMA CELLS

O.Kocgirli[1], V. Balz[2] ,M. Bas[2], G. Kojda[1]

Increasing evidence suggest a causative role for bradykinin B2-receptors in the development of hereditary and iatrogenic angioedema. For example, the specific bradykinin B2-receptor (BK-B_2-R) antagonist has been shown to be clinically valuable drug for the treatment of hereditary angioedema (Allergy. 2006 Dec;61(12):1490-2.). The aim of this study is to evaluate a method to quantitate BK-B_2-R in human tissues. To accomplish this, we used various human head and neck squamous carcinoma cell lines which were established in our laboratory. The ability of these cells to express BK-B_2-R was tested in variety of 25 different cell lines by reverse transcriptase polymerase chain reaction and we chose one cell line expressing BK-B_2-R (UDSCC-2) and another without BK-B_2-R expression (UDSCC-3) for all further experiments. Cells were lysed in a saline Tris-buffer (pH 8.0) containing the detergent NP-40 (1 %), SDS (0.1 %) and a mixute of proteinase inhibitors, exposed to ultrasound (50 W) for 5 min and centrifuged at 1.000xg for 20 min. The supernatant was subjected to the deglycosylation procedure either with or without the use of N-glycosydase F (PNGase, EC 3.2.218). The protein of the resulting solutions (40 µg) was separated on SDS gels and blotted on PVDF membranes for detection of BK-B_2-R by a monoclonal and 4 different polyclonal (rabbit) antibodies. Non deglycosylized protein fractions of UDSCC-2 cells showed several non specific signals ranging between molecular weights of 37 kD-70kD. In striking contrast, declycosylated protein fractions of UDSCC-2 cells showed only one specific signal at 48 kD with all polyclonal antibodies and only one specific signal of 42 kD with the monoclonal antibody. These signal were completely absent in all protein fractions derived from UDSCC-3 cells indicating the BK-B_2-R-specificity of the signals. We suggest that the deglycosylation procedure improves the semiquantitative detection of BK-B_2-R in human tissues.

[1]Institut für Pharmakologie und Klinische Pharmakologie, [2]Hals-, Nasen- und Ohrenklinik, Universitätsklinikum, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany

57

ESTABLISHMENT OF A SENSITIVE CANNABINOID RECEPTOR ANALYSIS SYSTEM

K. Nickl[1], E. Schneider[2], J. Heilmann[1], R. Seifert[2]

Two cannabinoid receptors are known to date, the cannabinoid receptor 1 (CB_1R) and the cannabinoid receptor 2 (CB_2R). The CB_1R is predominantly expressed in the CNS and influences appetite, memory and learning, whereas the CB_2R is mainly found in the periphery on cells of the immune system. However, no sensitive test system to compare the CB_2R with the CB_1R at a molecular level exists. In order to establish such a test system, we expressed the human CB_1R and the human CB_2R (hCB_2R) using the baculovirus/Sf9 cell expression system. hCB_2R was co-expressed with different G-protein subtypes ($G\alpha_o$, $G\alpha_{i2}$, $G\beta_1\gamma_2$) and different Regulators of G-Protein Signaling (RGS-4, GAIP). To compare the potencies and efficacies of ligands in the different hCB_2R membrane preparations, we used the steady-state GTPase assay. We studied anandamide (arachidonylethanolamide), an endogenous partial agonist and CP 55,940 (5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-phenol), a synthetic agonist derived from the classic full agonist, Δ^9-THC (tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol). hCB_2R membrane preparations co-expressing $G\alpha_o\beta_1\gamma_2$ with RGS4 supported a much higher CP 55,940 (10 µM)-stimulated GTP hydrolysis (61 ± 6%) than membranes expressing either $G\alpha_o\beta_1\gamma_2$ alone (30 ± 7%) or co-expressing $G\alpha_oG\beta_1\gamma_2$ and GAIP (32 ± 5%). In contrast, $G\alpha_{i2}\beta_1\gamma_2$ efficiently supported a strong agonist signal (76 ± 6%) even without a RGS-protein. Thus, we have established a sensitive system to analyse the pharmacological profile of hCBRs. Using the GTPase assay we ensure assessment of ligand effects at a very proximal point of the signaling cascade under steady-state conditions.

[1] Department of Pharmaceutical Biology, University of Regensburg
[2] Department of Pharmacology and Toxicology, University of Regensburg

58

ACCESSORY PROTEINS INTERACTING WITH THE C-TERMINUS OF THE A2A ADENOSINE RECEPTOR

O.Kudlacek, I.Gsandtner, I.Ostrouska, J.Zezula, C.Nanoff

The A2A adenosine-receptor has a long carboxyl-terminus (c-tail), which consists of 122 amino acids. Truncation of the last 100 amino acids had little effect on localization, dimerization and signalling of the receptor in heterologous expression systems except for the receptors ability to target ERK (extracellular signal regulated kinase) and for the constitutive activity of the receptor. To further investigate the physiological role of the c-tail we screened a brain cDNA library in a yeast-2-hybrid hunt to identify proteins that bind to the c-tail of the A2A-receptor. Among these we isolated SAP102, a protein that belongs to the MAGUK (membrane associated guanylate kinase-like domain) family of proteins. After narrowing down the interacting region by using truncated versions of the receptor c-tail we mutated candidate motifs in the A2A-receptor to verify their importance in binding the identified proteins. By mutating the DVELL motif in the A2a-receptor to RVRAA we disrupted the association of SAP102 with the receptor. In addition we found that SAP102 interacts with its MAGUK domain with the receptor. However, the function of SAP102 on the A2A-receptor remains unclear. SAP102 did not influence the receptor's ability to bind ligand and to stimulate the effector adenylyl cyclase. SAP102 has been shown to influence the membrane localization of the NMDA-receptor, which is in agreement with its being a structural component of the postsynaptic density. It is likely that SAP102 exerts a similar effect on the A2A-receptor given that this receptor may be localized in dendritic spines of nerve cells. We want to test this hypothesis in SH-SY5Y neuroblastoma cells and in primary hippocampal nerve cells where the receptor was targeted to peripheral neurite extensions. This pattern depends on the c-tail because a truncated version of the receptor was restricted to the soma and to adjacent neurite stretches. Hence, we believe that subcellular targeting is due to receptor retention by a resident structural protein to which it is linked via the c-tail.

Institute of Pharmacology, Center for Biomolecular Medicine and Pharmacology, Währinger Straße 13a; A-1090 Vienna, Austria

59

OVEREXPRESSION OF EP_3-RECEPTOR REDUCESES OXIDATIVE DAMAGE IN CARDIAC MYOCYTES

C. Schooß, K. Schrör, T. Hohlfeld, J. Meyer-Kirchrath

Prostaglandin EP_3-receptors have been identified in the heart and have been implicated in E-type prostaglandin-mediated protection against ischemia-induced myocardial injury. Previous investigations showed an anti-ischemic activity of the EP_3-receptor in isolated Langendorff-perfused hearts from transgenic mice with cardiospecific overexpression of the porcine EP_3-receptor under the control of the α-MHC promoter. In present study we investigated the involvement of antioxidative mechanisms in this effect. Cardiomyocyte viability after H_2O_2-induced oxidative damage was assessed with SYTOX Green. Treatment with 200 µM H_2O_2 for 10 minutes, led to a greater degree of cell death in wildtype myocytes (45 ± 22 fold vs. control) compared to EP_3-overexpressing cardiomyocytes (9 ± 2 fold increase vs. control; both n=7-8; p< 0.05). To identify genes potentially involved in the anti-ischemic activity of the EP_3-receptor, we performed microarray-analysis of transgenic mouse hearts. Two regulated genes of interest were identified. Thioredoxin interacting protein (TXNIP), a regulator of the ubiquitously expressed thioredoxin-system (a control element of the redox-balance), was significantly downregulated in transgenic mice in comparison to wildtype. Cardiac ankyrin repeat protein (CARP), which may attenuate injury and apoptotic cell death under oxidative

conditions, was found to be markedly upregulated in transgenic hearts. The regulation of TXNIP and CARP was verified by semi-quantitative RT-PCR. In summary, we demonstrate that cardiac EP_3-receptor overexpression regulates TXNIP and CARP, which may contribute to the observed increased protection against oxidative damage in cardiac myocytes.

60

CONTRACTILE AND ELECTROPHYSIOLOGICAL FUNCTION IN SMALL ARTERIES FROM LOX-1 OVEREXPRESSING MICE

B. Eichhorn, G. Müller*, H. Morawietz*, U. Ravens

Endothelial cells clear oxidized low-density lipoproteins (oxLDL) from blood via the lectin-like oxLDL receptor-1 (LOX-1), however, unrestricted uptake of oxLDL via LOX-1 leads to endothelial dysfunction associated with decreased NO levels. Here we examined whether transgenic overexpression of bovine LOX-1 in mice (LOX-1 mice) affects the function of small arterial vessels (A. saphena, A. mesenterica). Male LOX-1 mice were fed with high-fat Western-type diet (F) for 10 weeks and similarly fed and age-matched C57BL/6 wildtype mice (WT) as well as normally fed animals served as controls. Endothelium-dependent relaxation of phenylephrine-preconstricted vessels in response to acetylcholine (ACh) was measured using a myograph. In both arterial vessels of mice on F, ACh responses were reduced, however, the potency of ACh was lower in arteries of LOX-1 mice+F compared with the other groups (see table).

Animals	ACh-induced relaxation [%]		-logEC$_{50}$ [M]		BK$_{Ca}$ - current density [pA/pF]	
	A.saphena	A.mes.	A.saphena	A.mes.	A.saphena	A.mes.
LOX-1 mice	3.5±1.7	2.1±1.9	7.0±0.1	6.8±0.1	239.4±40.1	452.7±16.5
LOX-1 mice+F	13.6±2.4	20.2±7.3	6.7±0.1	6.0±0.1	661.3±133.4	893.8±74.4
WT	2.8±1.8	2.7±1.7	7.1±0.2	6.8±0.1	334.9±89.8	837.0±102.6
WT+F	8.2±1.6	6.3±2.8	7.1±0.1	6.8±0.1	360.1±99.5	788.4±29.1

The potency and efficacy of the NO-donor sodium nitroprusside was similar in all animal groups. Opening of Ca^{2+}-activated K^+ channels with large conductance (BK_{Ca}) promotes membrane hyperpolarization, decreases open probability of L-type Ca^{2+} channels and thereby relaxes vascular smooth muscle cells (VSMC). Current density at -60 mV was increased in LOX+F. The differences in BK_{Ca} current densities are reflected in the resting membrane potentials of VSMC from the saphenous arteries: LOX: -71.5±0.9mV; LOX+F: -77.5±1.5mV ; WT: -71.8±1.0mV; WT+F: -73.3±0.5mV). In conclusion, overexpression of LOX-1 receptors in addition to a high-fat diet is associated with changes in endothelial responses of saphenous and mesenteric arteries. It is speculated that a putative "adipocyte-derived relaxing factor" may open BK_{Ca} channels, and the resulting hyperpolarization compensates for impairment of NO-mediated relaxation.

Department of Pharmacology and Toxicology, *Department of Vascular Endothelium and Microcirculation, Medical Faculty, University of Technology, Dresden, Germany

61

ALPHA-HYDROXY-CARBOXYLIC ACIDS ARE LIGANDS FOR THE HUMAN ORPHAN RECEPTOR GPR109B

K. Ahmed, S. Tunaru, S. Offermanns

The human orphan G-protein-coupled receptor GPR109B (HM74) is a close relative of the nicotinic acid receptor, GPR109A (HM74A). The two receptors are highly homologous, displaying 96% amino acid identity, and are both expressed in human adipose tissues. GPR109B, which has no orthologue in rodents, can bind nicotinic acid, but only with very low affinity. Here we show that α-hydroxycarboxylic acids activate GPR109B, but not GPR109A. α-hydroxyoctanoic acid and α-hydroxyhexanoic acid elicit calcium mobilization via activation of GPR109B with half-maximal concentrations of 23.7 and 78.9 μM, respectively. Furthermore we show that the activation of GPR109B by α-hydroxycarboxylic acids leads to a pertussis toxin-sensitive inhibition of cyclic AMP production and stimulation of mitogen-activated protein kinase demonstrating that GPR109B couples to the $G_{i/o}$ pathway. In addition, we demonstrate that α-hydroxyoctanoic acid inhibits isoproterenol-induced lipolysis in human adipocytes. To investigate the molecular determinants of GPR109B-ligand selectivity we performed site-directed mutagenesis of putative ligand binding residues and generated chimeric GPR109A/B constructs. We show that Tyr86/Ser91 (extracellular loop 2) and Val103, Phe107 and Arg111 (transmembrane helix 3) are important amino acids for receptor activation. In addition, our data suggest that Arg111 functions as the critical residue for binding of carboxylic acid ligands, similar to data of nicotinic acid binding to GPR109A. We also determined the structural requirements for GPR109B-ligands and found that α-hydroxyoctanoic acid has the highest affinity and efficacy on GPR109B. Maximal receptor activation requires a carboxylic acid function, a H-bond donor/acceptor in its close proximity and a carbon chain length that is restricted to 6-8 carbon atoms. These data provide the basis for a rational design of new GPR109B-selective agonists that could be useful to further elucidate the utility of GPR109B as a target for antilipolytic drugs.

University of Heidelberg, Institute of Pharmacology, Im Neuenheimer Feld 366, 69120 Heidelberg

62

SENSITISATION BY ACETYLSALICYLIC ACID AND ACETIC ANHYDRIDE OF CAPASAICIN-INDUCED CURRENTS IN OOCYTES FROM *XENOPUS LAEVIS* EXPRESSING rTRPV1

S.D. Vulcu, I. Beck

TRPV1 is a ligand-gated cation channel, which is activated by various substances like capsaicin and resiniferatoxin, protons, and noxious heat. TRPV1 is predominantly found in primary sensory neurones and is thought to play a major role in the detection of pain-producing heat stimuli. PGE_2 and PGI_2 have been described to sensitise rTRPV1-mediated membrane currents (Moriyama et al., (2005), Mol Pain, 1, 3). Greffrath et al. (2002) have shown that micromolar concentrations of acetylsalicylic acid decrease noxious heat responses in isolated rat dorsal root ganglia cells (Neurosci Lett, 320, 61-64). We have studied the effects of acetylsalicylic acid, diclofenac, and acetic anhydride on membrane currents in oocytes from *Xenopus laevis* expressing rTRPV1. The two-electrode voltage-clamp technique was used (clamp potential: -60 mV). The experiments were carried out in modified Ringer's solution (composition in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, and HEPES 10; pH 7.4; temperature was held at 20° C). Capsaicin 1 μM induced rapid developing inward currents, which slightly inactivated to a new steady state within 30 min. In the presence of acetylsalicylic acid 1 mM, peak inward currents were increased from -126 ± 14.5 to -199 ± 25.8 nA, and steady state inward currents were increased from -101.9 ± 13.5 to -158 ± 20.2 nA (means ± SEM; $p < 0.05$). The EC_{50}-value of acetylsalicylic acid amounted to 0.11 ± 0.01 mM (mean ± SEM). Diclofenac 0.1 mM exerted no significant effects on capsaicin-induced currents. Acetic anhydride 1 mM increased the capsaicin-induced inward currents from -138 ± 27.2 nA to -1046 ± 357 nA (means ± SEM; $p < 0.001$). We tentatively propose that acetylsalicylic acid and acetic anhydride may sensitise rTRPV1-mediated currents by acetylation.

Department of Pharmacology, Johannes-Gutenberg-Universität, Mainz, Germany

63

INHIBITORY CROSSTALK BETWEEN NICOTINIC ACETYLCHOLINE AND GABA-A RECEPTORS

K. Schicker[1] and S. Boehm[1,2]

Inhibitory interactions between different classes of ligand gated ion channels have been demonstrated for the following pairs of transmitters: ATP-ACh, ATP-GABA, ATP-5HT, and GABA-Glycine. However, the functional basis for these effects remained controversial. Here, we first show a novel type of interaction between GABA and ACh receptors and then investigate the underlying mechanisms. In perforated as well as whole-cell patch-clamp recordings from rat superior cervical ganglion neurons, inward currents evoked by the combined application of 0.3 mM GABA and nicotine at a potential of -70 mV amounted to only 56.2±6.0 % (n=25) of the linear sum of the individual currents (% of prediction). This lack of additivity was not due to saturation of the current response, as it was also observed at agonist concentrations of 0.02 and 0.1 mM. The EC_{50} value for nicotine-induced currents was 26 μM and 8 μM for the decrease in the % of prediction caused by increasing nicotine concentrations in combination with 0.3 mM GABA. Vice versa, the EC_{50} value for GABA was 31 μM for the induction of currents and 14 μM for the decrease in the % of prediction in combination with 0.3 mM nicotine. The crossinhibitory effects did not involve GABA-B receptors, since baclofen did not alter nicotine-evoked currents, but they were attenuated by antagonists at nicotinic and $GABA_A$ receptors, respectively. GABA-induced currents showed a linear current-voltage relation between -100 and +25mV, whereas nicotine-evoked currents displayed clearcut inward rectification. The sub-additivity was observed at all voltages tested. When experiments were performed with solutions giving different equilibrium potentials for nicotine(0mV)- and GABA(-70mV)-evoked currents, mutual crossinhibition was even observed when one of the agonists did not induce significant currents. These results demonstrate inhibitory interactions between nicotinic acetylcholine and GABA-A receptors and suggest that the crosstalk does not depend on ion fluxes through, but only on gating of, the receptors.

Supported by the FWF (P 17611).

[1]Institut für Pharmakologie, Medizinische Universität Wien, Währingerstrasse 13a, A-1090 Wien, und [2]Institut für Experimentelle und Klinische Pharmakologie, Medizinische Universität Graz, Universitätsplatz 4, A-8010 Graz.

64

SLC10A4: A NEW ORPHAN TRANSPORTER EXPRESSED IN CHOLINERGIC NEURONS

C. Fernandes[1], J. Geyer[1], J. Godoy[1], S. Rafalzik[2], R. Gerstberger[2], E. Petzinger[1]

The solute carrier family 10 (SLC10) is known as the 'family of sodium/bile acid cotransporters' (SBATs). SBATs maintain the enterohepatic circulation of bile acids between the liver (via NTCP; SLC10A1) and the ileum (via ASBT; SLC10A2) and participate in the homoeostasis of cholesterol. A further member of this transporter family was cloned in 2004 and is referred to as sodium-dependent organic anion transporter (SOAT; SLC10A6). In contrast to NTCP and ASBT, SOAT does not transport bile acids but steroid sulfates in a sodium-dependent manner. Recently, we cloned another new member of the SLC10 family which is referred to as P4 (SLC10A4) [GenBank accession no. AY825923]. Phylogenetic analyses showed that P4 and NTCP emerged from a common ancestor gene which is different from that of ABST and SOAT. Tissue expression pattern of rat and human SLC10A4 were analyzed by northern blot and quantitative real-time PCR and revealed highest expression in the brain. A polyclonal rabbit antibody was generated against rat SLC10A4 and used for immunoblot and immunohistochemical analysis. SLC10A4 specific immunoreactivity was detected in cholinergic regions throughout the rat central nervous system. Co-localization studies with the cholinergic markers VAChT and CHT1 confirmed the presence of SLC10A4 in cholinergic neurons, whereas SLC10A4 expression did not merge with tyrosine hydroxylase, a marker protein for catecholaminergic neurons. Although of high phylogenetic relationship to the liver bile acid carrier NTCP, SLC10A4 showed no transport activity for bile acids and sulfated steroids, but also not for choline and acetylcholine when expressed in *X. laevis* oocytes and HEK293 cells. We regard SLC10A4 as new marker protein of cholinergic neurons, although its functional properties are so far unknown.

[1] Institute of Pharmacology and Toxicology, Frankfurter Str. 107, and [2] Institute of Physiology, Frankfurter Str. 100, Faculty of Veterinary Medicine, Justus-Liebig University of Giessen, 35392 Giessen

65

ALTERED AIRWAY M_2-RECEPTOR-G_i-PROTEIN INTERACTION IN ANIMAL MODELS OF ASTHMA

G. Abraham, P. Hajek, F.R. Ungemach

Among the five known muscarinic acetylcholine receptor subtypes (MAChR) the M_2-receptor subtype which preferentially couple to $G_{\alpha i/i}$-protein is predominantly expressed in the airways of all mammalian species. In bronchial hyper-reactivity such as asthma little is known about changes in the presence, localization and function of these receptors. Thus, the focus of present work is to investigate the relative contribution of the interaction between M_2-subtype and the respective G-protein in asthma pathophysiology. We assessed segment-dependent distribution of MAChRs in the equine asthmatic airway tissues ([N-methyl-^{3}H]-scopolamine (^{3}HNMS) binding

assays). The interaction between M_2-receptors and $G_{\alpha 0/i}$-protein was measured by [^{35}S]-guanosine-5'-O-(3-thio)-triphosphate ([^{35}S]-GTPgS) binding in the presence or absence of N-ethylmaleimide (NEM) or pertussis toxin (PTX). The membrane activity of adenylate cyclase (AC) and phospholipase C (PLC) was determined. The level of $G_{\alpha 0/i}$-protein was analyzed by western blotting, using β-actin as control. In asthmatic equine airways, we found no segment-dependent alteration in MAChR density and distribution. However, the presence of PTX and NEM increased the MAChR agonist-induced stimulation of [^{35}S]-GTPgS binding in bronchus and trachea, suggesting enhanced receptor-$G_{\alpha 0/i}$-protein-interaction in bronchial hyper-reactivity. Moreover, the adenylate cyclase activity was diminished in asthmatic tissues, indicating also increased G_i-coupling or expression; indeed, we found only a trend in changes in $G_{\alpha 0/i}$-protein amount. The M_3-receptor-$G_{q/11}$-protein-PLC activity was not altered in asthmatic airways. In conclusion, in asthma, the density and subtype distribution were not altered, but the M_2-receptor-$G_{\alpha 0/i}$-protein interaction was enhanced and inhibited the adenylate cyclase coupling, thus, it can strongly be supposed that the β_2-adrenergic receptor signal-transduction mechanism is functionally opposed and might contribute to increased contractile responses of the airway smooth muscle in allergic asthmatic processes.

Institute of Pharmacology, Pharmacy and Toxicology, University of Leipzig, An den Tierkliniken 15, Germany

66

QUINAZOLINIMINES IN LOW DOSAGE ATTENUATE COGNITIVE IMPAIRMENTS IN RATS INDUCED BY SCOPOLAMINE AND ENHANCE SCOPOLAMINE ACTION AT HIGHER DOSAGE

[1]D Appenroth, [2]M Decker, [2]J Lehmann, [1]C Fleck

Using alkaloids as lead structures for novel compounds for the therapy of dementia, tricyclic quinazolinimes have been identified as potent inhibitors of cholinesterases *in vitro* using structure-activity releationships. Functionalizing the imine-*N*-atom by introducing an additional benzene ring in varying distances from the heterocycle yielded micro- and submicromolar inhibitors either with activities at acetyl- (AChE) and butyrylcholinesterase (BChE) or at BChE alone. In order to further establish the antiamnesic properties of this class of compounds, an *in vivo*-test system has been established. Cognitive impairment in rats is reversibly induced by scopolamine and evaluated in an eight-arm radial maze. The established AChE-inhibitor rivastigmine attenuates the scopolamine-induced impairments. Experiments were done with adult female Wistar rats. The quinazolinimines and scopolamine as well were administered intraperitoneally. Cognition tests were done in the radial maze at 20, 60, and 180 minutes after scopolamine administration. A representative quinazolinimine showed attenuation of cognitive deficits in a dosage of 0.1 mg/kg, whereas at a dosage > 1 mg/kg the effect of scopolamine is markedly reinforced. Since the quinazolinimine itself does not influence cognition, the latter effect seems to be due to amplification of scopolamine action either by pharmacokinetic effects or allosteric modulation of mACh receptors.

[1]Institut für Pharmakologie und Toxikologie, FSU Jena, [2]Lehrstuhl für Pharmazeutische / Medizinische Chemie, FSU Jena

67

SYNTHESIS AND METABOLISM OF CATECHOLAMINES IN THE BRAIN OF α_{2A}, α_{2B} AND α_{2C} KO MICE

E. Moura[1,2], J. Afonso[1], M.P. Serrão[1], M.A. Vieira-Coelho[1,2]

Aim: Evaluate the activity of enzymes involved in monoamine synthesis - L-Amino Acid Decarboxylase (AADC) and Tyrosine Hydroxylase (TH), and metabolism – Monoamine Oxidase (MAO) and Catechol-O-methyltransferase (COMT), in the brain of knockout (KO) mice for each of the α_2-adrenoceptor subtypes: α_{2A}, α_{2B} and α_{2C}. Methods**:** Brains from α_{2A}, α_{2B} and α_{2C} KO mice and wild type (WT) mice (C57BL/6) aged 15 weeks were used. TH activity was measured using different concentrations of the substrate L-tyrosine and measuring the formation of L-DOPA or fixed concentration of the substrate and different concentration of cofactor (6-BH_4). AADC activity was measured using different concentrations of L-DOPA and measuring the production of dopamine. MAO activity was measured using different concentrations of dopamine and measuring the production of DOPAC. COMT activity was measured using different concentrations of adrenaline and measuring the production of metanadrenaline. The assay of catecholamines in samples was carried out by HPLC-ED. Results are given as arithmetic mean±sem. Statistical analysis was done by ANOVA.

Results: No differences were found in TH and MAO activity in all three α_2KO mice. AADC activity was significantly higher in α_{2A} and α_{2C}-KO mice compared to WT mice. COMT was significantly higher in all three α_2KO mice compared to WT mice.

	TH				AADC		MAO		COMT	
	L-Tyrosine		6-BH_4							
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
WT	2.6±0.1	78±7	2.5±0.1	106±7	40±1	1.6±0.1	4.5±0.1	54±4	2.0±0.0	2.0±0.1
α_{2A}KO	2.5±0.2	59±17	2.5±0.1	110±15	77±2*	3.6±0.2	4.4±0.1	50±5	2.4±0.1*	1.6±0.3
α_{2B}KO	2.4±0.2	52±12	2.4±0.2	117±5	40±1#	1.3±0.1	4.4±0.2	71±7	2.2±0.0*#	2.0±0.1
α_{2C}KO	2.6±0.1	56±10	2.5±0.1	93±7	50±1*#	1.9±0.1	4.6±0.2	62±5	2.2±0.1*#	2.2±0.2

V_{max} in nmol/mgprot/h and K_m values in µM except for AADC (in mM). *P<0.05 compared to WT; #P<005 compared to α_{2A}.

Conclusion: The activity of AADC and COMT is increased in mice lacking α_{2A} or α_{2C} adrenoceptors. In contrast, lack of α_{2B}-adrenoceptors did not influence catecholamine synthesis or metabolism.

[1]Institute of Pharmacology & Therapeutics (U-38), FMUP
[2]Institute of Cell and Molecular Biology, UP

68

TRANSGENIC MAPPING OF AUTO- VERSUS HETERORECEPTOR FUNCTION OF α_2-ADRENOCEPTORS

R. Gilsbach[1], C. Röser[2], N. Beetz[1], J.E. Moura[3], M.A. Vieira Coelho[3], L. Hein[1]

Previous studies revealed distinct physiological functions of the three adrenergic α_2-adrenoceptor subtypes ($\alpha_{2A,B,C}$-AR). Less is known whether these functions are mediated by autoreceptors, i.e. α_2-AR on adrenergic neurons, or heteroreceptors, i.e. α_2-AR on non-adrenergic neurons. To clarify this issue, we generated transgenic mice expressing α_{2A}-AR under control of the dopamine-β hydroxylase (DBH) promoter. These mice were backcrossed to onto a α_{2AC}-/- background. For our studies we compared the following mouse lines: 1) Mice with endogenous α_{2A}-AR (α_{2A}+/+). 2) Transgenic mice which express α_{2A}-AR only in nor/adrenergic (i.e. DBH positive) cells (DBH-α_{2A}TG). 3) Mice with a targeted disruption of α_{2A}-AR (α_{2A}-/-). Targeted deletion of the α_{2A}-gene and expression of the DBH-α_{2A} transgene were determined by PCR genotyping, by quantitative RT-PCR and by autoradiography with the α_2-AR antagonist [^{3}H]RX821002, respectively. Transgenic α_{2A}-AR under control of the DBH promoter were identified in peripheral (mRNA: superior cervical ganglia, adrenal gland) as well as in central adrenergic cells (mRNA and protein: brain stem, hippocampus, cortex). In order to compare the function of endogenous α_{2A}-AR with transgenic DBH-α_{2A} receptors, the following tests were performed: Superfusion experiments with [^{3}H]noradrenaline were performed in isolated tissues to determine feedback inhibition of noradrenaline release. Sedative and analgesic effects of α_2-agonists were assessed using a rotarod and a tail flick test, respectively. Cardiovascular parameters were measured during anesthesia using a Millar microtip catheter. α_2-AR agonists mediated feedback inhibition of noradrenaline release in vitro and sedation in α_{2A}+/+ and DBH-α_{2A}TG mice but not in α_{2A}-/- animals or tissues. In contrast, α_2-agonist mediated analgesia and bradycardia were only present in α_{2A}+/+ but not in DBH-α_{2A}TG and α_{2A}-/- mice. In conclusion, α_{2A} autoreceptors mediate the sedative effects of α_2-agonists and inhibit noradrenaline release from adrenergic neurons in vitro. α_{2A} heteroreceptors mediate analgesia as well as hypotension and bradycardia after application of α_2-agonists.

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, [2]Institut für Pharmakologie und Toxikologie, Universität Würzburg, [3]Institute of Pharmacology and Therapeutics, University of Porto, Portugal

69

DIRECT INHIBITION OF CARDIAC HCN PACEMAKER CHANNELS BY CLONIDINE

A. Knaus[1,2], X. Zong[3], N. Beetz[1], M.J. Lohse[2], M. Biel[3], L. Hein[1]

Inhibition of cardiac sympathetic tone represents an important strategy for treatment of cardiovascular disease, including arrhythmia, coronary heart disease and chronic heart failure. Activation of presynaptic α_2-adrenoceptors is the most widely accepted mechanism of action of the antisympathetic drug clonidine. However, other target proteins have been postulated to contribute to the in vivo actions of clonidine. In order to test whether clonidine elicits pharmacological effects independent of α_2-adrenoceptors, we have generated mice with a targeted deletion of all three α_2-adrenoceptor subtypes (α_{2ABC}-/-). α_{2ABC}-/- mice were completely unresponsive to the analgesic and hypnotic effects of clonidine. However, clonidine significantly lowered heart rate in α_{2ABC}-/- mice by up to 150 beats/min. Clonidine-induced bradycardia in conscious α_{2ABC}-/- mice was 32.3% (10 µg/kg) and 26.6% (100 µg/kg) of the effect in wild-type mice. A similar bradycardic effect of clonidine was observed in isolated spontaneously beating right atria from α_{2ABC}-KO and wild-type mice. Clonidine inhibited the native pacemaker current (I_f) in isolated sinoatrial node (SAN) pacemaker cells as well as the I_f-generating HCN2 and HCN4 channels in transfected HEK293 cells. As a consequence of blocking I_f, clonidine reduced the slope of the diastolic depolarization and the frequency of pacemaker potentials in SAN cells from wild-type and α_{2ABC}-KO mice. Direct inhibition of cardiac HCN pacemaker channels contributes to the bradycardic effects of clonidine gene-targeted mice in vivo and thus clonidine-like drugs represent novel structures for future HCN channel inhibitors.

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, [2]Institut für Pharmakologie und Toxikologie, Universität Würzburg, [3]Department Pharmazie, LMU München,

70

α_{2B}-ADRENERGIC RECEPTORS REGULATE ANGIOGENESIS

V. Muthig, M. Haubold, R. Gilsbach, L. Hein

α_2-adrenoceptors are essential presynaptic feedback regulators of norepinephrine release from sympathetic nerves. Previous studies in mice with targeted deletions in the three α_2-adrenoceptor genes have indicated that these receptors are essential for embryonic development by regulating the formation of blood vessels in extraembryonic tissues. The responsible subtype for this defect in angiogenesis seems to be the α_{2B}-receptor, because the α_{2B}-receptor was the most expressed α_2-receptor subtype in placenta, yolk sac and embryo. Congenic C57BL/6-α_{2B}-receptor-KO mice showed a similar placental phenotype like α_{2ABC}-KO mice with a lack of blood vessels and endothelial cells and an increased number of trophoblasts. Western Blot analysis showed a reduced activity of MAP kinases ERK1/2 in the placenta and yolk sac at E10.5. The purpose of the present study was to identify the molecular mechanism of α_2-adrenoceptor-mediated angiogenesis in the placenta. A whole genome transcriptome analysis of WT- versus α_{2B}-KO-placentae revealed 179 genes which were significantly up- or downregulated >1.5-fold in the α_{2B}-deficient placenta. The vascular endothelial growth factor receptor type 1 (VEGFR-1 or Flt-1), which is coexpressed with α_{2B}-receptors in the placenta was found to be upregulated 2.3-fold in the α_{2B}-deficient placenta. Furthermore, α_{2B}-receptor deletion resulted in upregulation of the soluble splice variant sFlt-1 of the VEGFR-1. Neutralization of VEGFR-1 and sFlt-1 by a specific antibody in vivo prevented the vascular defect in α_{2B}-KO placentae at E10.5 and normalized placental ERK1/2-phosphorylation. In order to investigate angiogenesis in the adult organism, an femoral occlusion model was used. This experiment resulted in a decreased capillary density in α_{2B}-KO mice as compared with WT mice. In conclusion, vascular α_2-adrenoceptors are not only involved in the acute regulation of the vascular tone, but they are also responsible for the angiogenesis during embryogenesis and in the adult organism. In the placenta, α_{2B}-adrenoceptors prevent upregulation of the angiogenic VEGF receptor type-1 at the interface between maternal and fetal tissues.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg

71

α_{1D}-ADRENOCEPTOR SUBTYPE REGULATES THE EXPRESSION OF THE CARDIAC GAP JUNCTION PROTEIN CONNEXIN 43 (CX43)

D.M. Rojas-Gomez, J.S. Schulte, C. Frenzel, S. Holtz, A. Salameh, F.-W. Mohr, S. Dhein

Background: Connexin43 is the predominant intercellular gap junction protein in the heart providing intercellular communication for the cell-to-cell transfer of electrical activation. It has been found that in cardiac hypertrophy Cx43 is up-regulated, but very often inserted at the lateral borders of the cells instead of the cellular poles thereby making the heart more prone to arrhythmia. Since noradrenaline plays a major role in cardiac pathophysiology and seems to be involved in hypertrophic signalling, the goal of our study was to find out, whether α1-adrenoceptor stimulation can affect Cx43 expression and function, and which α1-adrenoceptor subtype might be involved. Methods: Cultured neonatal rat cardiomyocytes were exposed to various concentrations of noradrenaline or phenylephrine (0.1 – 1000 nM) for 24 hours (n=6). Thereafter cells were harvested, and after lysis, Cx43 content was determined using SDS-PAGE and Western blot. Results were normalised to GAPDH. Finally, we determined the effect of this treatment on intercellular gap junction conductivity using dual whole cell voltage clamp. Similarly, we tested the effect of an additional treatment with either 10 nM prazosin, or in order to assess the subtypes, 10 nM of the α_{1A}-antagonist RS17053 (n=6), 500 nM of the α_{1B}-antagonist AH1111OA (n=6), or 50 nM of the α_{1D}-antagonist BMY7378 (n=6). Results: Noradrenalin and phenylephrine led to enhanced Cx43 expression with a pEC50 of 9±0.1 and 8.17±0.14, respectively. The other cardiac connexins, Cx40 and Cx45, were not affected. This increase in Cx43 expression resulted in enhanced gap junction conductance (43±4 nS versus 29±4 nS). As expected the increased Cx43-expression could be antagonized by prazosin. Moreover, it was nearly completely inhibited by BMY7378, but was not significantly affected by RS17053. AH1111OA lead to a moderate but incomplete inhibition. Conclusions: The low pEC50 value of about 1 nM for noradrenalin fits well to the hypothesis of an effect mediated predominantly via α_{1D}-adrenoceptors, which is further supported by the finding of a nearly complete antagonisation of the phenylephrine effect by BMY7378. Thus, we conclude that cardiac Cx43 expression seems to be regulated via α_1-adrenoceptors predominantly by subtype α_{1D}-adrenoceptors.

University of Leipzig, Heart Centre, Clinic for Cardiac Surgery; Struempellstr.39, 04289 Leipzig

72

ELECTROPHYSIOLOGIC AND FUNCTIONAL EFFECTS OF LEONURUS CARDIACA, A MEDIEVAL PLANT REMEDY

S. Dhein, M. Ritter, K. Melichar, J.W. Rauwald*, E. Cerbai[#], A. Mugelli[#], F.-W. Mohr

Background: *Leonurus cardiaca* was first mentioned in *Hortus sanitatis Lipsiae* in 1485, and was used for centuries as a remedy against cardiac palpitations. However, until today there is no scientific proof, whether this plant may contain pharmacologically effective substances. Therefore, we wanted to know whether extracts from *Leonurus cardiaca* exert functional or electrophysiological effects on isolated hearts.

Methods: An aqueous extract from L.c. was prepared, followed by a three step purification and characterization (HPLC, DC). This extract was infused (0.1 – 3.0 mg/ml) intracoronarily in isolated rabbit hearts perfused according to the Langendorff technique. Left ventricular pressure (LVP), heart rate (HR) and coronary flow (CF) were measured. Moreover, we registered 256 unipolar electrograms from the ventricular surface and calculated activation-recovery-intervals (ARI), dispersion of ARI (=SD($ARI_{1..256}$), total activation time (TAT), and atrioventricular conduction time. Direct electrophysiological effects were investigated using voltage clamp technique. We measured I(Ca.L) I(Na) [rat ventricular myocytes], I(f), and I(K.r.) [guinea pig cardiomyocytes] according to standard protocols under the influence of 2 mg/ml.

Results: The extract significantly and concentration-dependently reduced LVP by -15±5mm Hg and HR by 14±1 bpm, enhanced relative CF by 10±5% and atrioventricular conduction time by 5±1 ms. The ventricular propagation velocity was not affected. ARI, and QTc were prolonged (QTc: from 0.24±0.01 to 0.26±0.01) while dispersion was reduced by 23±18% (values for 1 mg/ml). Other, non aqueous extracts, were not effective or toxic. All experiments were carried out with n=7.

Perforated patch voltage clamp experiments revealed that the aqueous extract (2 mg/ml, superfusion) inhibited I(Ca.L) by 62±2%, while I(Na) was not altered. I(K.r.) was also inhibited and the time constant of activation of I(f) was increased by 30% at -80 mV. Using bridge mode in additional experiments we recorded action potentials, which were prolonged by the extract (by about 10%).

Conclusions: These results show for the first time, that *Leonurus cardiaca* indeed is pharmacologically active. The extract exerts calciumantagonistic effects, class III like antiarrhythmic effects by I(K.r.) blockade and shifts the activation of I(f). This might be of therapeutic interest in antiarrhythmic therapy or in therapy of coronary heart disease.

University of Leipzig, Heart Centre, Clinic for Cardiac Surgery; Struempellstr.39, 04289 Leipzig;
* Inst. für Pharmazeutische Botanik, Johannisallee 21-23, 04103 Leipzig,
[#]Dipartimento di Farmacologia Preclinica e Clinica, Firenze, Italia

73

CANNABINOID CB_1 RECEPTOR IN THE GUINEA-PIG: PARTIAL SEQUENCING AND IDENTIFICATION OF AN ATRIAL PRESYNAPTIC RECEPTOR

C.M. Kurz, M. Kathmann, E. Schlicker

In guinea-pig atrial pieces preincubated with [^{3}H]-noradrenaline, the cannabinoid receptor agonist WIN 55,212-2 1µM inhibited the electrically (3 Hz) evoked tritium overflow by 46 and 30% at Ca^{2+} concentrations of 1.63 and 3.25 mM, respectively. Subsequent experiments were carried out using 3.25mM Ca^{2+}. The concentration-response-curve of WIN 55,212-2 was shifted to the right by the CB_1 receptor antagonist rimonabant with an apparent pA_2 of 8.4 (0.1µM) and 8.2 (0.032µM), which, by itself, did not affect tritium overflow. To compare the results with other presynaptic inhibitory receptors, the effects of the muscarine receptor agonist oxotremorine and of prostaglandin E_2 (PGE_2) on tritium overflow have also been studied. Oxotremorine inhibited tritium overflow maximally by 55% and its concentration-response curve was shifted to the right by the M_2/M_4 receptor antagonist AF-DX 384 0.1µM with an apparent pA_2 of 8.5. An inhibition by 54% could be shown for PGE_2 1µM. Unlike in the atrium, WIN 55,212-2 1µM had no effect on tritium overflow in guinea-pig renal cortex pieces. Oxotremorine reduced tritium overflow maximally by 80% and PGE_2 1µM reduced it by 36%. The concentration-response curve of oxotremorine was shifted to the right by AF-DX 384 with an apparent pA_2 of 8.6. Since the guinea-pig proves to be suited for the identification of presynaptic CB_1 receptors, we examined its nucleotide sequence, using primers of the mountain paca (*Agouti taczanowskii*), a closely related species, the partial sequence of which has been published by Murphy et al. (2001) (GenBank AY011590). We determined a partial sequence (330 amino acids) of the guinea-pig (*Cavia porcellus*) including the 1st to 6th transmembrane domain (TMD) (GenBank DQ355990). The homology was 99% (mountain paca), 95% (man) and 96% (rat or mouse). In conclusion, noradrenaline release from the guinea-pig atrium, but not the kidney, is subject to inhibition by presynaptic CB_1 receptors. The guinea-pig CB_1 receptor shows a high homology to the CB_1 receptor of humans and rodents.

Institut für Pharmakologie, Uni Bonn, Reuterstr. 2b, 53113 Bonn

74

COMPARISON OF THE INFLUENCES OF MAGNESIUM, MENTHOL AND VERATRIDINE ON CATECHOLAMINE RELEASE IN DUCTUS DEFERENS AND ADRENAL MEDULLA

S. Jost, N. Kretschmer, W. Vierling

A change in transmitter release of the sympathetic nervous system could have important effects in several diseases, for example cardiac arrhythmias, heart failure and bronchial asthma. Therefore, we investigated the effects of magnesium, menthol and veratridine on catecholamine release. All these substances influence neuronal ion channels and thereby neurotransmitter release. We studied the influence on catecholamine release in ductus deferens of the rat (by an indirect method, i.e. measuring contraction) and in slices of rat adrenal medulla (by a direct method, i.e. measuring catecholamine release voltammetrically with carbon fibre microelectrodes). Magnesium and menthol, known blockers of neuronal calcium channels reduced electrically induced catecholamine release in ductus deferens. The effect of magnesium was especially pronounced in the therapeutically relevant concentration range between 0.6 and 1.2 mM while the most effective concentration of menthol was 100 µM. Magnesium exerted a stronger maximal effect. In contrast, low concentrations (0.5 µM) of veratridine increased catecholamine release in the absence of magnesium, probably due to its enhancing influence on sodium channels and elevating effect on intracellular calcium concentration. Already 1.2 mM magnesium abolished the effect of veratridine. Direct determination of catecholamine release from adrenal medulla confirmed the magnesium-induced inhibition of release. 1.2 mM Magnesium reduced potassium- as well as carbachol-induced catecholamine release. However, in contrast to the results in ductus deferens, 100 µM menthol increased the potassium-induced release. In accordance with the results at the ductus deferens, 0.5 µM veratridine has an enhancing effect on potassium-induced release. Also in the adrenal gland, this enhancing effect was abolished by magnesium. In contrast, the carbachol-induced release was decreased by veratridine, suggesting a different mechanism of action. The results show that magnesium exerts similar effects in ductus deferens and adrenal medulla. However, for menthol and veratridine, there seem to be important differences depending on the investigated tissue and the kind of stimulation of the release.

Institute of Pharmacology and Toxicology, Technical University Munich,
Biedersteiner St. 29, 80802 Munich

75

CHARACTERIZATION OF POTENT AND HIGHLY SELECTIVE ANTAGONISTS FOR THE HUMAN 5-HT_{5A} RECEPTOR

F.J. Garcia-Ladona, W. Amberg, A. Kling, U. E.W. Lange, W. Hornberger, W. Wernet, A. Netz, M. Ochse, M. Mezler, A. H. Meyer, A. Hahn, H. Hillen, G. Gross, A. Beyerbach, S. Bhowmik, H. Schoemaker, [1]J. P. Sullivan

Serotonin 5A (5-HT_{5A}) receptors are G-protein-coupled receptors classified as a singular subgroup of the 5-HT receptor family. Gene expression studies indicate that in both, humans and rats, 5-HT_{5A} receptors are localized exclusively in the central nervous system (CNS). The physiological function of 5-HT_{5A} receptors has remained elusive so far, but brain distribution and genetic linkage studies suggest an important role in higher CNS functions. Thus, 5-HT_{5A} receptors have been suggested to play a role in schizophrenia, bipolar disorders, autism, migraine and pain. The present study characterizes new potent and highly selective 5-HT_{5A} receptor antagonists from different chemical series. A-843277, A-833551 and A-763079 bind with high affinity (pKi=8.1, 8.7, 8.5, respectively) to h5-HT_{5A} receptors recombinantly expressed in HEK293 cells. None of these compounds modified basal GTPγS binding in HEK293 cells expressing h5-HT_{5A} receptors. However, all potently inhibited the 5-HT response (pKb = 7.8, 8.4, and 7.9, respectively) suggesting pure antagonist properties. All compounds displayed at least a 150-fold selectivity for the 5-HT_{5A} receptor in comparison to a large panel of receptors, transporters and ion channels, with the exception of A-763079 (pKi 5-HT_7 = 6.9). Pharmacokinetic studies in rats showed that, administration of A-843277, A-833551 and A-763079 at doses of 10 mg/kg (i.p.) achieved a plasma Cmax of 0.47, 0.45 and 0.44 µg/ml, respectively. High brain penetration was observed for all three compounds (brain/plasma ratios up to 5.81). Thus, A-843277, A-833551 and A-763079 are valuable tools to elucidate both, 5-HT_{5A} receptor function and the therapeutic utility of 5-HT_{5A} receptor antagonists for CNS disorders (see companion communication).

Neuroscience Research, GPRD, Abbott, Ludwigshafen, Germany, and [1]Abbott Park, IL,USA

76

PHARMACOLOGICAL CHARACTERIZATION OF 5-HT_{5A} ANTAGONISTS: EFFECT ON SEROTONIN AUTORECEPTORS AND ACETYLCHOLINE RELEASE

K.U. Drescher[1], W. Amberg[1], A. Kling[1], K. Wicke[1], G. Gross[1], H. Schoemaker[1], J.P. Sullivan[2], D. Żelaszczyk[3], E. Schlicker[3], M. Göthert[3], F.J. Garcia-Ladona[1]

Serotonin 5-HT_{5A} receptors are exclusively expressed in the brain, e.g. in hippocampus, amygdala and frontal cortex. So far, their function could not be investigated due to the lack of selective ligands. In the present study we used the new potent and selective 5-HT_{5A} receptor antagonists, A-843277, A-833551 and A-763079, to study the role of 5-HT_{5A} receptors in i) presynaptic serotonergic functions in different brain areas and ii) the cortical cholinergic system.

The effects of A-763079 on the presynaptic serotonergic system were studied in mouse cerebrocortical and hippocampal slices preincubated with ^{3}H-serotonin. A-763079 1 µM did not affect the electrically evoked overflow by itself nor did it influence the inhibitory effect of the unselective serotonin receptor agonist 5-carboxamidotryptamine on tritium overflow. By

contrast, metitepine (via 5-HT_{1B} receptors) increased tritium overflow and attenuated the effect of 5-carboxamidotryptamine. In anesthetized rats, doses of 0.06-16 mg/kg A-763079 i.v. had no significant effect on the firing rate of serotonergic raphe neurons. The effect of 5-HT_{5A} receptor antagonists on extracellular levels of acetylcholine was investigated by microdialysis in the medial prefrontal cortex (mPFC) of freely-moving rats. A-763079, A-843277 and A-833555, administered i.p. 120 min after the start of sampling induced a dose-dependent and significant increase in acetylcholine levels. Our study demonstrates that 5-HT_{5A} receptor antagonists do not affect presynaptic 5-HT autoreceptors and have no effect on the firing rate of serotonergic brain stem neurons, but they enhance extracellular acetylcholine levels in the mPFC although the localization of these 5-HT_{5A} receptors involved in this modulatory effect remains to be clarified. Such an effect has previously been described for atypical antipsychotics. This finding, together with the recently communicated behavioral data (Jongen-Relo 2006, Society for Neuroscience 529.26) suggests that 5-HT_{5A} antagonists might be of therapeutic value for psychiatric disorders, in particular for schizophrenia with cognitive deficits.

Neuroscience Research GPRD, Abbott, [1]Ludwigshafen, Germany and [2]Abbott Park, IL, USA, and [3]Institute of Pharmacology, University of Bonn, Germany

77

DIFFERENT CYTOTOXIC EFFECTS OF REFERENCE TOXIN B AND VARIANT TOXIN B FROM *CLOSTRIDIUM DIFFICILE*

J. Huelsenbeck, S. Dreger, F. Hofmann, I. Just, H. Genth

Clostridium difficile, the causative agent of the antibiotic-associated diarrhoea, produces two toxins, toxin A (TcdA) and toxin B (TcdB). Both toxins glucosylate RhoA, Rac1, and Cdc42 while the homologous toxin B from "variant" strain 1470 serotype F (TcdB-F) glucosylates Rac1 and R-Ras but neither RhoA nor Cdc42. Accordingly, Rac1 was glucosylated by TcdB and TcdB-F in rat basophile leukaemia (RBL) cells. The glucosylation of Rac1 correlated with the actin re-organization ("cytopathic effect") caused by either toxin. RhoA and Cdc42 were degraded in TcdB- but not in TcdBF-treated cells, suggesting that the degradation was due to their glucosylation by TcdB. Furthermore, the immediate early gene product RhoB was strongly up-regulated in TcdB- but not in TcdB-F-treated cells. To address the question, whether induction of apoptosis ("cytotoxic effect") depended on the substrate specificity of the glucosylating toxins, RBL cells were synchronized by the thymidine double block technique prior to toxin treatment. RBL cells were most sensitive to the cytotoxic effect of TcdB during S-phase, as analysed in terms of caspase-3 activation, phosphatidyl serine exposure, and nuclear fragmentation. In contrast, TcdB-F induced only a marginal cytotoxic effect suggesting that inactivation of RhoA was required for the cytotoxic effect. Correspondingly, the viability of TcdB-treated RBL cells was reduced but remained unchanged in TcdB-F-treated cells. In conclusion, the protein substrate specificity of the glucosylating toxins determines their biological activity. These results offer an explanation why variant *C. difficile* strains such as strain 1470 producing TcdBF exhibit reduced virulence in animals and humans.

Institut für Toxikologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

78

PERIPHERAL SEROTONIN AND THE CONTROL OF FOOD INTAKE

B. Bert, H. Fink, D. J. Walther[1], J.-P. Voigt[2]

Introduction. Brain serotonin (5-HT) is involved in the regulation of emotions, circadian rhythm and food intake. Pharmacological studies have also suggested a satiating effect of peripheral 5-HT. Recently, two isoforms of the tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT synthesis, were detected. TPH2 seems to be predominantly expressed in the brainstem, whereas TPH1 appears in the gut, pineal gland, spleen, and thymus. In TPH1 deficient mice (*Tph1-/-*) relatively normal 5-HT levels were detected in the brain, whereas in the periphery low 5-HT levels were found (Walther et al. 2003 *Science* 299:76). Therefore, these mice seem to be a useful tool to further elucidate the role of peripheral 5-HT in satiety. Methods. Initially, food intake, body weight and activity of *Tph1-/-* mice were recorded. In order to distinguish between central and peripheral effects on food intake 5-HT was administered systemically, since it is unable to pass the blood-brain-barrier. Fenfluramine (i.p.), a 5-HT_{2C} receptor agonist and 5-HT releaser, was used as a positive control because of its primarily centrally mediated satiating effect. Results. *Tph1-/-* mice exhibited higher body weight, probably caused by increased food intake but not due to lower activity. The satiating effect of systemically administered 5-HT was more prominent in *Tph1-/-* mice, whereas fenfluramine induced hypophagia likewise in *Tph1-/-* and in control mice. *Conclusion.* The increase in food intake and body weight of *Tph1-/-* mice underlines the relevance of peripheral serotonin satiety mechanisms. Moreover, the pronounced effect of 5-HT in food intake of *Tph1-/-* mice argues for adaptive changes of the peripheral 5-HT receptors. The data further suggest a relatively independent action of peripheral and central 5-HT with regard to the control of feeding and satiety.

Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, 14195 Berlin, Germany; [1]Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany; [2]University of Nottingham, School of Veterinary Medicine and Science, Sutton Bonington Campus, Loughborough, Leics, LE12 5RD, UK

79

MOLECULAR EVIDENCE FOR THE INVOLVEMENT OF AMPA RECEPTOR SUBTYPES IN PAIN STATES

B. Hartmann[1], S. Ahmadi[2], C. Schott[5], T. Borchardt[6], I. Tegeder[3], K. Sluka[4], G.F.Gebhart[5], P.H. Seeburg, H.U.[6], Zeilhöfer[2], R. Sprengel[6] and R. Kuner[1]

We aim at addressing the functional role of the AMPA subtype of glutamate receptors in mechanisms of pain plasticity using genetically-modified mice. Although AMPA receptors are known to mediate fast synaptic transmission in pain pathways, not much was known so far about their potential involvement in the regulation of pain and nociceptive plasticity. We observed that the number of calcium-permeable AMPA receptors as well as AMPA receptor-mediated synaptic currents is dramatically decreased in the spinal dorsal horn of GluR-A knock-out (KO) mice. These mice demonstrated a loss of nociceptive plasticity and a reduction in inflammatory pain behaviours. In contrast, the loss of the GluR-B subunit was found to result in an increase in the number of spinal calcium-permeable AMPA receptors and AMPA receptor-mediated synaptic currents in the spinal dorsal horn. In line with these changes, GluRB KO mice showed enhanced nociceptive plasticity and inflammatory hyperalgesia.In a second approach, we are using the Cre-loxP system to create tissue-specific knock-out mice which lack the AMPA receptor subunits either only in the dorsal root ganglia (DRG) or in the forebrain. Preliminary analyses show that peripheral as well as cortical AMPA receptors contribute to inflammatory pain. Taken together, these data suggest that AMPA receptors are not mere determinants of fast synaptic transmission underlying basal pain sensitivity as previously thought, but are critically involved in activity-dependent changes in synaptic processing of nociceptive inputs.

[1]Pharmacology Institute, University of Heidelberg;
[2] Institute of Pharmacology and Toxicology, University of Zurich;
[3] Institute of Clinical Pharmacology, University of Frankfurt / Main;
[4] Graduate Program in Physical Therapy & Rehabilitation Science, University of Iowa;
[5]Department of Medicine, Gastroenterology, University of Pittsburgh;
[6]Max-Planck Institute for Medical Research, Heidelberg

80

REELIN INDUCES MEMBRANE MOTILITY IN CORTICAL NEURONS

J. Leemhuis[1,2], C. Schwan[1,2], F. Henle[1,2], M. Frotscher[1,3], J. Herz[1,4], D. K. Meyer[1,2] and H. H. Bock[1,5]

Reelin, a large secreted signaling protein, regulates neuronal positioning during neurodevelopment and modulates synaptic plasticity in the adult brain. It binds to the very low-density lipoprotein receptor and apolipoprotein E receptor 2, thereby activating Src family kinases resulting in the tyrosine phosphorylation of the cytoplasmic adaptor protein Disabled-1 (Dab1) and activation of phosphatidylinositol 3-kinase (PI3K). By controlling the stability and assembly of the actin cytoskeleton PI3Ks regulate neuronal morphogenesis and migration. Dendrite morphogenesis is highly dynamic and characterized by the addition and elongation of processes and also by their selective maintenance, retraction, and elimination. We report here that Reelin increases membrane motility in dendrites of cultured stage III embryonic cortical neurons of the mouse. Pretreatment of cortical neurons with pharmacological inhibitors of PI3K or genetic ablation of the adapter protein Dab1 abolished the effect of Reelin on membrane motility. Since glutamate modulates membrane motility via N-methyl-D-aspartic acid (NMDA) receptors in a PI3K-dependent manner, we tested the effect of NMDA and Reelin in combination, which resulted in an additive effect. Our results suggest a role of the Reelin signaling pathway in modulating the actin cytoskeleton, which results in an increased membrane motility.

This work was supported by the Deutsche-Forschungs-Gemeinschaft (SFB505 and BO1806/2-1) and the Humboldt-Foundation.

1: Center for Neuroscience Albert-Ludwigs-Universität Freiburg; 2: Institut für Experimentelle und Klinische Pharmakologie Albert-Ludwigs-Universität Freiburg, 3 Institute of Anatomy and Cell Biology Department of Neuroanatomy Albert-Ludwigs-Universität Freiburg , 4: Department of Molecular Genetics, UT Southwestern Medical Center at Dallas, USA 5: Department of Medicine II Albert-Ludwigs-Universität Freiburg

81

MOLECULAR MECHANISMS OF NOCICEPTIVE ACTIVITY-DEPENDENT CHANGES IN CALCIUM DYNAMICS OF SPINAL SENSORY NETWORKS

C. Luo, B. Hartmann, A. TappeTheodor, R. Kuner

Various converging lines of evidence have revealed the importance of glutamatergic signaling mechanisms and intracellular calcium mobilisation in the development of nociceptive plasticity which underlie chronic, pathological pain. It is known that activation of synaptic glutamate receptors can modulate calcium levels in neurons via activation of ionotropic receptors. Furthermore, a variety of metabotropic receptors which couple with G_q and G_{11} G-proteins can mediate calcium mobilisation from intracellular stores in spinal neurons upon activation. However, the relative contributions of these diverse receptors and signaling pathways to nociceptive activity-induced calcium dynamics in the spinal neuronal networks have not been systematically delineated. We have applied calcium imaging on populations of spinal dorsal horn neurons in acutely prepared mouse spinal cord slices to understand the spatiotemporal patterns and nature of calcium alterations upon activation of nociceptive primary afferent fibers. Acute spinal cord slices attached with dorsal root were prepared and loaded with Fura-2 and calcium responses were imaged in the superficial spinal laminae upon activation of nociceptors. We found that a large population of Ca^{2+} transients was evoked in the spinal dorsal horn by stimulation of the dorsal root at the intensity sufficient to activate C- and A-delta fibers, in a frequency-dependent manner. Using genetically-modified mouse mutants and pharmacological antagonists, we have elucidated the functional significance and relative contribution of NMDA receptors, calcium-permeable AMPA receptors, calcium channels as well as metabotropic Gq/11-coupled receptors to activity-induced changes in calcium signals in normal and chronic inflammatory pain states.

Pharmacology Institute, University of Heidelberg, Im Neuenheimer Feld 366,
Heidelberg 69120, Germany

82

ANALYSIS OF THE POTENCIES OF CANGRELOR AND REACTIVE BLUE 2 AT WILD TYPE AND MUTANT HUMAN P2Y12-RECEPTORS

K. Hoffmann, I. von Kügelgen

The P2Y12-receptor plays a crucial role in platelet aggregation. Previously we have identified a number of amino acid residues which are important for receptor function. In cells expressing R256A, R256D, H256A/R256A, Y259D or K280A mutant receptors the agonist potency of 2-methylthio-ADP was markedly reduced or abolished. In the present study, we now analyzed the effects of P2Y-receptor-antagonists at wild type and mutant P2Y12-receptors. Wild type and mutant P2Y12-receptors were stably expressed in 1321N1 astrocytoma cells or CHO Flp-In cells. Immunofluorscene staining with a monoclonal antibody against a receptor epitope revealed similar expression levels for cells transfected with wild type or mutant P2Y12-receptor constructs. Receptor function was assessed by measuring the 2-methylthio-ADP induced inhibition of cAMP-accumulation. At wild type P2Y12-receptors, 2-metylthio-ADP inhibited the cAMP-accumulation with an EC_{50}-concentration of 1 nM. The competitive nucleotide P2Y12-antagonist cangrelor (AR-C69931MX) shifted the concentration-response-curve of 2-methylthio-ADP to the right with a pK_B-value of 9.1. In contrast to the marked reduction in potency of the agonist 2-methylthio-ADP (EC_{50} = 23 nM) at the R256A mutant receptor, there was no change in the pK_B-value of cangrelor (pK_B = 9.0) at this mutant. Interestingly, cells expressing the S101A

mutant receptor showed no differences in potency of 2-methylthio-ADP (EC_{50} = 1.5 nM), whereas the antagonism of cangrelor at this mutant receptor was non-surmountable (apparent pK_B = 10.7). Next, we analyzed the effects of the non-nucleotide P2Y-antagonist reactive blue 2. At wild type P2Y12-receptors, reactive blue 2 had a pK_I-value of 6.2, and in cells expressing R256A mutant receptors we found a pK_B-value of 6.3. These data indicate that amino acid residues which seem to be of great impact for agonist function at this receptor play a minor role in binding of the antagonists to the P2Y12-receptor. In contrast, residues which have no effects on agonist function are important for antagonist binding.

Department of Pharmacology, University of Bonn, Bonn, Germany

83

MOLECULAR MECHANISMS OF ACTION OF THIOL REAGENTS AT THE HUMAN PLATELET P2Y12 RECEPTOR.

Algaier, I. von Kügelgen

Clopidogrel blocks platelet aggregation. The active metabolites of clopidogrel and other thienopyridine compounds, but not the compound themselves, inactivate the platelet P2Y12 receptor. It has been proposed that the active metabolites and other thiol compounds such as pCMBS (p-chloromercuribenzene sulfonic acid) interact with extracellular cysteine residues of the receptor. However, direct experimental evidence for this suggestion is lacking. In the present study we now searched for the residues involved in the interaction of pCMBS with the hP2Y12 receptor. Cys97 and Cys175 form one disulfide bridge which is essential for receptor function. A second disulfide bridge between Cys17 and Cys270 is not essential. For our experiments the extracellular cysteine residues of the P2Y12-receptor were replaced by alanine by site-directed mutagenesis. Receptor constructs were stably expressed in 1321N1-astrocytoma or CHO Flp-In cells. Changes in receptor function were assessed by measuring the 2-methylthio-ADP (0.01 nM to 1.0 µM)-induced inhibition of cellular cAMP accumulation. In cells expressing the wild type hP2Y12 receptor pCMBS blocked the responses to receptor stimulation with an EC50 of 0.9 µM. The effect was irreversible. In order to identify the interaction site of pCMBS, protection experiments with ATP and suramin were performed. Cells were preincubated with ATP (10 µM to 100 µM) and suramin (100 µM to 300 µM) for 20 min before the addition of pCMBS for 5 min. Then, all compounds were removed. Protection of the receptor by ATP and suramin abolished the inhibitory effects of pCMBS. In cells expressing Cys17Ala/Cys270Ala mutant receptor constructs, pCMBS blocked the effects to receptor stimulation with an identical EC50-concentration (1.0 µM). In contrast, in cells expressing Cys97Ala mutants any action of pCMBS (10 µM) was lost. In conclusion, pCMBS did not interact with Cys17 or Cys270 but with Cys97 (and possibly C175). The same may be true for active metabolites of thienopyridine compounds such as clopidogrel. Our results also suggest that the interaction site of thiol reagents is located near of the ligand binding site.

Department of Pharmacology, Universität of Bonn, 53113 Bonn, Germany.

84

CLONING AND FUNCTIONAL EXPRESSION OF A NOVEL G_I PROTEIN-COUPLED RECEPTOR FOR ADENINE FROM MOUSE BRAIN

von Kügelgen I[1], Schiedel AC[2], Hoffmann K[1], Alsdorf BBA[2], Abdelrahman A[1], Müller CE[2]

An orphan G protein-coupled receptor from the rat has recently been demonstrated to act as a transmembrane receptor for the nucleobase adenine. The receptor is likely to be involved in nociception. In the present study we searched for a receptor for adenine in mouse tissues. Using RT-PCR we found a murine sequence encoding a second member of the family of GPCRs for adenine. mRNA for this receptor was detected in mouse brain and in the mouse neuroblastoma x rat glioma hybrid cell line NG108-15. The mouse protein sequence shares 76 % identity with that of the rat suggesting that the receptors are not species homologues but distinct receptor subtypes. In human 1321N1 astrocytoma cells stably expressing the mouse adenine receptor, adenine and 2-fluoroadenine inhibited the isoproterenol-induced cAMP formation with IC_{50} concentrations of 8 and 15 nM, respectively. The adenosine receptor antagonist dipropylcyclopentylxanthine (DPCPX, 1 µM) as well as the P2-receptor antagonist suramin (300 µM) failed to change the responses to adenine indicating that adenine did not act via DPCPX-sensitive P1-receptors or suramin-sensitive P2Y-receptors. Pre-treatment of cells with pertussis toxin abolished the effect of adenine. When the mouse adenine receptor was expressed in Sf21 insect cells, a specific binding site for [^{3}H]adenine was detected. In competition assays adenine exhibited an IC_{50} value of 63.5 nM versus 10 nM [^{3}H]adenine. The rank order of potency was identical to that obtained in membranes from NG108-15 cells and rat brain cortex (adenine > 2-fluoroadenine > 1-methyladenine >> N^6-dimethyladenine). In summary, our data show that a second mammalian DNA sequence encodes for a G_I-coupled GPCR activated by low, nanomolar concentrations of adenine. The novel receptor may belong to an even larger, yet unknown family of receptors for purine nucleobases.

1 Department of Pharmacology and Toxicology, University of Bonn, Bonn, Germany; 2 Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany

85

BIOCHEMICAL EVIDENCE AGAINST $P2X_2$ AND $P2X_3$ RECEPTOR REGULATION BY DIRECT PKC-MEDIATED PHOSPHORYLATION

C. Franklin, G. Schmalzing and R. Hausmann

P2X receptors assemble from three identical or homologous membrane-bound subunits, surrounding a central ion pore, and act as ligand-gated, cation-selective ion channels. A common characteristic of all seven P2X family members ($P2X_1$-$P2X_7$) is a conserved consensus sequence (TXR/K) for protein kinase C (PKC)-mediated phosphorylation in the intracellular N-terminal domain. Both site-directed mutagenesis experiments and treatment of cells with the general PKC activator phorbol-12-myristate-13-acetate (PMA) lend support to the conclusion that phosphorylation controls the electrophysiological kinetics of the $P2X_2$ and $P2X_3$ receptors (Boué-Grabot et al., JBC 2000; Brown and Yule, BBA 2006). Here we show that perturbation of the predicted PKC site by mutating ^{20}K to ^{20}T changes the electrophysiological phenotype of the rat $P2X_2$ receptor from non-desensitizing to fast desensitizing. To examine biochemically whether wild type $P2X_2$ or $P2X_3$ receptors serve as substrates for PKC or other kinases, detergent lysates of *X. laevis* oocytes or HEK293 cells expressing recombinant $P2X_2$ or $P2X_3$ receptors were prepared before and after PMA treatment and resolved by SDS-PAGE. Probing with phospho-specific antibodies (anti-Phospho-Threonine-X-Arginine and anti-Phospho-Threonine) gave no signal despite the presence of significant amounts of P2X receptors as visualized by immunoblotting with specific antibodies. Phosphorylation could also not be demonstrated by an in vitro kinase assays using [γ-^{32}P]ATP and affinity-purified $P2X_2$ and $P2X_3$ receptors as substrates of purified rat brain PKC (catalytic subunit). Also phosphorylation experiments in intact cells with ^{32}P-orthophosphate showed no ^{32}P incorporation in $P2X_2$ and $P2X_3$ receptors. In contrast, the splicing factor SF3B1 and a PKC-specific substrate peptide used as control substrates were efficiently phosphorylated under otherwise identical conditions in the in vitro assay as well as in intact cells. Taken together, our experiments show that site-directed perturbation of the predicted PKC site dramatically changes the desensitizing properties of the rat $P2X_2$ receptor. However, a mechanism other than direct PKC-mediated threonine phosphorylation must be responsible for the desensitizing effect of the PKC site mutations.

Molecular Pharmacology, RWTH Aachen University, 52074 Aachen.

86

FUNCTIONAL AND BIOCHEMICAL EVIDENCE FOR AN INTER-SUBUNIT LOCALISATION OF THE ATP BINDING SITE IN P2X RECEPTORS LOCALIZATION

B. Marquez-Klaka[1], J. Rettinger[2], and A. Nicke[1]

P2X receptors (P2XRs) represent a family of neurotransmitter-gated cation channels. They are activated by extracellular ATP and assembled as homo- or heterotrimers of homologous subunits. Each subunit contains two transmembrane domains linked by a large extracellular loop. So far, there is no direct evidence whether the agonist binding site is formed within one subunit, as in the ionotropic glutamate receptors, or at the interface of two neighbouring subunits, as in the Cys-loop receptor family. Here we used a disulfide crosslinking approach to identify pairs of residues that are in close proximity within the ATP binding site of the $P2X_1R$. Eight amino acid residues presumed to contribute to ATP binding (K68, K70, F185, K190, F291, R292, R305, K309) were substituted by cysteine residues and the respective mutant subunits were pairwise coexpressed in *Xenopus laevis* oocytes. Non-reducing SDS-PAGE analysis of the purified receptors revealed a spontaneous and specific disulfide bond formation between the K68C and F291C mutants. An almost complete cross-link into trimers was achieved with the K68C/F291C double mutant, suggesting an inter-subunit disulfide crosslink. To analyze the functional consequences of this crosslink we introduced the respective mutations in a non-desensitizing $P2X_{2\text{-}1}$chimera. Two-electrode voltage-clamp analysis of the K68C/F291 double mutant revealed only small ATP-activated currents which increased about 60-fold upon reduction by DTT. This current increase was reversible under oxidizing conditions. In support of an inter-subunit localisation of the ATP binding site, disulfide bond formation between neighbouring subunits was prevented in the presence of ATP. Furthermore, a non-functional K68C/K309C double mutant could be functionally rescued by coexpression with non-mutated subunits. In summary, these data provide first direct evidence for the spatial arrangement of these functionally important residues and for an inter-subunit localisation of the ATP-binding site in P2XRs. Based on these findings, strategies to investigate the activation mechanism and heteromerization of P2XRs will be discussed.

[1]Max Planck Institute for Brain Research, Dept. of Neurochemistry, Frankfurt, Germany
[2]Max Planck Institute of Biophysics, Dept. of Biophysical Chemistry, Frankfurt, Germany

87

A MEMBRANE NETWORK OF RECEPTORS AND ENZYMES FOR ADENINE NUCLEOTIDES AND NUCLEOSIDES REVEALED BY FRET MICROSCOPY

S. Hussl[1], H.H. Sitte[1], S. Boehm[1,2].

ATP and related nucleotides are present in and released from all types of cells. Membrane receptors for adenine nucleotides and nucleosides are widely distributed, and in the membranes there also are ectonucleotidases which degrade the nucleotides and thereby generate nucleosides. To search for a specific arrangement of these proteins at the cell surface, fusion proteins of several P2 receptors, adenosine receptors and NTPDases1 and -2 with yellow (YFP) and/or cyan (CFP) fluorescent proteins were expressed in tsA 201 cells, and their interactions where investigated by fluorescence resonance energy transfer (FRET) microscopy. $P2X_2$ receptor subunits tagged with CFP and YFP, respectively, showed a strong interaction resulting in normalized FRET values (N_{FRET}, Xia and Liu, Biophys J 81, 2395, 2001) of 0.148$\pm$0.008 (n=60), thus corroborating oligohomomeric assembly. The N_{FRET} values for separate YFP and CFP (0.033$\pm$0.004; n=77) served as negative control. Co-expression of $P2X_2$ and NTPDase1 gave negative N_{FRET} of 0.041$\pm$0.005 (n=59; p>0.05 vs CFP/YFP). In contrast, $P2Y_1$ and NTPDase1 gave positive N_{FRET} of 0.143$\pm$0.008 (n=81; p >0.05 vs. $P2X_2$/$P2X_2$). However, N_{FRET} for $P2Y_1$ and NTPDase2 were negative (0.039$\pm$0.006; p>0.05 vs CFP/YFP). In analogous measurements, $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$, A_1 and A_{2A} receptors were also found to give positive N_{FRET} values with NTPDase1, but not NTPDase2. For $P2X_2$ receptors, negative N_{FRET} values were obtained together with all P2Y receptors and NTPDase2. In contrast, expression of $P2Y_1$ tagged with CFP and YFP, respectively, and co-expression with the other P2Y receptors gave positive N_{FRET}, thus indicating homo- and heterooligomerization of P2Y receptors. However, with mGluR5 used as a control, $P2Y_1$ and NTPDase1 yielded negative values. These results indicate that P2Y and adenosine receptors may interact with each other and with ectonucleotidases to form a sophisticated network of nucleotide and nucleoside binding and degrading sites at the membrane.

Supported by the FWF (P 17611).

[1]Institut für Pharmakologie, Medizinische Universität Wien, Währingerstrasse 13a, A-1090 Wien, und [2]Institut für Experimentelle und Klinische Pharmakologie, Medizinische Universität Graz, Universitätsplatz 4, A-8010 Graz.

88

ROLE OF PROTEINKINASE C IN THE DESENSITIZATION OF STIMULATORY AND INHIBITORY EFFECTS OF B2 BRADYKININ RECEPTORS IN RAT SYMPATHETIC NEURONS

K. Kosenburger[1], K. Schicker[1], H. Drobny[1], and S. Boehm[1,2]

Bradykinin is known to exert two opposing actions in rat sympathetic neurons via B_2 receptors: an inhibition of M type K^+ currents (I_M) which leads to a stimulation of transmitter release and an inhibition of N-type Ca^{2+} currents (I_{Ca}) which leads to a presynaptic inhibition of transmitter release Here, we investigated mechanisms underlying the desensitization of these two types of responses. K^+-evoked release of previously incorporated [^{3}H] noradrenaline from primary cultures of dissociated rat superior cervical ganglia was inhibited by 1µM bradykinin in a time-dependent manner: 28.1±3.4% inhibition, when the peptide was present for 1min prior to the K^+ stimulus, 15.2±3.1 and 4.5±4.0% inhibition, when it was present for 3 and 7min, respectively. In the presence of the protein kinase C inhibitor bisindolylmaleimide I (0.3µM), the values of inhibition after 1, 3, and 7min amounted to 46.6±4.4, 32.7±2.4, and 12.7±3.1%, respectively. When cultures had been treated with phorbol-12-myristate-13-acetate (PMA; 1µM for 24h), these values were 59.2±4.4, 55.0±2.8, and 30.2±1.0%. Bradykinin (1µM) reduced I_{Ca}, yielding a maximum inhibition of 40.5±16.2% after about 1min; after 2min the inhibition had decreased to 21.6±17.7%, and after 7min to 9.7±14.4%. In the presence of bisindolylmaleimide I, the values of inhibition after 1, 2, and 7min were 60.0±7.0, 41.7±9.3, and 22.4±11.2%; in PMA-treated cells, they were 66.7±6.6, 49.9±9.6, 33.0±16.1%. Conversely, a 10min application of PMA accelerated the decline in the inhibition of I_{Ca}. Bradykinin (1µM) also reduced I_M, but the maximum inhibition was only achieved after 2min and declined slowly. The time course of I_M inhibition was not significantly altered by either of the above manipulations. These results show that inhibitory and stimulatory actions of B_2 bradykinin receptors in sympathetic neurons desensitize with different time courses, and PKC is only involved in the desensitization of the inhibitory actions.

Supported by the Austrian Science Fund, FWF (P17611).

[1]Institut für Pharmakologie, Medizinische Universität Wien, Währingerstrasse 13a, A-1090 Wien, und [2]Institut für Experimentelle und Klinische Pharmakologie, Medizinische Universität Graz, Universitätsplatz 4, A-8010 Graz.

89

ROLE OF EGF RECEPTORS AND C-CBL IN OPIOID-INDUCED ERK ACTIVATION

D.A. Eisinger, H. Ammer

δ-Opioid receptor (DOR) activation is linked to stimulation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), members of the mitogen-activated protein (MAP) kinase family. Recent studies indicate that individual opioid compounds may differ significantly in the duration of the ERK signal elicited after chronic agonist treatment. Whereas the high-efficacy opioid etorphine produces only transient stimulation of ERK1/2, the partial agonist morphine is able to induce long lasting stimulation of the MAP kinase signalling pathway. In order to identify the cellular mechanism underlying the differences in ERK1/2 dynamics, we compared the intracellular signalling pathways associated with both opioid ligands. Using stably DOR transfected human embryonic kidney cells (HEK/DOR), we show that opioid-induced ERK1/2 activation is mediated by epidermal growth factor receptors (EGFRs), since stimulation of ERK phosphorylation by maximum effective etorphine (100 nM) and morphine (1 µM) concentrations was blocked by the selective EGFR inhibitor 4-3-chloroanilino-6,7-dimethoxyquinazoline (AG1478; 5 µM). Moreover, EGFRs appear to contribute to the different kinetics of opioid-induced MAP kinase stimulation, because prolonged exposure of HEK/DOR cells to etorphine (100 nM; 60 min), which rapidly desensitizes ERK1/2 activation, also impairs stimulation of ERKs upon EGF exposure (10 ng/ml; 5 min). In contrast, EGF-induced MAP kinase stimulation was not affected by pre-treatment of the cells with morphine (1 µM, 60 min). The opioid-specific effects on EGFR activity might be attributed to c-Cbl, an ubiquitin ligase terminating EGFR signalling. Whereas chronic morphine treatment results in down-regulation of c-Cbl as determined by Western blot technique, exposure to etorphine had no effect on overall c-Cbl abundance in HEK/DOR cells. The finding that chronic morphine-induced down-regulation of c-Cbl is sensitive to 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2; 10 µM), a potent and selective inhibitor of the Src-family tyrosine kinase, further suggests that degradation of c-Cbl by a Src-kinase dependent mechanism and thus sustained activation of EGFRs may account for prolonged stimulation of ERKs by morphine-activated DORs.

Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Koeniginstrasse 16, D-80539 Munich, Germany

90

CHARACTERIZATION OF OPIOID RECEPTORS IN MURINE TUMOR CELL LINES

J.F. Renner, H. Ammer

Opioids are frequently used to provide adequate pain-management in cancer therapy. Besides their analgesic activity, recent studies reported controversial effects of opioids on tumor progression. Thus, the present study was designed to examine the effects of selective [mu]-, [delta]-, and [kappa] opioid receptor (OR) agonists and morphine on tumor cell proliferation. Among several cell lines tested, we found that the selective [delta]OR agonist deltorphine II (Delt II) and the selective [kappa]OR agonist [(−)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl] benzeneacetamide] (U50-488H) both significantly increased proliferation of B16 melanoma cells over a period of 5 days. In contrast, C26 colon carcinoma cells responded with an enhanced cell growth to the selective [mu]OR agonist sufentanil and [kappa]OR agonist U50-488H. In each case, colchicine was used as a negative control. Interestingly, the non-selective opioid agonist morphine as well as diverse inverse opioid agonists failed to affect cell proliferation. The presence of the respective opioid receptors was verified by radioligand binding studies using [3H]diprenorphine as the tracer. The functional integrity of ORs was further characterized by determination of their capacity to regulate intracellular cAMP production as well as extracellular signal-regulated protein kinase (ERK1/2). Activation of [delta]- and [kappa]ORs both significantly influenced intracellular cAMP production, but failed to regulate ERK1/2 activity in B16 melanoma cells. In contrast, activation of [mu]- and [kappa]ORs had no effect on adenylyl cyclase activity, but strongly stimulated ERK1/2 phosphorylation in C26 colon carcinoma cells. These results demonstrate that both B16 and C26 malignoma cell lines carry functionally G protein-coupled opioid receptors and that chronic opioid treatment results in enhanced cell proliferation.

Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Koeniginstrasse 16, D-80539 Muenchen, Germany

91

cAMP-INDEPENDENT REGULATION OF OPIOID RECEPTOR FUNCTION BY ADENYLYL CYCLASES

A. E. Seuberth, H. Ammer

The cAMP second messenger system plays a critical role in the development of opioid tolerance, dependence and addiction. Recent studies indicate that in addition to its catalytic activity, type V adenylyl cyclase (AC) may also function as a GTPase-activating protein accelerating the GDP-exchange of both inhibitory and stimulatory G[alpha] subunits. In the present study, we examined whether regulatory domains of distinct AC isoforms might possibly produce feedback regulation of mu-opioid receptor (MOR) activity after acute and chronic agonist treatment. For this, we generated fusion proteins consisting of the intracellular C1ab or C2ab domains of adenylyl cyclase types II and V, respectively, and the transmembrane portion of the CD8 T cell receptor as a membrane anchor. These constructs lack catalytic activity, but still retain their G[alpha] and G[beta/gamma] binding sites allowing the identification of cAMP-independent AC effects on intracellular signal transduction. The functional integrity of the constructs was tested after transfection into NG108-15 cells. We found that the C1ab domain of AC type V (ACV-C1), representing the G_i[alpha] binding site, completely blocks both acute and chronic opioid regulation of AC activity. Expression of the C2ab domain of AC type II (ACII-C2), which acts as a G[beta/gamma] scavenger, had no effect on acute opioid inhibition of AC activity but prevented the development of AC supersensitivity after chronic morphine treatment. For examination of cAMP-independent effects, the constructs were transiently transfected into COS-7 cells stably expressing the rat MOR. These cells lack an endogenous AC isoform that is subject to regulation by G_i-coupled receptors. Using this model system, ACV-C1 was found to enhance MOR activity in chronically morphine treated cells as determined by an increased capacity of [D-Ala2, D-Leu5]-enkephalin to stimulate GTP[gamma]S binding, activation of extracellular signal-regulated protein kinase (ERK1/2) and agonist-induced receptor internalization. In contrast, ACII-C2 accelerated desensitization of MOR function after chronic morphine treatment. These findings demonstrate signalling properties of distinct AC isoforms independent of their catalytic activity.

Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Koeniginstrasse 16, D-80539 Muenchen, Germany

92

EXPRESSION OF CYCLOOXYGENASES AND PROSTAGLANDIN RECEPTORS IN ARTICULAR CARTILAGE OF YOUNG RATS – AN IN SITU STUDY

C. Brochhausen[1], G. Klaus[2], R. M. Nüsing[3], C. J. Kirkpatrick[1]

Background: Cyclooxygenases are involved in various physiological and pathophysiological conditions as well as in developmental processes. Cyclooxygenase-1 plays a role as a housekeeping enzyme and is detectable in nearly all tissues, whereas cyclooxygenase-2 is inducible, for example, in inflammation, cancerogenesis but also in organ growth during fetal development. The effects of prostaglandins are mediated by four isoforms of prostaglandin receptors (EP-1 - EP-4). The role of cyclooxygenases in bone development and metabolism is already known from various *in vitro* systems. Our group has demonstrated the role of cyclooxygenases in the growth plate *in situ*. However, the expression and distribution of cyclooxygenases in articular cartilage is not yet known. In the present study we examined the expression and spatial distribution of COX-1, COX-2 and EP1 – EP-4 in the articular cartilage of the tibia in 6 weeks old Sprague Dawley rats.

Material and Methods: Frozen sections (4µm) from 4 week old (60-80g) Sprague Dawley rats were analysed by the APAAP-method with polyclonal rabbit antibodies directed against COX-1, COX-2 and prostaglandin receptors (EP-1 – EP-4).

Results: Articular cartilage of young Sprague Dawley rats showed expression of COX-1 and COX-2 as well as EP-1 – EP-4 receptors with a characteristic distribution. In particular, the apical cell layers are negative for these antigens.

Conclusion: Further studies by molecular techniques are needed, including microdissection to verify the special distribution of cyclooxygenases and prostaglandin receptors. Since in the literature discrepancies in COX expression in different species are described, it has to be verified if COX-expression is species-specific, or if the discrepancies could be explained on the basis of different age of analysed specimens. Furthermore, the expression pattern of COX and EP-receptors in human embryonic tissue and tissue from children are ongoing to analyse the role of cyclooxygenases and prostaglandin receptors in the development and metabolism of human articular cartilage with respect to possible side effects of COX-inhibition.

[1]REPAIR-lab, Institute of Pathology, Johannes Gutenberg-University, Mainz, Germany
[2]Department of Pediatrics, Philipps University, Marburg, Germany
[3]Department of Clinical Pharmacology, Johann Wolfgang von Goethe-University, Frankfurt/Main, Germany

93

The non COX-2 inhibitor methylcelecoxib (DMC) inhibits PGE_2 production by influencing mPGES-1 activity and expression in HeLa cells

I. Wobst, S. Schiffmann, T. J. Maier, A. Janssen, R. Schmidt, C. Angioni, G. Geisslinger and S. Grösch.

Methylcelecoxib (4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene-sulfonamide) as a derivative of celecoxib is well known as non COX-2 inhibitor at pharmaceutically reasonable concentrations. Surprisingly, we found that DMC inhibits PGE_2 production (IC_{50} = 5 µM) in IL-1beta and TNF-alpha stimulated HeLa (human cervix carcinoma cells) and A549 (human lung carcinoma cells) cells. Because this substance was reported to have no impact on COX-2 activity at these concentrations we looked for downstream enzymes for PGE_2 production, the prostaglandine E_2 synthases (PGES). HeLa cells express the cytosolic PGES (cPGES) and the microsomal PGES-2 (mPGES-2) constitutively whereas mPGES-1 is

highly inducible in these cells after treatment with IL-1beta and TNF-alpha. After stimulation, mPGES-1 is therefore the most responsible enzyme for PGE_2 production. In a cell free assay we found that DMC inhibits mPGES-1 activity but only at rather high concentrations (IC_{50} = 12 µM) and up to 60 % of total activity. Therefore, we further analysed mPGES-1 mRNA and protein expression after treatment of HeLa cells with DMC. Simultaneous treatment of HeLa cells with IL-1beta and TNF-alpha as well as DMC diminished (mRNA) and prevented (protein) mPGES-1 upregulation, respectively. However, these effects again take place at rather high concentrations(20-40 µM) which may not explain the low IC_{50} for PGE_2 inhibition. Expression of cPGES or mPGES-2 was not influenced by DMC. In conclusion, we were able to show that the non COX-2 inhibitor methylcelecoxib inhibits PGE_2 production in part by inhibition of mPGES-1 activity and expression in human HeLa cells. On the basis of the concentrations necessary for PGE_2 inhibition and mPGES-1 inhibition we conclude that methylcelecoxib has also an impact on further mechanisms which until now are not known.

The work is supported by the Deutsche Forschungsgemeinschaft (European Graduate School "Roles of Eicosanoids in Biology and Medicine" GRK 757).

pharmazentrum frankfurt, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany

94

OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION IN DIETARY INDUCED IRON OVERLOAD IN RATS

Schwedhelm E, Kom GD, Khaljani E, Jaoulak H, Sultan K, Heeren J, Böger RH, Beisiegel U, Nielsen P

Background: We have previously reported increased oxidative stress in patients with iron overload disease (hemochromatosis). Whether oxidative stress is associated with altered endothelial function is subject to recent investigation.

Methods: Using a well-known animal model for severe experimental iron overload (feeding of 0.5 % TMH-ferrocene), the impact of iron on endothelial function was studied in rats. Female Wistar rats were fed a control diet or high ferrocene diet (HFE) for 10 weeks (n=6 in each group). Endothelium –dependent and -independent relaxation of isolated aortic rings was measured in organ bath experiments. Iron overload was examined in the liver. 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in urine was quantified by gas chromatography-mass spectrometry (GC-MS) as a reliable marker of oxidative stress.

Results: Feeding a diet enriched with TMH-ferrocene resulted in a severe liver siderosis in the HFE-group compared to controls ($p<0.01$ for hepcidin antimicrobial peptide(HAMP) expression and liver iron). The endothelium –dependent and -independent relaxation were substantially abolished in HFE vs. control ($p<0.01$, both). Urinary 8-iso-$PGF_{2\alpha}$ excretion was increased in HFE vs. control (3.53 ±0.85 vs. 0.70 ±0.19 ng/mg creatinine, mean ±SEM, $p=0.01$).

Conclusion: The TMH-ferrocene model of dietary iron overload in rat results in a marked vascular endothelial dysfunction which is probably caused by iron-induced oxidative stress *in vivo*.

Institute of Experimental and Clinical Pharmacology and Toxicology, and Department of Biochemistry and Molecular Biology II: Molecular Cell Biology, Center of Experimental Medicine, University Medical Center Hamburg-Eppendorf, Germany

95

THE MODULATOR OF VASCULAR OXIDATIVE STRESS AND APOPTOSIS, PARAOXONASE 2, INTERACTS WITH THE TRANSCRIPTION FACTOR TFII-I

S. Horke[1]*, I. Witte[1], P. Wilgenbus[1], D. Strand[2] and U. Förstermann[1]

The pathophysiology of diseases such as atherosclerosis and diabetes is causally linked to formation of reactive oxygen species (ROS). In the vascular wall, it has been shown that ROS production occurs in all layers, intima, media and adventitia. Enhanced vascular ROS can damage cells directly and / or promote oxidation of e.g. low density lipoprotein particles. These may accumulate in the subendothelial space and enhance plaque formation and progression. Lipid accumulation at these sites causes macrophage infiltration. The macrophages will take up oxidized lipids and lipid overload of the cells will induce stress reactions in the endoplasmic reticulum (ER). This induces a pathway known as the unfolded protein response (UPR), which may promote apoptosis of macrophages and secondary necrosis. We recently found that the human enzyme paraoxonase 2 (PON2) significantly reduces vascular ROS formation in cells characteristic of the intima, media and adventitia. Because substantial amounts of PON2 are found in the ER, we analyzed its putative involvement in UPR signaling. PON2 was upregulated at the transcriptional level by ER stress. Functionally, we provide evidence that PON2 controls caspase activity stimulated by ER stress. Thus, PON2 reduces vascular ROS and UPR mediated apoptosis, hence representing a potential anti-atherogenic principle. To address pathways involved, we investigated interactions of PON2 with other proteins and found transcription factor TFII-I. TFII-I is a multifactorial transcription factor, which facilitates communication between the basal machinery assembled at core promoters of various genes and the activator complexes assembled at upstream regulatory site(s). The interaction of PON2 and TFII-I is not altered by ER stress. However, upon UPR provoked by disturbed Ca^{2+} homeostasis, i.e. thapsigargin treatment PON2 translocates to the nucleus in a TFII-I dependent fashion. Functional consequences of this protein-protein interaction and PON2 translocation are currently being investigated.

Departments of [1]Pharmacology and [2]Internal Medicine I, University of Mainz, Germany

96

EFFECT OF CHRONIC TREATMENT WITH PENTAERYTHRITYL TETRANITRATE AND OF INCREASED ENDOGENOUS NITRIC OXIDE FORMATION ON THE EXPRESSION AND ACTIVITY OF EXTRACELLULAR SUPEROXID DISMUTASE IN-VIVO.

M. Oppermann[1], V. Balz[2], T. Suvorava[1], Z. Hassan-Oglu[1], G. Kojda[1]

Studies in mice suggest that endogenous nitric oxide (NO) stimulates vascular expression of the antioxidative enzyme extracellular superoxide dismutase (ecSOD) in-vivo. For example, ecSOD expression significantly increased with an elevated expression and activity of endothelial NO-synthase (eNOS) induced by exercise training. Likewise, ecSOD expression in eNOS-knockout mice was strongly reduced as compared to controls. We investigated whether changes in ecSOD expression and activity occur after chronic treatment with the NO-donor pentaerythrityl tetranitrate (PETN) and in transgenic mice characterized by a vascular specific overexpression of eNOS ($eNOS^{++}$-mice) exhibiting 3.5-fold aortic eNOS-levels and elevated vascular NO levels (49.4±19.7 vs. 19.1±8.7 pmol NO/µg protein/min as measured by electron spin resonance) compared to transgene negative mice. C57BL/6 mice were randomly divided into four groups (n=7 each) which received either placebo (PETN-0), 6mg (PETN-6), 60mg (PETN-60) or 300mg PETN/kg BW/day (PETN-300) for four weeks. Resorption of PETN was controlled by measuring plasma levels of the mono- and dinitrate metabolites. Expression of ecSOD was determined by western blot analysis of lung cytosols. EcSOD expression was increased in all of the PETN-treated groups: protein levels increased 3-fold in PETN-6, 6-fold in PETN-60 and 5-fold in PETN-300, each compared to PETN-0 ($P<0.05$). Similar, western blots of $eNOS^{++}$ mice lung cytosols showed an increase of ecSOD expression to 160±23% as compared to transgene negative littermates (n=4, $P<0.05$). EcSOD activity in lung homogenates was assayed by spectrophotometric measurement of xantine oxidase inhibition rate after separation on concanavalin A-sepharose. There was a significant increase of ecSOD activity in $eNOS^{++}$ (729±32 U/mg total protein) compared to transgene negatives (592±32 U/mg, $P<0.05$). These data suggest that pharmacologically induceable chronic increase of vascular NO bioavailability, as well as an increase of endogenous NO formation result in significant upregulation of ecSOD in-vivo. Thus, ecSOD upregulation may contribute to the antioxidative effects of NO.

[1]Institut fuer Pharmakologie und klinische Pharmakologie, [2]Hals-, Nasen- und Ohrenklinik, Universitätsklinikum Düsseldorf, Moorenstr. 5, 40225 Duesseldorf

97

RECIPROCAL REGULATION OF ENOS AND NADPH OXIDASE BY BETULINIC ACID IN HUMAN ENDOTHELIAL CELLS

K. Steinkamp-Fenske, L. Bollinger, Y. Yao, H. Xu, U. Förstermann, and H. Li

Nitric oxide (NO) produced by endothelial NO synthase (eNOS) is a protective principle in the vasculature. Many cardiovascular diseases are associated with reduced NO bioactivity and eNOS uncoupling due to oxidative stress. Compounds that reverse eNOS uncoupling and increase eNOS expression are of therapeutic interest. *Zizyphi spinosi semen* (ZSS) is one of the most widely used traditional Chinese herbs with protective effects on the cardiovascular system. In human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells, an extract of ZSS increased eNOS promoter activity, eNOS mRNA and protein expression, as well as NO production in a concentration- and time-dependent manner. Major ZSS constituents include saponins such as jujuboside A and B and triterpenoids such as betulin and betulinic acid. Jujuboside A, jujuboside B, or betulin had no significant effect on eNOS expression whereas betulinic acid increased eNOS mRNA and protein expression in HUVEC and EA.hy 926 cells. Interestingly, betulinic acid also attenuated the expression of NADPH oxidase subunits Nox4 and p22phox, thereby reducing oxidative stress and improving eNOS function. Consequently, betulinic acid-treated endothelial cells showed an increased production of bioactive NO (as indicated by a higher efficacy in stimulating cGMP generation in RFL-6 reporter cells). Thus betulinic acid possesses combined properties of eNOS upregulation and NADPH oxidase downregulation. Compounds such as betulinic acid may have a therapeutic potential in cardiovascular disease.

Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany

98

ENOS-UPREGULATING COMPOUNDS FROM "VASOACTIVE" CHINESE HERBS

H. Li, K. Steinkamp-Fenske, L. Bollinger, Y. Yao, H. Xu, R. Bauer, and U. Förstermann

Cardiovascular diseases are associated with reduced NO bioactivity and enhanced oxidative stress. Therefore, a combined upregulation of eNOS and downregulation of NADPH oxidase may have therapeutic potential. The purported effects of "circulation-improving" herbs according to the Traditional Chinese Medicine show striking similarities with the vascular actions of eNOS-derived NO. In the present study, we tested extracts of 17 Chinese herbs known to have potential effects on the vasculature for their effect on eNOS gene expression. The results demonstrated that aqueous extracts of *Salvia miltiorrhiza* L., *Prunella vulgaris* L., and *Zizyphus jujuba spinosa* significantly increased eNOS promoter activity, eNOS mRNA and protein expression, as well as NO production in EA.hy 926 cells, a cell line derived from human umbilical vein endothelial cells (HUVEC). We then analyzed the effects of known constituents of these three herbs on eNOS expression. Among such compounds, ursolic acid, betulinic acid, luteolin and cynaroside were capable to increase eNOS expression in HUVEC and EA.hy 926 cells. Interestingly, ursolic acid and betulinic acid also attenuated the expression of NADPH oxidase subunit Nox4, thereby reducing oxidative stress and improving eNOS functionality. Consequently, ursolic acid- or betulinic acid-treated endothelial cells showed an increased production of bioactive NO (as indicated by a higher efficacy in stimulating cGMP generation in RFL-6 reporter cells). Thus, some natural compounds from Chinese herbs possess dual protective effects: upregulation of eNOS and a parallel downregulation of NADPH oxidase. The resulting increase in bioactive NO could mediate some of the beneficial effects of such medicinal plants.

Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany; Department of Pharmacognosy (R.B.), Karl Franzens University, Graz, Austria

99

PIOGLITAZONE UPREGULATES GLUTATHIONE PEROXIDASE 1 EXPRESSION IN VITRO AND IN VIVO

G. Spanier, H. Xu, V. Ochsenhirt, U. Gödtel-Armbrust, S. Deng, L. Wojnowski, M. Torzewski, K.J. Lackner, U.Förstermann, and H. Li

Glutathione peroxidase 1 (GPx1) is a key antioxidant enzyme in many cells including endothelial cells. Using glutathione as its substrate, GPx1 reduces hydrogen peroxide and lipid peroxides and acts as a peroxynitrite reductase. A low GPx1 activity is associated with an increased risk for cardiovascular events in patients with documented coronary artery disease, and an increase in GPx1 activity has been shown to reduce cardiovascular risk. Therefore, a pharmacologic enhancement of GPx1 expression and/or activity may be of prophylactic and therapeutic interest. Treatment of human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 endothelial cells with pioglitazone (1 – 100 micromol/L) resulted in a concentration-dependent increase in GPx1 mRNA and protein expression. Pioglitazone is an agonist of peroxisome proliferator-activated receptor gamma (PPARgamma). The compound is used for the treatment of type 2 diabetes mellitus, based on its ability to reduce glucose and lipid levels in

diabetic patients. The effect of pioglitazone on GPx1 expression was prevented by the PPARgamma antagonists GW9662 (10 micromol/L) and T0070907 (1 micromol/L). In human endothelial cells, GPx1 mRNA had a half-life of about 20 hours and treatment with pioglitazone did not enhance GPx1 mRNA stability. Thus, the increased mRNA expression of GPx1 in response to pioglitazone is likely a result of an enhanced gene transcription. Treatment of wild type C57BL/6 mice with pioglitazone (20 mg/kg, ≥2 weeks) increased GPx1 mRNA expression and GPx1 activity in the heart and in red blood cells. This effect was also seen in mice deficient of endothelial NO synthase, indicating that the effect of pioglitazone on GPx1 expression is NO-independent. In conclusion, our data demonstrate that pioglitazone enhances GPx1 expression and activity in a PPARgamma-dependent manner. This novel property of pioglitazone may contribute to the pleiotropic protective effects of this drug observed in clinical studies.

Department of Pharmacology and Institute of Clinical Chemistry and Laboratory Medicine, Johannes Gutenberg University, Mainz, Germany

100

ROLE OF ENDOGENOUS HYDROGEN PEROXIDE IN EXERCISE-INDUCED MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS

T. Suvorava, S. Kumpf, V. Adams*, R. Hambrecht*, G.Kojda

Bone marrow-derived endothelial progenitor cells (EPCs) characterized by coexpression of vascular endothelial growth factor receptor 2 (FLK-1, KDR) and the hematopoeitic stem cell markers (CD34 and CD133) have been shown to augment neovascularization of tissue after ischemia and contribute to reendothelization and improvement of organ perfusion.

We thought to determine whether hydrogen peroxide, an important component of vascular oxidative stress influences EPC mobilization from bone marrow. Transgenic mice with a vascular-specific overexpression of catalase and reduced vascular levels of hydrogen peroxide (cat^{++}) were assigned to a sedentary group and a group undergoing moderate exercise training (15 m/min, 30 min, 5 days a week, 3 weeks) and compared to their non-transgenic littermates (catn) undergoing the same protocol. Fluorescence-activated cell sorter analysis of peripheral blood of cat^{++} and cat^{n} revealed no effect of catalase overexpression on the basal level of circulating EPCs counted as CD3 negative and CD34/Flk-1 and CD133/Flk-1 double positive cells. Inhibition of catalase by 3 week treatment with catalase inhibitor aminotriazole (670 mg/kg dissolved in drinking water) strongly reduced the number of endothelial progenitors in peripheral blood of sedentary cat^{n}, and to the lesser extend also in cat^{++} ($P<0.05$, n=5-8). Three weeks of subischemic training failed to mobilize EPCs in cat^{n} confirming earlier reports showing that ischemic stimuli are important for exercise-induced EPC release. When mice with vascular specific overexpression of catalase were subjected to the same exercise protocol, the number of circulating EPCs was strongly increased ($P<0.05$, n=4). Furthermore, exercise-induced increase of circulating EPCs was completely reversed in cat^{++} treated with the aminotriazole.

Taken together, these data suggest that endogenous hydrogen peroxide, a non-radical reactive oxygen species contributing in vascular oxidative stress inhibit the exercise-induced mobilization of EPCs. Therefore, continuously high level of vascular oxidative stress may impair important stem cell-induced endogenous vascular repair mechanisms and thus promote cardiovascular morbidity and mortality.

*Heart Center Leipzig, Universitaet Leipzig, Germany; Institut fuer Pharmakologie und Klinische Pharmakologie, Universitaetsklinikum Duesseldorf, Moorenstr. 5, 40225 Duesseldorf

101

HEME OXIDISED SOLUBLE GUANYLATE CYCLASE: A NOVEL TARGET FOR REGIO-SPECIFIC VASORELAXATION

Schmidt HHHW, Luk JTY, Stasch JP, Kemp-Harper BK

The heme-containing nitric oxide (NO) receptor, sGC can exist in the NO-sensitive reduced (Fe^{2+}) state and NO-insensitive oxidized (Fe^{3+})/heme free form. Indeed, the redox state of sGC can be probed using the NO-independent sGC activator, BAY 58-2667, which preferentially targets sGC in its oxidized states. We hypothesised that in the resistance vasculature, sGC exists in the Fe^{2+}, Fe^{3+} and heme-free forms and the ratio of these redox states is altered under conditions of oxidative stress. Small mesenteric arteries (~300μm diam) from male Sprague Dawley rats were mounted in small vessel myographs and isometric force measured. Vessels were precontracted (~50%) with U46619 and cumulative concentration-response curves to sGC activators which target sGC in its Fe^{2+} (DEA/NO), Fe^{3+} (BAY 58-2667) and heme-free states (BAY 58-2667, PPIX) were examined. BAY 58-2667 was the most potent vasodilator studied (pEC_{50}=12.82±0.13, -log M) with a maximal relaxation of 91.9±1.9% (n=6). Its potency was increased up to 200-fold ($P<0.0001$) upon oxidation of sGC by ODQ (10μM, n=6) and in the presence of the NO synthase inhibitor, L-NAME (100μM, n=4). Similarly, ODQ enhanced vasorelaxation to PPIX yet impaired the response to DEA/NO (control pEC_{50}=7.24±0.11 vs ODQ pEC_{50}=5.68±0.08, n=5-10, $P<0.0001$). Zn-PPIX (3&10μM), an inhibitor of heme-free sGC, caused a concentration-dependent right-ward shift in the relaxation response to BAY 58-2667 such that the potency was decreased 30- ($P=0.0001$, n=6) and 3000-fold ($P<0.0001$, n=6), respectively. The response to PPIX (pEC_{50}=5.31±0.57, R_{max}=55.5±5.8%) was abolished in the presence of 3μM Zn-PPIX ($P<0.0001$) yet vasorelaxation to DEA/NO was unchanged. The peroxynitrite donor, SIN-1 (1mM) decreased the sensitivity to DEA/NO 15-fold ($P=0.0003$, n=4) yet the response to BAY 58-2667 was unaffected. The vasodilator efficacy of BAY 58-2667 and PPIX in small mesenteric arteries suggests that a relatively enhanced pool of oxidized/heme-free sGC exists in the resistance vasculature under physiological conditions and this pool may be increased during oxidative stress and in the absence of endogenous NO. This establishes sGC activation as a novel pharmacological approach for both disease- and region-specific vasodilator therapy.

Department of Pharmacology & Centre for Vascular Health, Monash University, Melbourne, Australia; Bayer HealthCare, Wuppertal

102

FIBER-FREE DIET RESCUES MICE LACKING NO-SENSITIVE GUANYLYL CYCLASE FROM FATAL GASTROINTESTINAL DYSMOTILITY

A. Friebe, D. Groneberg, P. König*, and D. Koesling

By catalyzing the production of the intracellular signaling molecule cGMP NO-sensitive guanylyl cyclase (NO-GC), as the major receptor for NO, has a key function within the NO/cGMP cascade. The pharmacological importance of the enzyme is reflected by NO donors used for the therapy of coronary heart disease. NO-GC is made up of one β subunit and one α subunit. As there are two α subunits (α_1 and α_2), two different GC isoforms are known to exist ($\alpha_1\beta_1$ and $\alpha_2\beta_1$). In the cardiovascular system, vasorelaxation and inhibition of platelet aggregation are mediated by the $\alpha_1\beta_1$ GC. As the α_2 subunit is mainly found in nerve cells of the CNS, the $\alpha_2\beta_1$ heterodimer is believed to participate in synaptic plasticity. The role of NO-GC in the gastrointestinal tract is still unclear. NO is contributing to non-adrenergic non-cholinergic (NANC) relaxation of gastrointestinal smooth muscle. sing mice deficient in the β_1 subunit we investigated the role of NO-GC in intestinal tissues. These mice do not express any of the α subunits and reveal no detectable cGMP synthesis upon NO stimulation. Thus mice lacking the β subunit are in fact total NO-GC knock out mice. Whereas mice heterozygous for the β_1 subunit of NO-GC were phenotypically undistinguishable from WT, homozygous GC-KO mice died prematurely and revealed two phases of mortality: 80% of born KO mice died within the first two days whereas most of the remaining mice died between day 18 and 31. 3-week-old homozygous GC-KO mice exhibit considerable growth retardation shown by a 40% reduced body weight. Here we show that KO mice surviving until day 18+ die from gastrointestinal dysmotility leading to ileus and perforation. By substituting normal rodent chow with fiber-free diet we are able to rescue GC-KO mice. With these rescued mice, further studies are being conducted in order to elucidate the role of NO and NO-GC in the gastrointestinal tract.

Institut für Pharmakologie und Toxikologie, Ruhr-Universität Bochum, 44780 Bochum, Deutschland
*Institut für Anatomie, Universität Lübeck, 23538 Lübeck, Deutschland

103

IMPAIRMENT OF PROTEINKINASE G STIMULATION BY VASCULAR SPECIFIC OVEREXPRESSION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE

S. Kumpf, M. Oppermann, T. Suvorava, G. Kojda

We generated mice with a vascular-specific overexpression of endothelial NO•-synthase (eNOS) using the Tie 2 Promotor and backcrossed these mice to C57BL/6 background. Western blot proved a 3.5 times higher vascular expression of eNOS in transgenic mice ($eNOS^{++}$) as compared to transgene negative littermates ($eNOS^{n}$). Electron spin resonance measurements using $Fe^{2+}(DETC)_2$ revealed an increased concentration of NO• in the aorta of $eNOS^{++}$ (49.4 ± 19.7 pmol NO•/μg protein/min, n=6) vs. $eNOS^{n}$ (19.1 ± 8.7 pmol NO•/μg protein/min, n=6). Systolic blood pressure is decreased in $eNOS^{++}$ (105.0 ± 3.0 mmHg, n=6, $p<0.01$) as compared to $eNOS^{n}$ (118.1 ± 1.4 mmHg, n=4). Treatment with the NOS-inhibitor L-nitroarginine abolished this difference. Thus, the reduction of blood pressure is likely caused by an increased NO•-bioavailability. The response to acetylcholine (1 μM) in organ bath experiments showed a reduction of endothelium dependent vasorelaxation in $eNOS^{++}$ (68.4 ± 6.5 % of maximal dilation) compared to $eNOS^{n}$ (97.7 ± 7.9 %) indicating endothelial dysfunction. To examine basal proteinkinase G (PKG) activity, phosphorylation of vasodilator active stimulated protein (VASP) at serine 239 sites compared to total VASP was measured. Western blots showed decreased levels of VASP phosphorylation in $eNOS^{++}$ in aorta (69 ± 5 %, n=7, p=0.0004) and lung (71 ± 7 %, n=6, p=0.0085) as compared to $eNOS^{n}$ (100%). However neither expression of vascular soluble guanylyl cyclase nor vasorelaxation to exogenous NO• (S-Nitroso-N-acetyl-D,L-penicillamin) was changed in $eNOS^{++}$ as compared to $eNOS^{n}$. We conclude that continuously high concentrations of endogenous NO• may impair PKG activity by desensitisation of sGC but appear to have no net effect on the vasodilatory efficacy of NO•. However, this desensitisation might protect $eNOS^{++}$ from lethal reduction of blood pressure. Furthermore, endothelial dysfunction in $eNOS^{++}$ is not mediated by an impairment of NO-induced vasodilation.

Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum, Heinrich-Heine-Universität Düsseldorf, Moorenstraße 5, 40225 Düsseldorf, Germany

104

NEW MOUSE MODELS FOR THE ANALYSIS OF cGMP/cGKI SIGNALING IN THE BRAIN

S. Feil[1], P. Franken[2], S. Langmesser[3], F. Hofmann[4], K. Weindl[5], S.M. Hölter[5], W. Wurst[5], Y. Emmenegger[2], M. Tafti[2], U. Albrecht[3], R. Feil[1]

The cGMP-dependent protein kinase type I (cGKI) is an attractive candidate mediator of cGMP signaling in the CNS. The analysis of mouse mutants that lack cGKI globally in all cells or specifically in the hippocampus or cerebellum showed that this protein kinase modulates various forms of neuronal plasticity, ranging from axonal pathfinding during embryogenesis to the adaptation of synaptic activity during learning and nociception. Recent immunohistochemical data indicate that the distribution and functional relevance of the cGMP-cGKI pathway in the mammalian brain is even broader than previously thought. Conventional cGKI null mutants are not appropriate to study the role of cGKI in the behaviour of adult animals, because they have multiple defects and a short life expectancy. We have developed new cGKI-deficient mouse models that lack the protein in the nervous system and can be studied throughout adulthood. The analysis of these mouse mutants indicates that cGKI is indeed involved in complex behavioural processes, such as object recognition, social discrimination and the regulation of sleep-wake activity.

[1] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
[2] Center for Integrative Genomics, University of Lausanne, Switzerland
[3] Institute of Biochemistry, University of Fribourg, Switzerland
[4] Institut für Pharmakologie und Toxikologie, TU München, Germany
[5] Institute of Developmental Genetics, GSF - National Research Centre for Environment and Health, Neuherberg, Germany

105

POST-TRANSCRIPTIONAL REGULATION OF HUMAN INOS EXPRESSION BY THE JUN N-TERMINAL KINASE

R. Korhonen[1,2], K. Linker[1], A. Pautz[1], U. Förstermann[1], E. Moilanen[2] and H. Kleinert[1]

The expression of human inducible nitric oxide synthase (iNOS) is regulated both by transcriptional and post-transcriptional mechanisms. We have recently shown that the RNA binding protein tristetraprolin (TTP) is a major regulator of human iNOS expression. Induction of iNOS expression by treatment of cells with cytokines also promoted an increase in TTP expression. TTP was found to interact with the KH-type splicing regulatory protein (KSRP), a protein bound to the iNOS 3'-UTR. By this interaction, TTP prevented the recruitment of the iNOS mRNA to the exosome, a protein complex responsible for the 3'→ 5' exonucleolytic degradation of mRNAs. Thereby, the cytokine-induced increase of TTP expression enhances iNOS mRNA stability and protein expression. In human A549/8 cells, both inhibition of Jun N-terminal kinase (JNK) by the JNK inhibitor SP600125 or siRNA-mediated down-regulation of JNK protein expression resulted in a reduction of iNOS mRNA and protein expression. However, iNOS promoter activity was not affected by these treatments. Therefore, JNK seems to regulate iNOS expression post-transcriptionally by stabilizing iNOS mRNA. Since TTP is an important post-transcriptional regulator of iNOS expression, we investigated the effect of JNK inhibition by SP600125 or down-regulation by siRNA on TTP expression. Both SP600125 and siRNA-mediated reduction of JNK protein expression resulted in a decrease of TTP protein expression without affecting the amount of TTP mRNA. These data suggest a post-transcriptional control of TTP expression by JNK. Moreover, the modulation of JNK signaling by SP600125 or siRNA did not change p38 phosphorylation. In summary, our results show that JNK regulates human iNOS expression by stabilizing iNOS mRNA in a TTP-dependent mechanism.

[1]Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, D-551131 Mainz, Germany (R.K., K.L., A.P., U.F., H.K.);
[2]The Immunopharmacology Research Group, University of Tampere, FIN-33014 University of Tampere and Tampere University Hospital, Tampere, Finland (R.K., E.M.)

106

THE MATRICELLULAR PROTEIN SMOC-1 IS DOWNREGULATED BY IL-1β AND NO IN RAT MESANGIAL CELLS

Dreieicher E, Plešková M, Pfeilschifter J, Beck KF

Cytokines like IL-1β and TNFα are able to force rat renal mesangial cells to produce and to liberate high amounts of inflammatory mediators. Nitric oxide (NO) represents one of these inflammatory products that exerts detrimental effects resulting in cell death by necrosis or apoptosis, moreover, NO is able to trigger fine-tuned signalling cascades that result in changes of the gene expression pattern. We are interested in the cross-talk of cytokines and nitric oxide on gene expression. To analyse IL-1β-mediated effects on the gene expression pattern, quiescent rat renal mesangial cells were stimulated with or without IL-1β [1 nM] for 16 hours and the effects of IL-1β were analysed using the mRNA arbitrarily primed reverse transcription polymerase chain reaction (RAP-PCR). A band that appeared to be decreased in the cDNA from cells treated with IL-1β compared to vehicle-treated controls was identified to represent a cDNA for "secreted modular calcium binding protein 1" (SMOC-1). SMOC-1 is a matricellular protein localised preferably in basement membranes. Its biological function is not yet clear. Northern blotting experiments confirmed a time- and dose-dependent downregulation of SMOC-1 mRNA expression by IL-1β. To test if this effect is due to NO we performed experiments using the unspecific NOS inhibitor monomethyl L-arginine (L-NMMA) to inhibit IL-1β-induced iNOS activity and we observed a partial reversion of the IL-1β effect. Remarkably, administration of the NO donor DETA-NO revealed only little alteration of the SMOC-1 mRNA steady-state levels indicating that NO alone has less effect on SMOC-1 expression but modulates cytokine-triggered signalling pathways. To analyse the regulation of SMOC-1 at the protein level, we established an antibody specific for SMOC-1 protein. Experiments using this antibody for ELISA revealed that NO/IL-1β affected SMOC-1 mRNA expression is followed by changes at the protein level. The mechanistic details of this effect and its relevance *in vivo* have to be elucidated in further experiments.

pharmazentrum frankfurt, Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

107

LACK OF NO-MEDIATED NANC RELAXATION IN MICE DEFICIENT IN NO-SENSITIVE GUANYLYL CYCLASE

D. Groneberg, D. Koesling, and A. Friebe

The NO/cGMP signal transduction pathway plays an important role in the control of smooth muscle function. NO-sensitive guanylyl cyclase (NO-GC) is the main target of the messenger molecule NO produced by NO synthases. Stimulation of NO-GC by NO results in the synthesis of the second messenger cGMP. cGMP-mediated activation of cGMP-dependent protein kinase leads to the phosphorylation of various target proteins which finally induce the relaxation of smooth muscle. Several smooth muscle-containing tissues are innervated by non-adrenergic non-cholinergic (NANC) neurons. Release of NO from these neurons is known to mediate relaxation. Recently, we deleted NO-GC on the genomic level in mice. Lack of GC-NO abolishes NO-dependent relaxation of smooth muscle resulting in hypertension and gastrointestinal dysfunction. The GI dysmotility reduces the life expectancy as many mice die due to peritonitis and perforation of the gastrointestinal wall. We hypothesized that the absence of NO-GC as the receptor for the NANC transmitter NO is the reason for this phenotype. The importance of NO-GC in NANC relaxation was investigated in organ bath experiments using a pharmacological approach and electrical field stimulation (EFS). Neither physiological concentrations of NO donors nor EFS at low frequencies led to NANC relaxation of gastric fundus or sphincters (pylorus and lower esophageal sphincter) from GC-KO mice. This lack of NO-mediated NANC relaxation was corroborated in non-gastrointestinal tissue using penile corpus cavernosum. Taken together, we think that NO-GC is indispensable for NO-mediated NANC relaxation.

Institut für Pharmakologie und Toxikologie, Ruhr-Universität Bochum, 44780 Bochum, Germany

108

NO MEDIATES INHIBITORY EFFECTS ON MURINE PLATELETS SOLELY VIA NO-SENSITIVE GUANYLYL CYCLASE.

O. Dangel, D. Koesling, and A. Friebe

Signalling via NO/cGMP regulates diverse physiological processes. NO produced in the endothelium activates the NO-sensitive guanylyl cyclase (NO-GC) which catalyses the formation of the second messenger cGMP. In platelets, cGMP formation leads to the activation of cGMP-dependent protein kinase (PKG) and the phosphorylation of several target proteins. This results in the reduction of intracellular calcium release, reduced adhesion and finally in the inhibition of platelet aggregation. In addition to this well characterized cascade, NO has been postulated to inhibit platelet aggregation and adhesion via cGMP-independent mechanisms. Mice lacking NO-GC are a suitable system to differentiate between cGMP-dependent and -independent effects of NO. Here, we show that NO signalling leading to inhibition of agonist-induced platelet aggregation is totally abrogated in platelets from mice deficient in NO-GC. None of a variety of NO donors inhibited collagen-induced aggregation of GC-KO platelets. Furthermore, neither ADP-induced adhesion nor thrombin-induced calcium release in GC-KO platelets was influenced by NO. However, cAMP-mediated signalling is still functional. These in vitro data are completed by a reduced bleeding time in vivo corroborating the physiological importance of NO. We conclude that NO-GC is the only NO receptor in murine platelets mediating the inhibition of platelet adhesion and aggregation.

Institut für Pharmakologie und Toxikologie, Medizinische Fakultät, Ruhr-Universität Bochum, 44780 Bochum, Germany

109

LYSOPHOSPHATIDIC ACID INDUCES NO-DEPENDENT AORTAE VASORELAXATION VIA THE LPA_3-RECEPTOR SUBTYPE

K. Zimmermann, H.H. Pertz, B. Kleuser

The lipid mediator lysophosphatidic acid (LPA) has been known to mediate a variety of biological responses. Here we show that LPA induces vasorelaxation of percontracted isolated mice aortae. This effect was due to the stimulation of endothelial nitric oxide synthase (eNOS) as LPA in eNOS-deficient mice was not capable to induce vasorelaxation. Therefore, we further examined in bovine aortic endothelial cells BAEC how LPA induces eNOS activation and NO formation. LPA signals through its G protein-coupled receptors (GPCR) $LPA_{1\text{-}5}$ activating distinct downstream pathways. We performed PCR to identify the expressed receptors in BAEC, detecting LPA_2 and LPA_3. To elucidate the role of these receptors in eNOS activation, we compared the effects of LPA and the LPA_3 agonist 1-oleoyl-2-*O*-methyl-*rac*-glycerophosphothionate (OMPT). OMPT showed a similar activation of eNOS, detected by phosphorylation at Ser^{1179}. In addition, we examined the response towards LPA in the presence of diacylglycerol pyrophosphate 8:0 (DGPP), an antagonist on LPA receptor subtypes 1 and 3. DGPP abolished the phosphorylation of eNOS evoked by LPA. This effect proved to be specific for LPA, since sphingosine-1-phosphat transduced eNOS phosphorylation was not affected. These findings on DGPP and OMPT indicate a dependence of eNOS activation on the LPA_3 receptor. To further substantiate these results, we downregulated LPA receptors using antisense oligodesoxynucleotides (ODN). Indeed, application of LPA_3-ODN revoked the LPA-transmitted activation of eNOS, whereas downregulation of other LPA receptors showed no effect. We elucidated LPA_3 as the responsible receptor subtype for activation of eNOS in BAEC leading to a subsequent release of NO which is the crucial mediator of LPA-induced vasorelaxation in mice aortae.

Freie Univerität Berlin, Institut für Pharmazie, Königin-Luise-Str. 2+4, 14195 Berlin

110

ANTIAPOPTOTIC ACTION OF SPHINGOSINE 1-PHOSPHATE IN HUMAN KERATINOCYTES IS MEDIATED VIA THE $S1P_3$ RECEPTOR SUBTYPE AND eNOS SIGNALLING

M. Schüppel, B. Kleuser

Keratinocytes are exposed to many environmental insults leading to cell damage and apoptotic signalling. Sphingosine 1-phosphate (S1P) has been shown to modulate apoptosis after cell injury. The most prominent sources of S1P are human platelets suggesting its potential role in regulation of wound healing after cell damage. Indeed, S1P effectively protects keratinocytes from apoptosis, but the molecular mechanism of the antiapoptotic action of S1P is not well characterised. It has been well established that S1P achieves most of its action after ligation to its specific S1P receptors. To date five receptor subtypes, named $S1P_{1\text{-}5}$, have been identified. To figure out, which receptor subtype is implicated in the S1P-mediated antiapoptotic action, keratinocytes were treated with specific antisense oligonucleotides against $S1P_{1\text{-}5}$ receptors. Interestingly, the reduced expression of $S1P_3$ exclusively attenuates the antiapoptotic action of S1P. We next proved whether nitric oxide (NO) is involved in the $S1P_3$-mediated protective action of S1P as it has been suggested that NO exerts both pro- and antiapoptotic effects. For this purpose, we examined the expression status of NO synthase (NOS) isoformes in primary human keratinocytes. RT-PCR reveals that endothelial NOS (eNOS), neuronal NOS, and inducible NOS mRNA are present in primary human keratinocytes. Most interestingly, the inhibition of NO formation by N^{ω}-nitro-L-argininemethylester abrogated the antiapoptotic effect. Indeed, stimulation of S1P receptor induces activation of eNOS measured as increased eNOS phosphorylation and augmented NO formation. Moreover, S1P clearly induces eNOS mRNA. Thus, these results suggest a role of this isoform in the antiapoptotic effect of S1P. In agreement, downregulation of $S1P_3$ abrogated the ability of S1P to stimulate eNOS-activation. These results indicate that S1P protects primary human keratinocytes from apoptosis via the activation of eNOS. Moreover this effect is mediated by the $S1P_3$ receptor subtype.

Freie Universität Berlin, Institut für Pharmazie, Königin-Luise-Str. 2+4, 14195 Berlin

111

NITRIC OXIDE INHIBITS THE TPA-INDUCED MATRIX METALLOPROTEINASE-9 EXPRESSION IN HUMAN BREAST CANCER CELLS

C. Kurowski, A. Doller, P. Gutwein, J. Pfeilschifter and W. Eberhardt

MMPs are a family of enzymes which can promote angiogenesis and tumour cell invasion due to their ability of degrading extracellular matrix and basement membrane components. By contrast, MMPs are also capable of proteolytically generate endogenous factors indicating a more complex role of MMPs in tumour metastasis than

simply matrix degradation. Nitric oxide (NO) is a proinflammatory mediator which is assumed to exert both, inhibitory and tumour promoting activities, depending on the concentration and cell type used. The aim of the present study was to examine the influence of NO on the phorbolester (TPA) -induced MMP-9 expression in the breast cancer cell lines MCF-7 and MDA-231. Using gelatine zymography, we demonstrated that different exogenously applied NO donors caused a decrease in the extracellular MMP-9 content. Concomitantly, the intracellular level of MMP-9 was negatively affected by NO-donors. Using cycloheximide and lactacystin experiments we further revealed that the effects on MMP-9 were mainly due to an increased degradation of the MMP-9 protein. Additionally NO caused a marked reduction of the MMP-9 mRNA level within the polysomal fraction. Finally, realtime-PCR and reporter gene analysis demonstrated that treatment with NO attenuated the steady-state MMP-9 mRNA level correlating with a reduction in TPA-induced MMP-9 promoter activity. From these data, we suggest that NO can interfere with both, the transcriptional and posttranscriptional mechanisms of MMP-9 regulation. NO triggered suppression in MMP-9 synthesis may reflect an important regulatory process in the MMP-9 dependent tumorigenesis.

pharmazentrum frankfurt, ZAFES; Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, 60590 Frankfurt, Germany

112

REGULATION OF HUMAN INOS EXPRESSION BY THE RNA BINDING PROTEIN AUF1

S. Altenhöfer, S. Heil, A. Pautz, K. Linker and H.t Kleinert

Human iNOS expression is regulated both at the level of transcription and mRNA stability. The 3'-unstranlated region (3'-UTR) of the human iNOS mRNA, which is able to destabilize heterologous mRNAs, contains five AU-rich elements. These AREs regulate iNOS mRNA stability by interaction with RNA binding proteins. We have shown previously that both stabilizing factors like HuR, TIAR, hnRNP A1 and TTP as well as destabilizing RNA binding proteins like KSRP are involved in a complex network regulating human iNOS mRNA stability.

The RNA binding protein AUF1 (AU binding factor 1) has been described to participate in the regulation of the stability of several ARE-containing mRNAs (coding for pro-inflammatory proteins, cytokines, chemokines and growth factors). AUF1 exists in four isoforms (p37-, p40-, p42- and p45-AUF1) generated by differential splicing of a common hnRNA. In recent years conflicting results for the involvement of AUF1 in the regulation of mRNA stability have been described, indicating that AUF1 can obtain stabilizing as well as destabilizing properties. Moreover, different roles for the four isoforms of AUF1 have been proposed. In this study, we aimed to analyze if AUF1 regulates human iNOS expression and if the four isoforms perform different actions. For this purpose, we generated stably transfected DLD-1 cells overexpressing each isoform as fusion protein with the enhanced green fluorescent protein (EGFP). In all these cells cytokine-induced iNOS expression was markedly reduced. Accordingly, siRNA-mediated downregulation of the expression of the different AUF1 isoforms resulted in enhanced cytokine-induced iNOS expression in all cases. UV-cross linking experiments showed that the different isoforms interacted with the same AREs in the human iNOS mRNA 3'-UTR. In conclusion, our data indicate that AUF1 contributes to the regulation of iNOS expression by destabilization of the iNOS mRNA. Thereby, all four isoforms seem to act in a similar mechanism.

Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Str. 67, 55101 Mainz

113

NUMBER OF NITRATE GROUPS DETERMINES REACTIVITY AND POTENCY OF ORGANIC NITRATES: A PROOF OF CONCEPT STUDY IN ALDH-2$^{-/-}$ MICE

S. Göbel, P. Wenzel, U. Hink, M. Oelze, A. Seeling, T. Isse, K. Bruns, L. Steinhoff, K. Lange, H. Weiner, J. Lehmann, K. Lackner, T. Kawamoto, T. Münzel and A. Daiber

Background and purpose: Mitochondrial aldehyde dehydrogenase (ALDH-2) has been shown to provide a pathway for bioactivation of organic nitrates and to be prone to desensitization in response to highly potent, but not to less potent nitrates. We therefore sought to strengthen the concept, that bioactivation by ALDH-2 critically depends on the amount of nitrate groups within the nitrovasodilator. Experimental approach: Nitrates with one (PEMN), two (PEDN; GDN), three (PETriN; glycerlyl trinitrate, GTN) and four (pentaerithrityl tetranitrate, PETN) nitrate groups were investigated. Vasodilatory potency was measured in isometric tension studies using isolated aortic segments of wild type (WT) and ALDH-2$^{-/-}$ mice. Activity of the cGMP-dependent kinase-I (reflected by levels of phosphorylated VAsodilator Stimulated Phosphoprotein, P-VASP) was quantified by Western Blot analysis, mitochondrial dehydrogenase activity by HPLC. Following incubation of isolated mitochondria with PETN, PETriN-chromophore and PEDN, metabolites were quantified using chemiluminescence nitrogen detection and mass spectrometry. Key results: Compared to WT, vasorelaxation in response to PETN, PETriN and GTN was attenuated about 10fold in ALDH-2$^{-/-}$ mice, identical to WT vessels preincubated with inhibitors of ALDH-2. Reduced vasodilator potency correlated with reduced P-VASP formation and diminished biotransformation of the tetranitrate- and trinitrate-compounds. Intriguingly, none of these findings were observed for PEDN, GDN and PEMN. Conclusions and implications: Our results support the crucial role of ALDH-2 in bioactivating highly reactive nitrates like PETN and PETriN. ALDH-2-mediated relaxation by organic nitrates therefore mainly depends on the number of nitrate groups. Less potent nitrates like PEDN, GDN and PEMN are apparently biotransformed by alternative pathways.

II. Medizinische Klinik der Johannes Gutenberg-Universität Mainz, Germany (S.G., P.W., U.H., M.O., L.S., T.M., A.D.), Department of Environmental Health, University of Occupational and Environmental Health, Yahatanishi, Kitakyushu, Japan (T.I., T.K.), Department of Biochemistry, Purdue University, West Lafayette, IN, USA (H.W.), Institut für Pharmazie, Lehrstuhl für pharmazeutische Chemie, Friedrich-Schiller-Universität Jena, Germany (A.S., K.L., J.L.), Institut für Klinische Chemie und Laboratoriumsmedizin, Klinik der Johannes-Gutenberg-Universität Mainz, Germany (K.B., K.L.)

114

IN-VIVO EFFECTS OF PENTAERYTHRITOL TETRANITRATE ON VASCULAR EXPRESSION OF SOLUBLE GUANYLYL CYCLASE?

T. Dao, M. Oppermann, M.Bas, M.Wuttke, T.Suvorava, G.Kojda

It is not known whether long-term treatment with NO-donors might desensitize vascular NO signal transduction. We investigated whether high dose treatment with the NO-donor pentaerythritol tetranitrate (PETN) has any influence on the expression and activity of vascular sGC and on aortic relaxation to NO. A total of 60 C57BL/6 mice were fed with either a standard diet (PETN 0) or a diet containing 6 (PETN 6), 60 (PETN 60), or 300 (PETN 300) PETN/kg BW/day for 4 weeks. Treatment with PETN resulted in plasma concentrations of pentaerythritol dinitrate (PEDN) in PETN 6 (17.3±3.3), PETN 60 (120.8±32.7), PETN 300 (498.5±96.6) and of pentaerythritol mononitrate (PEMN) in PETN 6 (167±104.8), PETN 60 (242±48.4), PETN 300 (798.4±89.5). Western blot analysis of cytosolic fractions prepared from the lungs (n=8/group) showed similar protein expression for the alpha (sGC alpha, p=0.0961, ANOVA) and the beta subunit of soluble guanylyl cyclase (sGC beta, p=0.3709, ANOVA). The activity of sGC stimulated by S-nitroso-N-acetyl-D,L-penicillamin (SNAP, 1 µM -1mM) was measured by conversion of [α-^{32}P]-GTP to [α-^{32}P]-cGMP in pmol cGMP/mg protein/min. There were no differences between the halfmaximal stimulatory concentration (PD_2-values in-log mol/l) of SNAP in PETN 0 (3.86±0.31), PETN 6 (3.84 ± 0.07), PETN 60 (3.89±0.15) and PETN 300 (3.73±0.14, each n=7, p=0.9412 ANOVA). Likewise, PETN treatment had no effect on the concentration-dependent relaxation response to SNAP (1nM – 10 µM) in endothelium intact aortic rings. Halfmaximal concentrations of SNAP (PD_2-values in -log mol/l) were similar in all four groups (PETN-0: 6.85±0.08, PETN-6: 6.28±0.06, PETN-60: 6.81±0.07 and PETN-300: 6.84±0.09, p=0.7217, ANOVA). These data suggest that long-term treatment treatment with PETN even in high doses does not impair vascular NO signal transduction.

115

CIGARETTE SMOKE CONDENSATE LOWERS ASYMMETRIC DIMETHYLARGININE CONCENTRATIONS BY INCREASING DIMETHYLARGININE DIMETHYLAMINOHYDROLASE 2 EXPRESSION

F. Schulze , K. Hamraz , E. Schwedhelm , R. Maas, R.H. Böger

Introduction: Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase and an independent cardiovascular risk factor. Only contradictory information is available about the influence of smoking on ADMA. The aim of this study was to investigate the effects of cigarette smoke condensate (CSC) on cultured endothelial cells with a focus on ADMA concentrations and gene expression of enzymes involved in the generation and metabolism of ADMA.

Methods: The experiments to evaluate the effect of CSC on endothelial cells were conducted with EA.hy926 cells. Cells were cultured in DMEM supplemented with 10% FCS and HAT. For the experiments was DMEM supplemented with 1% FCS, HAT and the CSC or vehicle. ADMA concentrations were determined in the cell culture supernatant by using an ELISA assay. Gene expression was determined by quantitative RT-PCR using the TaqMan 7900HT. NO-production was determined by use of a fluorescence based assay in viable cells.

Results: After incubation with CSC as well as vehicle for 48 h (n=12) ADMA concentrations in cell culture supernatants were significantly lower than under control conditions (35.02 ± 1.18 nmol/mg protein) when the cells were incubated with 1.0 µg/mL of CSC (25.07 ± 1.67 nmol/mg protein; p<0.01 vs. control) and 10 µg/mL of CSC (26.15 ± 1.13 nmol/mg protein; p<0.01 vs. control). We observed no effect with 0.1 µg/mL of CSC (34.99 ± 2.23 nmol/mg protein; p=0.99 vs. control). We observed an increased expression of dimethylarginine dimethylaminohydrolase 2 (DDAH2) after 48 h (1.0 µg/mL CSC: 46% induction, p=0.336; 10 µg/mL CSC: 88% induction, p=0.021). The decrease of ADMA concentrations was time-dependent (ANOVA; p=0.0035; n=6) with a maximum effect after 24 h. After incubation with CSC for 48 h we found an dose-dependent increase in NO-production (ANOVA; p<0.001; n=6).

Conclusion: We conclude from our data that ADMA concentrations can be influenced by cigarette smoke extract and that one mechanism might be an increased DDAH2 expression.

University Medical Center Hamburg-Eppendorf, Institute of Experimental and Clinical Pharmacology, Hamburg, Germany

116

SELECTIVE INHIBITION OF THE CD98-ASSOCIATED AMINO ACID TRANSPORTERS y$^+$LAT AND LAT BY IRON-DINITROSYL

A.L. Kleschyov[1], A. Rotmann[3], A. Simon[2], A. Habermeier[2], J. Rupp[2], H. Nawrath[2], T. Münzel[1] and E.I. Closs[2]

Cells expressing iNOS mobilize and release non-heme iron and generate iron-dinitrosyl species (detectable by electron paramagnetic resonance, EPR). The role of these species is still obscure. In contrast to common NO donors, a model dinitrosyl-iron-thiosulfate (DNIC) complex induces a non-selective cationic current, membrane depolarization (PC12cells) and Bcl-2/caspase-independent apoptosis (Jurkat cells). Iron-dinitrosyl species undergo ligand-exchange reactions and may resemble some heavy metals, such as Hg^{++}, known to activate a non-selective cationic current, inhibit amino acid transport and initiate "nutritional" apoptosis in rapidly growing cells. The mechanism of this action is thought to be due to a direct interaction of Hg^{++} with the transporter-associated transmembrane glycoprotein CD98 (4F2hc). We hypothesized that dinitrosyl-iron may mimic Hg^{++} and thus interact with CD98. Here we studied the effect of DNIC on CD98-associated y$^+$L and L amino acid transporters (y$^+$LAT and LAT) as well as on CD98-independent cationic amino acid transporters (CAT). In human glioblastoma cells, total L-arginine uptake was inhibited by DNIC in a concentration dependent manner (low µM range). This was antagonized by a metal chelator, MGD and could not be reproduced by common NO donors. DNIC inhibited specifically the leucine-sensitive (y$^+$LAT-mediated) L-arginine transport. In contrast, no specific effect of DNIC on CAT-mediated L-arginine transport was observed. Additionally, DNIC inhibited both the L-arginine-sensitive (y$^+$LAT-mediated) and L-arginine-insensitive (LAT-mediated) leucine uptake. EPR experiments (using 57Fe) demonstrated continuous generation of Fe-NO species by the activated macrophages RAW 264.7. Collectively, these data are consistent with the idea of iron-dinitrosyl targeting CD98. This may imply a novel role of this iNOS-derived species in inflammation.

[1] Klinikum der Johannes Gutenberg-Universität, II. Medizinische Klinik, Kardiologie, 55101 Mainz, Germany
[2] Institut für Pharmakologie, Johannes Gutenberg-Universität, 55131 Mainz
[3] Universitätsklinikum Heidelberg, Hygiene-Institut, Abteilung Parasitologie, 69120 Heidelberg, Germany

117

ACTIVATION OF G-PROTEIN CHIMERAS OF VARIOUS HETEROTRIMERIC G PROTEIN FAMILIES BY *PASTEURELLA MULTOCIDA* TOXIN

J. Orth, I. Preuß and K. Aktories

Pasteurella multocida toxin (PMT) is one of the strongest activators of $G\alpha_q$-dependent phospholipase C (PLC) β to induce inositoltrisphosphate production, Ca^{2+} mobilization and formation of diacylglycerol. The toxin also activates the small GTPase RhoA, resulting in formation of stress fibers and focal adhesions. The activation of RhoA depends on $G\alpha_q$ and $G\alpha_{12/13}$. Interestingly, $G\alpha_{11}$, a member of the G_q family is not stimulated by PMT. In addition, PMT induces MAP kinase and STAT activation. Using chimeric α-subunits, consisting of parts from PMT-responsive and non-responsive G proteins, we were able to stimulate different effector pathways by PMT. Chimeras of $G\alpha_{11}$, which is not a substrate of PMT, and $G\alpha_{13}$ were activated by PMT and stimulated PLCβ as measured by inositoltrisphosphate accumulation in $G\alpha_{q/11}$-deficient cells. By stepwise reducing the portion of $G\alpha_{13}$ within the $G\alpha_{11}/G\alpha_{13}$ chimera essential regions for PMT-induced activation of G proteins were determined. In addition, various chimeras of $G\alpha_q$ and $G\alpha_i$ were tested for response towards PMT. $G\alpha_{q/i}$ chimera were obtained, which after transfection and expression in HEK 293 cells, mediated PMT effects. Activation of these chimeras by PMT caused inhibition of receptor-stimulated adenylylcyclase activity as measured by cAMP accumulation. The data indicate that a specific region in G proteins is responsible for activation by PMT. Activation of G proteins by PMT appears to be largely independent on the type of G protein/effector interaction.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg

118

A G-PROTEIN REGULATOR CONTROLS BLOOD PRESSURE IN MAN AND MICE

N. Beetz[1], U. Broeckel[2], M. Harrison[2], M. Stoll[3], J.E. Moura[4], M.A. Vieira Coelho[4], X. Zong[5], M. Brede[6], M. Biel[5], M.J. Lohse[6], L. Hein[1]

Hypertension is one of the leading causes of morbidity and mortality in the world. Clarifying the role of genetic factors for a susceptibility of individuals to develop hypertension is an ongoing process. By genomic association studies and by means of an animal model, we have identified phosducin, a 33kDa retinal phosphoprotein, to be essential for the control of blood pressure and sympathetic tone in stress situations in humans and mice. We have previously generated mice carrying a targeted deletion in the phosducin gene (Pdc-/-). Pdc-/- displayed higher systolic and diastolic blood pressure than Pdc+/+ mice during acute measurements under anesthesia as well as after stress (e.g. cage change) during long-term monitoring by telemetry. Nighttime norepinephrine excretion in the urine was significantly enhanced in Pdc-/- vs. Pdc+/+ mice. Circulating norepinephrine levels in the plasma were increased 3.34-fold in Pdc-/- mice. Elevated tissue levels of pre- and postsynapatic norepinephrine metabolites in Pdc-/- tissues pointed towards enhanced sympathetic tone with increased transmitter release from postganglionic sympathetic neurons. Sympathetic neurons isolated from Pdc-/- superior cervical ganglia showed delayed desensitization of nicotinic currents and prolonged action potential firing as compared with Pdc+/+ neurons. In two human populations of African American and French Canadian origin, single nucleotide polymorphisms within the phosducin gene as well as in the 5' and 3' regions were significantly linked with elevated blood pressure during stress situations induced by a math test or by a change from supine to standing body position. We conclude that phosducin is an important blood pressure regulator in stress situations and that the phosducin-deficient mouse is an appropriate animal model to mimick the human disease.

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, [2] Human and Molecular Genetics Center, Wisconsin, Milwaukee, USA, [3]Leibniz-Institut für Arterioskleroseforschung, Universität Münster, [4]University of Porto, [5] Department Pharmazie, LMU München, [6]Institut für Pharmakologie und Toxikologie, Universität Würzburg

119

CONDITIONAL INACTIVATION OF $G\alpha_q/G\alpha_{11}$ IN MOUSE β-CELLS OF PANCREATIC ISLETS

A Sassmann, S Offermanns, N Wettschureck

Insulin secretion from pancreatic β-cells is modulated by a variety of G-protein coupled receptors, such as muscarinic acetylcholine recptors, adrenergic receptors, or receptors for gastrointestinal peptide hormones such as cholecystokinin or glucagon like peptide-1. To study the role of the $G_{q/11}$ family of heterotrimeric G-proteins in pancreatic β-cells, we generated β-cell specific $G_{q/11}$ double deficient mice by mating mice with floxed $G\alpha_q$ alleles to $G\alpha_{11}$ deficient mice and Rat Insulin Promoter Cre-transgenic mice (Rip-Cre). These β-cell specific $G_{q/11}$ double deficient mice are viable and fertile, and histologically their islets did not show any differences in size and number compared to Rip-Cre control mice. In addition, we did not find any alterations in insulin content, glucagon and GLUT2 expression in immunohisto-chemistry and by gene expression analysis using quantitative real time PCR. To test the functional relevance of $G_{q/11}$ family G-proteins under *in vivo* conditions, we performed oral and intravenous glucose tolerance tests. We found an impaired glucose tolerance in β-cell specific $G_{q/11}$ knockout mice, which was accompanied by a loss of the first peak of insulin secretion during glucose challenge. To investigate impaired insulin secretion from $G_{q/11}$ deficient β-cells in more detail, we performed *in vitro* studies on isolated islets of β-cell specific $G_{q/11}$ deficient mice. These islets did not respond to agonists of $G_{q/11}$-coupled receptors like carbachol or palmitic acid, while insulin secretion stimulated by agonists of G_s-coupled receptors, like glucagon like peptide-1 or pituitary adenylate cyclase activating polypeptide, was not altered. Surprisingly, we found that also glucose induced insulin secretion is reduced in β-cell specific $G_{q/11}$ deficient islets, and we are currently investigating the molecular mechanisms underlying this defect.

Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany

120

SIGNALING OF $G\alpha_{12}/G\alpha_{13}$ HETEROTRIMERIC G PROTEINS IN T CELL FUNCTION

S. Herroeder[1,2], S. Offermanns[1], N. Wettschureck[1]

Signaling of various G protein-coupled receptors is involved in regulation of leukocyte function, and thus the modulation of the inflammatory response. While the *in vivo* functions of G_i family G proteins have been studied extensively, the impact of other heterotrimeric G proteins on T cell function is unclear. To characterize the role of G_{12}/G_{13} family G proteins we used the Cre/LoxP system to generate mice that lack $G\alpha_{12}$ and $G\alpha_{13}$ selectively in their T cells. T cell specific $G\alpha_{12}/G\alpha_{13}$ double-deficient mice suffer from lymphopenia, yet absolute T cell numbers in primary and secondary lymphoid organs are increased, resulting in lymphadenopathy and thymic hyperplasia. Since both chemokines and sphingosine-1-phosphate (S1P) have been shown to direct T cell trafficking from and to lymphoid tissues, we performed transwell migration assays with $G\alpha_{12}/G\alpha_{13}$ deficient T cells. While chemokine-induced migration remained unaffected, migration of peripheral T cells in response to S1P was significantly impaired in the double deficient mice. These data do not only suggest $G\alpha_{12}$ and $G\alpha_{13}$ to be involved in S1P induced signal transduction, but may also explain lymphadenopathy of $G\alpha_{12}/G\alpha_{13}$ double-deficient mice. Decreased T cell egress may also contribute to thymic hyperplasia; however, the fact that not only single-positive, but also double-positive and double-negative thymocyte subpopulations were increased indicate that, in addition, abnormalities in proliferation and/or apoptosis exist. Whereas proliferation assays did not reveal major differences between the genotypes, apoptosis at the double-negative/double-positive maturation level was reduced in $G\alpha_{12}/G\alpha_{13}$ deficient thymocytes. Since impaired apoptosis during thymocyte selection might lead to an abnormal T cell repertoire, we are currently testing whether susceptibility towards autoimmune diseases is altered in T cell specific $G\alpha_{12}/G\alpha_{13}$ deficient mice.

[1]University of Heidelberg, Institute of Pharmacology, INF 366, 69120 Heidelberg
[2]University of Heidelberg, Department of Anesthesiology, INF 110, 69120 Heidelberg

121

$G\alpha_{12}/G\alpha_{13}$ AND DIA1 REGULATE MICROTUBULE CYTOSKELETON AND CELL POLARITY

P. Goulimari, H. Knieling, U. Engel, R. Grosse

Regulation of cell polarity is a process observed in all cells. During directed migration, cells orientate their microtubule cytoskeleton and the microtubule-organizing-center (MTOC) in a polar fashion. This process has been shown to involve glycogen synthase kinase 3β (GSK-3β) downstream of integrins and the small GTPase Cdc42. Here we report that in addition to Cdc42 the heterotrimeric GTPases $G\alpha_{12}$ and $G\alpha_{13}$ are necessary for MTOC reorientation based on studies using $G\alpha_{12/13}$-deficient mouse embryonic fibroblasts. Moreover, we found that MTOC polarity requires the $G\alpha_{12/13}$-interacting leukemia-associated RhoGEF (LARG), RhoA and the diaphanous-related formin Dia1 by a mechanism not involving GSK-3β regulation. Thus, $G\alpha_{12/13}$ and Dia1 are part of a signal transduction pathway that contributes to cell polarization. In line with this observation, EB1 dynamics are aberrant in $G\alpha_{12/13}$-deficient fibroblasts and a disorganized microtubule meshwork is evident when compared to wild type cells. We conclude that the heterotrimeric G-proteins $G\alpha_{12/13}$ regulate microtubule dynamics and are essential for cell polarity.

Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg

122

ATORVASTATIN REDUCES THE DESENSITIZING EFFECT OF CHRONIC ISOPRENALINE INFUSION ON β-ADRENERGIC SIGNALLING IN RAT HEART

A.Schmechel, M. Grimm, Alexander Schwoerer, Heimo Ehmke, T. Eschenhagen

Background: Desensitization of β-adrenergic (β-AR) signalling is a well-known key feature of chronic heart failure. We have previously shown that atorvastatin blunts the response of cultured neonatal rat cardiomyocytes to β-AR stimulation by reducing isoprenylation of the γ-subunit of heterotrimeric G proteins which finally causes a reduction in Gs. Here we examined (1) whether atorvastatin exerts similar effects in vivo and (2) whether this can prevent adverse effects of chronic catecholamine stimulation.

Methods: Male Wistar rats were treated with atorvastatin (ATOR 1 or 10 mg/kg/d) or H2O for 14 days per gavage and, for the last 4 days, with isoprenaline (ISO; 1 mg/kg/d) or NaCl via an osmotic mini pump. Heart rate was monitored under normal and stressed conditions by telemetry, contractile function of left atria and left papillary muscles was measured in organ bathes.

Results: ATOR treatment alone had no effect on basal, stress- or isoprenaline-stimulated heart rate or on heart weight. However, it was associated with a dose dependent decrease in the maximal inotropic effect of isoprenaline in isolated atria and papillary muscles (ATOR 10: -26 and -30%, respectively). These data mirror the effects on cultured cells. ATOR in combination with isoprenaline infusion reduced the isoprenaline-induced increase (+18% versus NaCl) in heart-to-body weight ratio to 15% and 8% at 1 and 10 mg/kg/d respectively; n=11). As expected chronic infusion of isoprenaline was associated with a marked reduction of the maximal inotropic response to isoprenaline in atria/papillary muscles (-60%/-41%). This desensitizing effect of chronic isoprenaline was dose-dependently attenuated by ATOR pretreatment (low dose: -54%/-15%*; high dose:-20%*/-8%*). *$p < 0.05$ vs. H2O).

Summary and Conclusion: A 14-day treatment with atorvastatin dose-dependently decreased the positive inotropic, but not chronotropic effects of β-adrenergic stimulation and, likely by this mechanisms, protected the heart from hypertrophic and desensitizing effects of chronic isoprenaline infusion. The data support the idea that statins act directly on the heart as mild β-blockers.

Institutes of Experimental and Clinical Pharmacology and Toxikology and Physiology, University Medical Center Hamburg-Eppendorf, Germany

123

REGULATION OF PERTUSSIS-TOXIN-INSENSITIVE SIGNALLING OF CC CHEMOKINE RECEPTOR 2b BY TUMORPROTEIN D52

B. Moepps, J. Schuhholz, G. Michels, and P. Gierschik

Chemokine receptors are members of the G protein-coupled receptor (GPCR) family and are coupled to pertussis-toxin-sensitive and, in certain cases, also to pertussis-toxin-insensitive G protein(s). The carboxyl-terminal portions of several GPCRs, including chemokine receptors, have been shown to interact with multi-protein complexes made up of heterotrimeric G protein subunits and non-G-protein-components. This interaction may affect intrinsic receptor functions, regulate the interaction of the receptors with G proteins, or give rise to non-canonical, i.e. G-protein-independent, signal transduction processes. We have previously identified the tumor protein D52 (TPD52) as a novel interaction partner of the intracellular carboxyl-terminal-most portion of the human CC chemokine receptors CCR2a and CCR2b and have found that coexpression of TPD52 together with CCR2b in transfected COS-7 cells resulted in a reduction of the MCP-1-dependent, CCR2b-mediated increases in inositol phosphate formation and serum response factor (SRF)-dependent transcriptional activation of a luciferase reporter gene. We now report that both responses, inositol phosphate formation and SRF-dependent gene transcription, were synergistically enhanced by coexpression of pertussis-toxin-insensitive $G\alpha_q$ and that this enhancement was sensitive to inhibition by TPD52. To further characterize the specificity of the stimulatory effect of $G\alpha_q$ among the members of the $G\alpha_q$ subfamily of G protein α subunits, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$, were expressed together with CCR2b in COS-7 cells. The results showed that activation of SRF-mediated gene transcription by CCR2b was specifically mediated by $G\alpha_q$ and $G\alpha_{14}$, but not by $G\alpha_{11}$, and $G\alpha_{16}$. The observation that other receptors, e.g. the virally encoded chemokine receptor homolog pUS28, is coupled in the same cellular system to SRF-mediated transcriptional activation through $G\alpha_q$ and $G\alpha_{11}$, but not through $G\alpha_{14}$ and $G\alpha_{16}$, suggests that the chemokine receptor CCR2b interacts with (i) a specific subset of $G\alpha_q$ subfamily members, $G\alpha_q$ and $G\alpha_{14}$, and that (ii) TPD52 exerts an inhibitory effect on this interaction, its downstream consequences, or both.

Institute of Pharmacology and Toxicology, University of Ulm, 89081 Ulm

124

GRK2/3 INCREASE THE DISTANCE BETWEEN Gαq AND Gβγ SUBUNITS DURING ACTIVATION

Schleifenbaum, J., Lohse M.J. and Bünemann, M.

G protein coupled receptor kinases 2/3 (GRK2/3) are kinases involved in the regulation of G protein coupled receptor (GPCR) signaling pathway. GPCR desensitization is initiated by GRK2/3 primarily by receptor phosphorylation of serine and threonine residues. It has been demonstrated that GRK2/3 is activated by binding of G protein βγ subunit to a pleckstrin homology domain at its C-terminus, thereupon translocates to the membrane and initiates receptor desensitization by phosphorylation. In addition GRK2/3 has been shown also to interact with Gα subunits of Gq proteins and by this regulate Gq signalling. Recently the structure of a GRK2 /Gαq/ $G\beta_1\gamma_2$ complex has been solved (Tesmer et al., Science 310, 1686 *2005*). This crystal structure demonstrated a substantial distance between Gβγ and Gαq subunits when simultaneously bound to GRK2. In order to address the relevance of Gq- GRK2/3 interaction in single living cells we established FRET based assays for Gq activation and studied effects of GRK2/3 expression on Gq FRET. In order to stimulate Gq proteins we activated coexpressed P_2Y_1 receptors with ATP. This resulted in an about 10% decrease of the FRET signal between YFP-labelled Gαq and CFP-labelled Gβγ subunits. Upon coexpression of GRK2 or GRK3 the FRET decrease upon Gq activation was increased by 2-3 fold. This increase in FRET change induced by GRKs was not dependent on kinase function. However, binding of Gβγ alone by the C-terminus of GRK2 was not sufficient to induce the observed effect on Gq-FRET, suggesting that both Gαq and Gβγ binding was required. Our results provide evidence for the existence of direct protein-protein interaction of GRK2/3 with both activated Gαq and Gβγ in living cells and incomplete separation of Gαq and Gβγ during activation when not bound to GRK2/3.

University of Würzburg, Department of Pharmacology and Toxicology, Würzburg, Germany.

125

FRAP MICOSCROPY REVEALS STABLE PRECOMPLEX FORMATION OF GIRK CHANNELS AND G PROTEIN SUBUNIT βγ

A. K. Kreile, M. J. Lohse, M. Bünemann

G protein-activated inwardly rectifying potassium (GIRK) channels are heterotetramers which are activated by the βγ subunit of G proteins. To characterize the spatio-temporal organisation of G protein-mediated GIRK channel activation, we employed fluorescence microscopy. Specifically, we followed the hypothesis that G proteins and GIRK channels are stably precomplexed. In addition, we were interested in the stability of the presumable precomplex. Therefore, we measured the lateral mobility of βγ in presence or absence of GIRK. By fluorescence recovery after photobleaching (FRAP), we assessed the diffusion coefficient at room temperature. Both, GIRK and βγ showed an immobile fraction of 25% for GIRK and 9% for βγ. The recovery curves displayed a biphasic run reflecting a mobile fraction and a fraction shifting between mobile and immobile. The mobile fraction of GIRK with a diffusion coefficient of 0.24μm^2/s comprised 23% and the shifting fraction of 52% exhibited a diffusion coefficient of 0.04μm^2/s. The mobile fraction of βγ with a diffusion coefficient of 0.40 μm^2/s comprised 35% and the shifting fraction of 56% exhibited a diffusion coefficient of 0.06μm^2/s. In cells stably expressing GIRK, the immobile fraction of βγ was increased to 22%, the mobile fraction of only 15% exhibited a diffusion coefficient of 0.40μm^2/s; the shifting fraction of 62% exhibited a diffusion coefficient of 0.07μm^2/s. We immobilized GIRK on the cell surface by biotinylation and avidin-crosslinking and thereby increased the immobile fraction of βγ to 75% in the presence of GIRK while mobile and shifting fraction were diminished to 6% and 20% with diffusion coefficients of 0.40μm^2/s and 0.04μm^2/s, respectively. These findings were supported by the observation of a strong retention of βγ in the endoplasmatic reticulum (ER) through GIRK1 whose retention signal sequence locates it to the ER in the absence of other GIRK channel subtypes. From our data we conclude a stable precomplex formation of G protein subunit βγ and GIRK channels.

University of Würzburg, Department of Pharmacology and Toxicology, Würzburg, Germany

126

SECOND MESSENGER ANALYSIS WITH MOBILITY-BASED FLUORESCENT BIOSENSORS BY REPETITIVE TWO-COMPONENT FRAP ANALYSIS

A. Tannert, S. Burgold, G. Schultz, M. Schaefer

Mobility of proteins within a cell mainly depends on their size and the viscosity of their environment. Exploiting a 10- to 30-fold difference in diffusion coefficients between soluble and membrane-associated proteins, we propose that membrane translocation of fluorescent biosensors could be sensitively detected and quantified by monitoring changes in the lateral mobility. We developed a convolution-based approach to analyze fluorescence redistribution after photobleaching (FRAP) assuming membrane-bound and cytosolic species that exhibit different velocities (two-component model). By applying repetitive FRAP experiments within single cells, changes in pools of membrane-bound and free molecules can be quantified in a time-resolved manner. To this end, we calculated the two diffusion coefficients that remain constant for every single FRAP measurement. Receptor stimulation leading to a protein translocation was analyzed by fitting the changing amounts of slow and fast fractions. We then analyzed the translocation of a variety of mobility-based GFP-fused biosensors and show that this method sensitively reports on various signal transduction pathways downstream of G-protein-coupled receptors or receptor tyrosine kinases. Applications include the activation of Gq leading to a phospholipase C-mediated hydrolysis of $PI(4,5)P_2$ and a release of PLCδ(PH) from the plasma membrane. Activation of Gi-coupled receptors was monitored by Gβγ-mediated activation of PI3-kinase γ, $PI(3,4,5)P_3$ formation and translocation of GRP1(PH) to the plasma membrane. Vasopressin V2 receptor-mediated activation of Gs leading to an increase in cAMP concentrations was followed by a dissociation of a membrane-associated CAAX box-modified regulatory and a YFP-fused catalytic PKA subunit. Receptor tyrosine kinase activation was monitored directly by recruitment of the SH2 domain-bearing adapter protein Grb2 or via class 1A PI3-kinases that mediate membrane recruitment of GRP1(PH).

In contrast to imaging- or FRET-based approaches, the FRAP-based approach is independent of protein concentrations, cellular movements and background fluorescence and allows a precise quantification of activated protein species. We conclude that two-component FRAP analysis is applicable to a wide range of existing or specifically designed translocating fluorescent biosensors.

Institut für Pharmakologie, Charité - Universitätsmedizin Berlin, Thielallee 67-73, 14195 Berlin

127

PREREQUISITES OF G_q FOR *PASTEURELLA MULTOCIDA* TOXIN-INDUCED ACTIVATION

I. Preuß, S. Lang, J. Orth and K. Aktories

The intracellularly acting *Pasteurella multocida* toxin (PMT) affects several signal transduction pathways by stimulating heterotrimeric G proteins. PMT has been shown to activate the small GTPase Rho, the MAP kinase ERK and STAT proteins via the stimulation of two G protein families, $G\alpha_q$ and $G\alpha_{12/13}$. PMT action also results in an increase in inositolphosphates, which is due to the stimulation of PLCβ. Recent studies indicate that PMT can distinguish between the highly related $G\alpha_q$ and $G\alpha_{11}$. IP_3 accumulation only depends on the activation of $G\alpha_q$.It was reported that PMT induces phosphorylation of tyrosine 356 in the C-terminal part of $G\alpha_q$. Here we studied the functional consequence of the mutation of this tyrosine residue on the activity of PMT and on the enhancement of the agonist-induced activation of $G\alpha_q$ by overexpressing $G\alpha_q^{Y356F}$ and $G\alpha_q^{Y356D}$ constructs in $G_{q/11}$-deficient MEF cells. These experiments were done by measurement of IP_3 accumulation and GTPγS-binding to $G\alpha_q$, respectively. Furthermore, mutation and amino acid replacement studies have shown that the extreme C-terminus of the $G\alpha_q$ subunit is involved in receptor-coupling. To clarify whether the binding to the receptor is required for PMT-induced G protein activation, we deleted the C-terminus of $G\alpha_q$ and measured the stimulation of PLCβ by IP_3 production. These experiments display structural and functional prerequisites of G_q which are important for the PMT-induced activation.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg

128

ROLE OF G-ALPHA-Q/11 AND G-ALPHA-12/13 IN THE REGULATION OF ENDOTHELIAL BARRIER FUNCTION

Korhonen H.-K., Moers A., Wettschureck N., Offermanns S.

A decreased endothelial barrier function resulting in an increased microvascular permeability is a key feature of various diseases, including inflammation or anaphylaxis. The opening of the endothelial barrier involves the regulation of junctional adhesions as well as endothelial cell contraction. Endothelial cell contraction is initiated by phosphorylation of the myosin light chain (MLC), a process that is dually regulated by myosin light-chain kinase (MLCK) and myosin light-chain phosphatase (MLCP). Various mediators, like histamine, lysophosphatic acid (LPA), or thrombin, which are able to increase vascular permeability, have in common that they act through receptors coupled to the G-proteins G_q/G_{11} and G_{12}/G_{13}. We generated endothelial cell specific double knock-out mice for the genes encoding the α-subunits of G_q/G_{11} and G_{12}/G_{13} using the Cre/LoxP system. The aim of this study was to characterize the role of G-protein mediated signalling involved in the regulation of endothelial functions by mediators acting through GPCR both under physiological and pathophysiological conditions. Endothelium specific loss of $G\alpha_{q/11}$ or $G\alpha_{12/13}$ did not appear to affect the basal function of endothelium. In a vascular permeability assay, endothelium specific $G\alpha_{q/11}$ knock-out mice showed hardly any plasma extravasation in response to LPA, and strongly reduced response to histamine, substance P, and PAR-1 peptide in the skin. In a model of local cutaneous anaphylaxis $G\alpha_{q/11}$ knock-out mice showed reduction in vascular leakage. In an *in vivo* mouse model of systemic anaphylaxis, $G\alpha_{q/11}$ knock-out mice show less severe hypothermia and arterial hypotension, and almost no rise in hematocrit compared to control mice. Histamine induced hypotension is clearly reduced in $G\alpha_{q/11}$ knock-out mice in telemetric blood pressure measurements. Thus, $G\alpha_{q/11}$ obviously play a crucial role in the regulation of vascular permeability. We are currently evaluating the role of the $G\alpha_{12/13}$- mediated pathway in endothelial function under pathological conditions.

University of Heidelberg, Institute for Pharmacology, Im Neuenheimer Feld 366, D-69120 Heidelberg

129

THE 5-HT$_{2A}$ RECEPTOR CONSTITUTIVELY ACTIVATES SERUM RESPONSE FACTOR-DEPENDENT GENE TRANSCRIPTION

G. Michels, E. Kuhn, P. Gierschik, and B. Moepps

As a major neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT) is involved in regulation of perception, pain, anxiety, aggression, and mood. In non-neural tissues, 5-HT plays a role as a regulator of smooth muscle growth and platelet aggregation. The cellular functions of 5-HT are mediated by 14 different 5-HT receptors (5-HTRs), which are classified into 7 subfamilies (5-HT$_{1-7}$). With the exception of the ligand-gated ion channel 5-HT$_3$R, all receptors belong to the family of G protein-coupled receptors (GPCRs). Interestingly, certain 5-HTRs, e.g. 5-HT$_{1A}$R and 5-HT$_{2C}$R, display a ligand-independent constitutive activity. For example, 5-HT$_{2C}$R constitutively couples to Gα_q and stimulates phospholipase C-β isoenzymes when expressed in mammalian cells. In contrast, stimulation of phospholipase C-β isoenzymes by 5-HT$_{2A}$R is 5-HT-dependent and the receptor shows little or no constitutive activation of inositol phosphate formation. We previously reported that the virally encoded chemokine receptor homologue pUS28 shows constitutive, G$\alpha_{q/11}$-dependent enhancement of (i) inositol phosphate formation and (ii) serum response factor (SRF)-mediated gene transcription when transiently expressed in COS-7 cells. To investigate whether 5-HT$_{2A}$R is also coupled to SRF-mediated gene transcription and, if so, is constitutively active, we transiently expressed the rat 5-HT$_{2A}$R in COS-7 cells and determined serum response factor (SRF)-dependent transcriptional activation of a luciferase reporter gene. The results showed that expression of 5-HT$_{2A}$R resulted in activation of SRF-mediated gene transcription even in the absence of 5-HT. To characterize the heterotrimeric G protein(s) mediating this constitutive activity of 5-HT$_{2A}$R, various members of the Gα_q subfamily of the G protein α subunits were expressed together with 5-HT$_{2A}$R in COS-7 cells. The results showed that activation of SRF-mediated gene transcription by HT$_{2A}$R was specifically enhanced by Gα_q, but not by Gα_{14} and Gα_{16}, Taken together, our findings indicate that expression of 5-HT$_{2A}$R in cultured cells causes a Gα_q-mediated constitutive activation of SRF-dependent gene transcription.

Institute of Pharmacology and Toxicology, University of Ulm, 89069 Ulm

130

RELATIVE QUANTIFICATION OF HIGHLY HOMOLOGOUS G PROTEIN β SUBUNIT TRANSCRIPTS – A NEW APPLICATION FOR THE PYROSEQUENCING PROCEDURE

C. Rimmbach, I. Geissler, H. Petersenn, D. Rosskopf

There are 5 isoforms of G protein β subunits in humans which exhibit an enormous sequence homology. This high homology has hampered exact quantification of Gβ subunit protein by immunostaining. Likewise, the quantification of transcript levels is also affected by the high homology. Here, we applied the pyrosequencing technique, a procedure in common use for quantitative genotyping. This procedure combines a sequencing step with the generation of luminescence that can be measured with high precision. Hence, the peak in the pyrogram is proportional to the incorporated number of nucleotides. G protein β transcript mRNA was amplified by standard RT PCR. Oligonucleotide primers were chosen from sequence motifs that were identical in the Gβ subunits Gβ1 - Gβ4. Upon amplification with these common primers, the resulting PCR product was pyrosequenced with a sequencing primer, again from an identical motif within all Gβ subunits. This primer was located adjacent to a motif that differed between Gβ1 - Gβ4. Quantitative sequencing of this motif served to quantify the relative content of Gβ transcripts. Analyses included specimens from human heart, brain, kidney, intestine, placenta, adipose tissue, fibroblasts, lymphoblasts and other common cell types. We observed highly distinctive signatures of Gβ transcript composition for different tissues. While the Gβ composition differed from tissue to tissue, for a given tissue or cell type, we observed highly constant signatures of these transcripts between different individuals. Activation of cells (e.g. lymphocytes) caused a significant change in the composition of Gβ mRNA transcripts, potentially associated with altered signalling functions of these stimulated cells. This is the first report demonstrating the possibility to quantify homolog transcripts with pyrosequencing.

Institut für Pharmakologie – Ernst-Moritz-Arndt Universität Greifswald

131

G$_{12/13}$-DEPENDENT GROWTH OF SMALL CELL LUNG CANCER

Grzelinski M, Büch T, Pinkenburg O, Aigner A, Gudermann T

The involvement of G$_{12/13}$ proteins in tumor growth has been intensively discussed since Gα_{12} protein was identified as a putative oncogene in soft tissue sarcoma. More recently, disruption of G$_{12/13}$ signaling has been shown to inhibit invasiveness, but not the proliferative capacity of breast and prostate cancer cells. In small cell lung cancer (SCLC), neuropeptide hormone receptors which couple to G$_{q/11}$ and G$_{12/13}$ are constitutively activated by auto- and paracrine stimulation. Thus, G$_{12/13}$-dependent pathways may also contribute to the malignant phenotype of SCLC. To test for this hypothesis, we transduced H69 cells (a human SCLC line) with shRNAs against Gα_{12}, Gα_{13}, or random shRNA using a lentiviral expression system. Indeed, transduction of specific shRNA specifically inhibited Gα_{12} or Gα_{13} expression and random shRNA was without effect. Interestingly, knock-down of Gα_{12} markedly inhibited proliferation of H69 cells in normal culture medium, whereas shRNA against Gα_{13} exhibited a weaker effect and random shRNA did not inhibit growth. However, using a colony-forming assay in semi-solid medium which more closely mimics an *in vivo* situation both targeting of Gα_{12} and Gα_{13} inhibited growth of H69 cells. Finally, we tested tumor growth of xenotransplanted cells in mice using *wild-type* H69 cells compared to tumor cells previously transduced with control shRNA or shRNA against Gα_{12}, Gα_{13} or both Gα_{12} and Gα_{13}. Interestingly, interference with Gα_{12} and Gα_{13} strongly inhibited tumor growth *in vivo* and double knock-down of Gα_{12} and Gα_{13} completely abolished tumorigenicity in mice, whereas cells transduced with random shRNA did not differ from *wild-type* H69 cells. In summary, proliferation of H69 cells *in vitro* and tumorigenicity *in vivo* critically depends on intact G$_{12/13}$ signaling thus, contrasting with previous findings in breast and prostate cancer cells where inhibition of these signaling molecules interfered with tumor invasiveness, but not with proliferation. Further elucidation of the role of G$_{12/13}$ in SCLC will extend the understanding of pathways involved in autonoumous growth of this tumor and may define novel therapeutic targets.

Institut für Pharmakologie und Toxikologie, Philipps Universität, Marburg

132

G PROTEIN SUBTYPES IN MAMMALIAN SPERMATOZOA: EXPRESSION PROFILE OF α-GUSTDUCIN AND ASSOCIATED Gβγ-SUBUNITS.

D. Meyer[1], J. Fehr[1], H. Borth[1], P. Widmayer[3], B. Wilhelm[2], T. Gudermann[1] and I. Boekhoff[1]

Sperm chemotaxis is a general guidance mechanism of spermatozoa to eggs throughout the animal kingdom. Since the G protein subunit α-gustducin is accepted as a marker of chemosensitive cells, attempts were made to explore whether α-gustducin and its associated Gβγ-dimer, originally discovered in a subset of taste cells on the tongue, are also expressed in spermatozoa of mammals. Using subtype-specific antibodies in immunohistochemical experiments we found that expression of Gustducin partners (Gβ$_3$γ$_{13}$) was not detectable in testicular derived tissue. However, anti-α-Gustducin–specific antibodies showed a strong labeling of differentiating spermatids localized in the lumen of the seminiferous tubules. To verify whether α-gustducin is still expressed in mature spermatozoa, mouse and rat sperm were subjected to immunocytochemistry as well as electron microscopy. We observed that α-gustducin is detectable in mature spermatozoa from mouse to humans and that the expression is notably enriched in the mitochondria-rich midpiece region as well as the proximal part of the principal region of the sperm tail. The observation that α-gustducin is expressed in the tail of mammalian spermatozoa may now fascilitate the identification of the corresponding signaling cascade, probably defining the functional role of α-gustducin in spermatozoa.

[1] Institut für Pharmakologie und Toxikologie und [2] Institut für Anatomie und Zellbiologie, Philipps-Universität Marburg; [3] Institut für Physiologie, Universität Stuttgart-Hohenheim

133

POSITIVE FEEDBACK BETWEEN DIA1, LARG AND RHOA IS REQUIRED FOR CANCER CELL INVASION

Kitzing, T., Sahadevan A., Brandt D.T., Knieling H., Hannemann S., Fackler O.T., Grosse R.

The RhoA-interacting formin Dia1 is involved in actin-dependent processes such as cytokinesis, SRF transcriptional activity and cell motility. Dia1 polymerizes actin through its formin homology (FH) 2 domain. Here we show that Dia1 can promote RhoA activity. RhoA activation by Dia1 is separable from its effects on actin nucleation and SRF activity. Dia1 binds the leukaemia-associated Rho-GEF LARG and this interaction is regulated by RhoA-dependent release of Dia1 auto-inhibition. The FH2 domain binds to the LARG C-terminus and stimulates guanine nucleotide exchange activity of LARG in vitro. Moreover, Dia1 is necessary for LPA-stimulated Rho/ROCK signaling. Consistent with this, Dia1 and LARG mediate ROCK-dependent bleb-associated tumor cell invasion. We propose that Dia1-dependent RhoA activation constitutes an essential feedback mechanism to modulate LPA signaling and tumor progression. These results identify formins as potential future drug targets and have implications for understanding invasive diseases.

Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg

134

DIA1 AND IQGAP1 INTERACT TO MEDIATE PHAGOCYTOSIS

Brandt D.T., Marion S., Kaibuchi K., Grosse R.

The Diaphanous-related formin Dia1 nucleates actin polymerization thereby regulating cell shape and motility. The mechanisms that control cellular location of Dia1 in order to spatially define actin polymerization are largely unknown. Here we identified the cytoskeletal scaffold protein IQGAP1 as a Dia1 binding protein that is necessary for its subcellular location. IQGAP1 interacts with Dia1 through a region within the Diaphanous-inhibitory domain (DID) in a serum-regulated fashion. Both proteins colocalize at the front of migrating cells but also at the actin rich phagocytic cup in macrophages. Interestingly, IQGAP1 interaction with Dia1 appears to be required for phagocytosis and phagocytic cup formation. Thus, we identify IQGAP1 as a novel component in immune function by mediating the localization of the actin filament nucleator Dia1.

Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg

135

INTESTINE-SPECIFIC DISRUPTION OF *ARFRP1* RESULTS IN IMPAIRED DIFFERENTIATION OF GOBLET AND PANETH CELLS

C. Zahn[1], A. Jaschke[1], A. Hommel[1], M. Moser[2], R. Kluge[1], R. Augustin[1], H.-G. Joost[1] and A. Schürmann[1]

ADP-ribosylation factor related protein 1 (ARFRP1) is a member of the ARF-family known to regulate intracellular protein trafficking. We have recently demonstrated that ARFRP1 plays an essential role for Golgi function by recruiting ARL1 and its effector Golgin-245 to the trans-Golgi network. Mice lacking *Arfrp1* are embryonic lethal, presumably due to defects in cell-cell adhesion. ARFRP1 interacts with the E-cadherin adhesion complex and is required for E-cadherin mediated cell-cell adhesion. Within the gut, ARFRP1 is expressed in the epithelium and the crypts of small intestine and colon. To address the function of ARFRP1 in organ development and tissue regeneration we used the Cre/loxP-recombination system to delete *Arfrp1* specifically in the gut. *Arfrp1*$^{flox/flox}$-mice with loxP-sites flanking exons 2 and 4 were crossed with transgenic mice expressing Cre recombinase under the control of the intestine-specific villin promoter. *Vil-Cre-Arfrp1*$^{flox/flox}$-mice were viable but showed postnatal growth retardation (weight reduction of about 40% in comparison to control littermates). *Arfrp1*-mutants exhibit markedly abnormal intestinal epithelium. Histological analysis demonstrated

strong vacuolization of enterocytes, indicating necrotic events. However, the polarization and sorting of epithelial cells appeared not to be affected but the shape of cells was altered, e.g. nuclei were irregularly arranged. Loss of *Arfrp1* induced extension of the crypts due to an increased cell proliferation. Furthermore, A*rfrp*1-deficiency specifically perturbs the differentiation of goblet and paneth cells, whereas the number of enteroendocrine cells and the distribution of the enterocytes were not affected. These data suggest that ARFRP1 is required for the differentiation events of a subset of secretory cells in the intestine.

[1]Department of Pharmacology, German Institute of Human Nutrition Potsdam-Rehbruecke
[2]Department of Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried

136

Lack of white adipose tissue in fat-specific *Arfrp1* null-mutants.

A. Hommel[1], C. Zahn[1], S. Schmidt[1], R. Kluge[1], R. Augustin[1], A. Jaschke[1], M. Moser[2], H-G. Joost[1] and A. Schürmann[1]

ADP-ribosylation factor related protein 1 (ARFRP1) is a ubiquitously expressed member of the ARF-family of GTPases. We have recently demonstrated that ARFRP1 is required for function and / or organization of the trans-Golgi network and that it is involved in the regulation of adhesion processes via controlling the targeting of the adhesion protein E-cadherin to the plasma membrane. Accordingly, deletion of *Arfrp1* in the mouse resulted in embryonic lethality during early gastrulation. In order to investigate the function of ARFRP1 in different tissues, the Cre/loxP-recombination system was used. *Arfrp1*$^{flox/flox}$-mice carrying loxP sites in intron 1 and intron 4 were crossed with transgenic mice expressing the Cre-recombinase under the control of the adipocyte-specific aP2 promoter. In contrast to the conventional *Arfrp1*$^{-/-}$ mouse, fat specific null-mutants (aP2-Cre-*Arfrp1*$^{flox/flox}$-mice) were viable and of normal size at birth. However, the survival rate of the aP2-Cre-*Arfrp1*$^{flox/flox}$-mice was reduced. Until the age of 21 days, at weaning, only 30% of aP2-Cre-*Arfrp1*$^{flox/flox}$-pups survived. At day 14 the nullmutants exhibit about a 50% reduced body weight (3.78g ± 1.12) in comparison to control littermates (*Arfrp1*$^{flox/flox}$: 6.9 g ± 0.14). aP2-Cre-*Arfrp1*$^{flox/flox}$-mice do not develop white adipose tissue and have markedly reduced amounts of brown fat. At day 7 and 14, brown adipose tissue of the interscapular region in aP2-Cre-*Arfrp1*$^{flox/flox}$-mice mutants show a marked reduction of lipid accumulation. As a physiological consequence, nullmutants show an early hepatic steatosis as detected by oil red O-staining of the livers. Our results demonstrate a severe failure in adipocyte differentiation and fat storage in aP2-Cre-*Arfrp1*$^{flox/flox}$-mice and suggest a critical role of ARFRP1 in adipogenesis.

[1]Department of Pharmacology, German Institute of Human Nutrition, 14558 Potsdam-Rehbruecke, Germany
[2]Department Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried

137

THE SPLIT PLECKSTRIN HOMOLOGY DOMAIN OF PHOSPHOLIPASE C-γ_2 MEDIATES ACTIVATION OF THE ENZYME BY Rac GTPases

Michael Retlich, Claudia Walliser, and Peter Gierschik

Phospholipases C (PLCs) catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate. Rho GTPases stimulate membrane PtdInsP_2 hydrolysis catalyzed by PLC isozymes. This novel mode of PLC activation was first reported by our group, and has recently been corroborated by the crystal structure of the Rho GTPase Rac1 bound to PLCβ_2. The structure confirms the amino-terminal pleckstrin homology (PH) domain of PLCβ_2 as the site of interaction between Rac1 and PLCβ_2.Besides PLCβ_2, PLCγ_2 is also activated by Rac. Both members of the PLCγ subfamily, PLCγ_1 and PLCγ_2, contain two PH domains, an amino-terminal one and one located between the two catalytic subdomains and split by a SH2-SH2-SH3 tandem. PLCγ_1 is not an effector for Rac, whereas activated Rac stimulates PLCγ_2 in intact cells and *in vitro*. To examine the relevance of the amino-terminal PH domain for PLCγ_2 activation by Rac, we constructed cDNAs encoding chimeras of PLCγ_1 and PLCγ_2 with their amino-terminal PH domains swapped. The sensitivity to Rac2 was not affected in these proteins, indicating that the amino-terminal PH domain is not involved in activation of PLCγ_2 by Rac2. We then investigated the effect of an exchange of the split PH domains between PLCγ_1 and PLCγ_2. Most interestingly, PLCγ_1 with its split PH domain replaced by that of PLCγ_2 was stimulated by Rac2, in contrast to wild-type PLCγ_1. Conversely, replacement of the split PH domain of PLCγ_2 by that of PLCγ_1 rendered the resulting chimera resistant to stimulation by Rac2. Taken together, the results suggest that the split PH domain of PLCγ_2 rather than its amino-terminal PH domain serves as an effector site for Rac and provide a first indication that the mechanisms of activation by Rac are distinct for PLCβ_2 and PLCγ_2. Recent findings suggesting that the split PH domain of PLCγ_2 is involved in heterodimeric interactions raise the possibility that Rac GTPases are involved in regulating these processes as well.

Institute of Pharmacology and Toxicology, University of Ulm, Germany

138

THE G$\alpha_{q/11}$ SIGNAL MEDIATOR P63RHOGEF: A NEW PLAYER IN THE DEVELOPMENT OF CARDIAC HYPERTROPHY

C. Vettel, A. Vogt, T. Wieland, S. Lutz

While it is nowadays well accepted that the activation of G$\alpha_{q/11}$ proteins contributes to the onset of cardiac hypertrophy, the importance of the different downstream signal cascades, e.g. PLCβ and RhoA activation, is still unclear. Therefore we investigated the role of the guanine nucleotide exchange factor p63RhoGEF, a selective mediator of G$\alpha_{q/11}$ dependent RhoA activation, in cardiac RhoA activity and hypertrophy. The activation of the ET_{AB} and α_1-adrenergic receptors with their respective agonists endothelin-1 (ET-1) and phenylephrine (PE) induced a moderate RhoA activation in isolated neonatal rat cardiomyocytes, which could be enhanced by the adenoviral mediated over-expression of p63RhoGEF. Moreover, expression of the dominant negative mutant p63ΔN, which specifically inhibits G$\alpha_{q/11}$ dependent RhoA activation, showed a 50% decrease in the ET-1 and PE dependent induction of RhoA activity. The residual RhoA activity was completely abolished by co-expression of PDZRhoGEF-RGS, a G$\alpha_{12/13}$ GTPase activating protein domain. The contribution of both G protein subfamilies, G$\alpha_{q/11}$ and G$\alpha_{12/13}$, to ET-1 and PE mediated RhoA signalling could also be confirmed in a RhoA dependent stress fibre formation assay. Furthermore the down-regulation of endogenous p63RhoGEF with specific siRNAs showed that about 50% of the ET-1 induced RhoA activation is p63RhoGEF-dependent. When cardiomyocytes were incubated with ^{3}[H] -Leucin to measure protein translation and hence the degree of hypertrophy, a significant increase due to PE or ET-1 stimulation could be detected. This effect was reversed by the application of C2IN-C3, a membrane permeable RhoA/B/C inactivating toxin. The over-expression of p63RhoGEF resulted in an increased protein translation on a basal level, which was comparable to ET-1 or PE stimulated cells. This data leads to the conclusion, that at least two different G protein subfamilies contribute to RhoA activation in PE or ET-1 stimulated neonatal rat cardiomyocytes and that p63RhoGEF is an important mediator in G$\alpha_{q/11}$ dependent cardiac RhoA activation and hypertrophy.

Institute of Clinical and Experimental Pharmacology and Toxicology, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Mannheim

139

RAC1 PHOSPHORYLATION PROTECTS FROM *CLOSTRIDIUM DIFFICILE* TOXIN A-INDUCED CYTOTOXICITY

J. Schoentaube, I. Just, R. Gerhard

Toxin A (TcdA) and toxin B (TcdB) from *Clostridum difficile* are causative agents of antibiotic-associated pseudomembranous colitis. They are single chain protein toxins that mono-glucosylate Rho GTPases. Glucosylation leads to functional inactivation of Rho GTPases and to subsequent breakdown of the actin cytoskeleton. It was shown that EGF-treatment of human colonic mucosa attenuated the toxin-induced damage as shown by decrease in transepithelial electrical resistance and loss of the epithelial barrier function. It is further known that Akt1, activated by EGF signaling, phosphorylates Rac1. Rac1, one of the major substrates of TcdA, possesses a phosphorylation site at serine 71, (Rho at serine 73) which is part of the consensus motif ^{64}YD**R**L**R**PL**SY**P^{73} recognized by the Akt kinase. In this study, we investigated the influence of Rac1 phosphorylation on the cytopathic effect of *Clostridium difficile* toxin A. EGF-treatment of CaCo-2 cells induced an Akt-catalysed phosphorylation of Rac1 in a time-dependent manner as shown by immunoblot analysis with phospho-Rac1 specific antibody. Preincubation of CaCo-2 cells with EGF also delayed the TcdA-induced decrease in transepithelial electrical resistance. Accordingly, glucosylation of Rac1 by TcdA was reduced in EGF-treated cells compared to control cells. To check whether phosphorylated Rac1 is substrate for TcdA we generated the phosphomimetic mutant Rac S71E. [^{14}C]-Glucosylation assay showed that wild type Rac but not Rac S71E was substrate for TcdA. However, Rac S71E competed concentration-dependently with wild type Rac1 and reduced glucosylation of the wild type Rac. To analyse the function of the phosphorylated Rac we performed pull down experiments. Rac wild type as well as Rac S71E bound to their effector protein (Pak) and inhibitor (GDI) in a nucleotide-dependent manner. Interestingly, phosphorylated Rac1 from EGF-treated cells preferentially bound to Pak-Crib domain but not GDI in a pull down assay indicating that phosphorylation does not inactivate Rac1 as it is reported for RhoA. From these data we can conclude that phosphorylation of Rac attenuates the effect of TcdA. Phosphorylated Rac is not glucosylated and it may sequester the catalytic domain of TcdA.

Institute of Toxicology, Hannover Medical School, Hannover

140

INACTIVATION OF H-/K-/N-RAS IS THE BASIS OF THE CYTOTOXIC EFFECT OF *CLOSTRIDIUM SORDELLII* LETHAL TOXIN

S. Dreger, M. Isermann, J. Huelsenbeck, F. Hofmann, I. Just, H. Genth

Clostridium sordellii lethal toxin (TcsL) and toxin B from variant *C. difficile* strain 1470 serotype F (TcdB-F) are glucosyltransferases inactivating small GTPases of the Rho and the Ras families. While both toxins glucosylate Rac1 and R-Ras, TcsL has been reported to additionally glucosylate H-/K-/N-Ras. Both TcsL and TcdB-F caused actin re-organization ("cytopathic effect") of rat basophilic leukaemia (RBL) cells. The cytopathic effect correlated with the glucosylation of the common substrate Rac1. Rac1 glucosylation seems to be the major determinant of the cytopathic effect. The pro-apoptotic immediate-early gene product RhoB was strongly up-regulated in TcsL- but not in TcdB-F-treated RBL cells, indicating that up-regulation of RhoB was due to inactivation of H-/K-/N-Ras but not Rac1. We analysed the ability of TcsL and TcdB-F to induce apoptosis ("cytotoxic effect") in synchronized RBL cells. TcsL caused the cytotoxic effect as determined by activation of caspase-3 and phosphatidyl serine exposure. In contrast, TcdB-F failed to do so. Furthermore, TcsL reduced the viability of RBL cells whereas the viability remained unchanged in TcdB-F-treated cells. In conclusion, both the cytotoxic effect of TcsL and the up-regulation of RhoB by TcsL were based on the inactivation of H-/K-/N-Ras. RhoB is most likely involved in the regulation of the cytotoxic effect.

Institut für Toxikologie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany.

141

DIFFERENT CYTOTOXIC EFFECTS OF REFERENCE TOXIN B AND VARIANT TOXIN B FROM *CLOSTRIDIUM DIFFICILE*

J. Huelsenbeck, S. Dreger, F. Hofmann, I. Just, H. Genth

Clostridium difficile, the causative agent of the antibiotic-associated diarrhoea, produces two toxins, toxin A (TcdA) and toxin B (TcdB). Both toxins glucosylate RhoA, Rac1, and Cdc42 while the homologous toxin B from "variant" strain 1470 serotype F (TcdB-F) glucosylates Rac1 and R-Ras but neither RhoA nor Cdc42. Accordingly, Rac1 was glucosylated by TcdB and TcdB-F in rat basophile leukaemia (RBL) cells. The glucosylation of Rac1 correlated with the actin re-organization ("cytopathic effect") caused by either toxin. RhoA and Cdc42 were degraded in TcdB- but not in TcdBF-treated cells, suggesting that the degradation was due to their glucosylation by TcdB. Furthermore, the immediate early gene product RhoB was strongly up-regulated in TcdB- but not in TcdB-F-treated cells. To address the question, whether induction of apoptosis ("cytotoxic effect") depended on the substrate specificity of the glucosylating toxins, RBL cells were synchronized by the thymidine double block technique prior to toxin treatment. RBL cells were most sensitive to the cytotoxic effect of TcdB during S-phase, as analysed in terms of caspase-3 activation, phosphatidyl serine exposure, and nuclear fragmentation. In contrast, TcdB-F induced only a marginal cytotoxic effect suggesting that

inactivation of RhoA was required for the cytotoxic effect. Correspondingly, the viability of TcdB-treated RBL cells was reduced but remained unchanged in TcdB-F-treated cells. In conclusion, the protein substrate specificity of the glucosylating toxins determines their biological activity. These results offer an explanation why variant *C. difficile* strains such as strain 1470 producing TcdBF exhibit reduced virulence in animals and humans.
Institut für Toxikologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

142

CNF1 OF *ESCHERICHIA COLI* AND CNFY OF *YERSINIA PSEUDOTUBERCULOSIS* USE A SIMILAR MODE OF CELL ENTRY

B. Blumenthal, Z. Knust, K. Aktories, and G. Schmidt

The cytotoxic necrotizing factors CNF1 of pathogenic *E. coli* strains and CNFy of *Yersinia pseudotuberculosis* belong to a family of Rho-activating toxins that constitutively activate small GTPases by deamidation of a specific glutamine residue which leads to a block of GTP hydrolysis. CNF1 activates RhoA, Rac, and Cdc42 whereas CNFy is a selective activator of RhoA (Hoffmann et al., 2004). CNFy shares 61 % homology on the amino acid level with the *E. coli* toxin. Therefore, we investigated the cellular entry of CNFy in comparison to CNF1. CNF1 has been suggested to enter cells via clathrin-independent and caveolin-independent endocytosis, upon binding to a cell surface receptor. After internalization, CNF1 is delivered to late endosomes by a microtubuli-dependent transport mechanism. It translocates into the cytosol in an acidic pH-dependent manner (Contamin et al., 2002). By inhibitor studies and transfection studies we demonstrated that CNFy uses a similar mode of cell entry. Furthermore, the same specific acidic residues in the translocation domains of CNF1 (Pei et al., 2001) and CNFy are important for the translocation process. In cell fractionation experiments a 55 kD fragment could be detected in the cytosolic fraction of intoxicated cells by immunoblotting with an anti-CNF1 antibody. This finding implicates that CNF1 is cleaved during its cellular uptake.

Contamin S., Galmiche A., Doye A., Flatau, G., Benmerah A., and Boquet P. (2002) Mol. Biol. Cell 11, 1775-1787
Hoffmann, C., Pop M., Leemhuis J., Schirmer J., Aktories K., and Schmidt G. (2004) J. Biol. Chem. 279, 16026-16032
Pei S., Doye A., and Boquet P. (2001) Mol Microbiol 41, 1237-1247

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg

143

THE PDZ DOMAIN OF LARG IS NECESSARY FOR ITS ACTIVATION BY G_q AND $G_{12/13}$ PROTEIN COUPLED RECEPTORS

P. Winkler, C. Vettel, T. Wieland and S. Lutz

The activation of the RhoA specific guanine nucleotide exchange factor Larg (Leukemia associated RhoGEF) by $G_{12/13}$ protein coupled receptors is nowadays widely accepted, whereas its activation by G_q coupled receptors (G_qPCR) is still a matter of debate. Our data demonstrate, that in HEK293 cells the co-expression of full length Larg with different G_qPCRs, like the histamine H_1 (H_1R) and the muscarinic M_3 acetylcholine (M_3R) receptors, synergistically stimulated the RhoA activation in the presence of the respective receptor agonists (100µM histamine, 1mM carbachol). Similar results were obtained by co-expressing the selectively $G_{12/13}$ coupled thromboxane A2 receptor (TxA_2R) and full length Larg in the presence of the specific agonist U-46619 (200nM). By using the more sensitive and quantitative serum response factor dependent reporter gene assay, we could further show that the synergistic action of the G_qPCR coupled receptors and Larg was agonist concentration dependent. To identify protein domains of Larg which are necessary for the G_qPCR induced RhoA activation we deleted the extreme N-terminus of Larg including a PDZ domain. This truncated Larg mutant (LargΔPDZ) was still able to activate RhoA as shown by precipitation of active RhoA and by the SRF reporter gene assay, but additional co-expression of G_qPCRs and stimulation with the respective agonist leads to no further increase in RhoA activation at neither agonist concentration. The cooperative RhoA activation of the TxA_2R and Larg was similarly abolished by the deletion of the PDZ domain. As we could not detect any synergistic RhoA activation by, nor a direct interaction of over-expressed constitutively active $G\alpha_{q/11}$ or $G\alpha_{12/13}$ with Larg or LargΔPDZ, we conclude that the GPCR dependent Larg activation requires an active receptor and the N-terminal PDZ domain of Larg.

Institute of Experimental and Clinical Pharmacology and Toxicology, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Mannheim

144

THE INTEGRITY OF THE EFFECTOR DOMAIN OF THE SMALL GTPase Rac2 IS NECESSARY FOR THE INTERACTION WITH ITS EFFECTOR PHOSPHOLIPASE C-γ_2.

C. Walliser, C. Christow, M. Retlich, and P. Gierschik

Rac2 is a hematopoietic-cell-specific member of the Rho GTPase family. Rho GTPases cycle between active GTP- and inactive GDP-bound forms and bind effector proteins only in the active, GTP-bound state. The exchange of GTP for bound GDP on Rho GTPases leads to conformational changes in two amino terminal regions termed switch regions I (e.g. residues 25-49 of Rac1) and II (e.g. residues 59-76 of Rac1). Switch I is important for proper interaction of Rho GTPases with effector molecules and is therefore referred to as "effector region". Mutations in this region prevent or impair the interaction of Rho proteins with some, but not all, effector targets. We have previously shown that activated Rac GTPases (Rac1/2/3) mediate stimulation of phospholipase C-γ_2 (PLCγ_2), thus identifying PLCγ_2 as a new effector of Rac. To characterize the molecular mechanisms of the interaction between Rac2 and PLCγ_2, we constructed cDNAs encoding Rac2 variants bearing mutations in switch I (V36A, F37A, D38A, Y40C, N43A) and II (R66A) or both regions (V36A/R66A). Wild-type and mutant Rac2 proteins were expressed in baculovirus-infected insect cells and functionally reconstituted in a cell-free system with recombinant PLCγ_2 produced in the same expression system. Introduction of alanine and cysteine at positions 37 and 40, respectively, fully and introduction of alanine at position 38 nearly abrogated Rac2-mediated stimulation of PLCγ_2, indicating that F37, D38, and Y40 in switch region I are essential for the Rac2/PLCγ_2 interaction. In contrast, the substitution N43A did not affect the ability of Rac2 to stimulate PLCγ_2. It has previously been shown that $Rac2^{V36A}$, $Rac2^{R66A}$, and $Rac2^{V36A/R66A}$ cannot interact with a negative regulator of Rac, RhoGDI (Rho guanine nucleotide dissociation inhibitor). However, these mutants clearly stimulated PLCγ_2 activity, albeit to a somewhat lower extent than wild-type Rac2. The results show that the integrity of the effector region of Rac2 is required for PLCγ_2 activation.

Institute of Pharmacology and Toxicology, University of Ulm, Germany

145

ACTIVATION OF RHO-A, -B, AND -C BY THE CONSTITUTIVELY ACTIVE, $G\alpha_q$-COUPLED VIRAL CHEMOKINE RECEPTOR HOMOLOG pUS28

M. Pfreimer, P. Gierschik, and B. Moepps

The human cytomegalovirus (HCMV) gene US28 encodes a chemokine receptor homolog, pUS28, which specifically binds CC chemokines like RANTES and MCP-1. As previously reported, COS-7 cells expressing pUS28 showed a constitutive, i.e. chemokine-independent production of inositol phosphates and a constitutive transcriptional activation of genes controlled by the serum response factor, SRF. The latter effect was specifically mediated by $G_q\alpha$ and $G_{11}\alpha$ and by an unidentified $G\alpha_{q/11}$-regulated Rho guanine nucleotide exchange factor (RhoGEF). Rho-GTPases were involved in the activation of SRF-mediated gene transcription by pUS28, since coexpression of *Clostridium botulinum* ADP-ribosyltransferase C3, which specifically inhibits RhoA, -B, and -C by ADP-ribosylation, abolished the stimulatory effect of pUS28. To further characterize the Rho isoform(s) involved in this process, RhoA, -B, and -C were expressed together with pUS28 in COS-7 cells and their effects on the activation of SRF-mediated gene transcription by pUS28 were determined. The results showed that coexpression of pUS28 with each of the three Rho isoforms synergistically enhanced the stimulatory effect of pUS28 on SRF-mediated gene transcription and that this enhancement was sensitive to *Clostridium botulinum* ADP-ribosyltransferase C3 in all cases. To investigate the role of $G\alpha_{q/11}$-regulated RhoGEFs in this process, the Rho-GEFs p63Rho-GEF and LARG were coexpressed together with RhoA, -B, or -C. Expression of both RhoGEFs led to a synergistic enhancement of SRF-mediated gene transcription, irrespective of the nature of the specific Rho isoform present. Taken together, our findings indicate that all three Rho isoforms, RhoA, -B, and -C, are capable of mediating pUS28/$G\alpha_{q/11}$/RhoGEF-induced activation of SRF-dependent gene transcription in COS-7 cells.

Institute of Pharmacology and Toxicology, University of Ulm, 89069 Ulm

146

ACTIVATION OF RAC BY CALCIUM IONOPHORE A23197 IN MURINE MAST CELLS

H. Stratmann, K. Aktories and J. Orth

Activation of mast cells via FcεRI receptors involves stimulation of the guanine nucleotide exchange factor Vav and activation of Rac, a small GTPase of Rho family. It has been reported that Rac is involved in regulation of PLCγ to induce formation of IP_3 and diacylglycerol. Here we studied the role of calcium ions in Rac activation and hexosaminidase release in bone marrow mast cells from mouse and peritoneal mast cells from rats. Incubation of mast cells with calcium ionophore A23187 stimulated hexosaminidase release and caused activation of Rac, which was determined by precipitation of the GTP-bound GTPase, with the Rac-binding domain of PAK. Treatment of mast cells with the intracellular calcium chelator BAPTA inhibited the A23187-caused release of hexosaminidase and inhibited Rac activation. *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B, which inhibit Rho GTPases by glucosylation, blocked A23187-induced activation of mast cells and prevented activated of Rac. These data indicate that calcium mobilization by A23187 induces activation of Rac. The findings suggest that Rac acts not only between Vav and PLCγ in signalling induced by stimulation of FcεRI receptors but also in calcium ionophore A23187-induced mast cell activation downstream of PLCγ.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg

147

A NOVEL SH3 DOMAIN CONTAINING PROTEIN INTERACTING WITH RAC GTPASES

K. Sandrock, G. Schmidt and N. Klugbauer

Rac proteins belong to the Rho family of small GTPases which comprises 20 different members. They play a critical role in diverse processes, such as control of cell morphology, actin dynamics, transcriptional activation and apoptosis signaling. As molecular switches, Rac GTPases cycle between an inactive GDP-bound state and an active GTP-bound state and are strictly controlled by three classes of regulatory proteins. The GTPase activating proteins (GAPs) accelerate the low intrinsic GTPase activity. The GDP/GTP exchange is promoted by the association of a guanine nucleotide exchange factor (GEF). Guanine nucleotide dissociation inhibitors (GDIs) are able to extract GTPases from the cellular membrane and create their cytosolic pool. The three distinct mammalian Rac proteins share very high sequence similarity but they are involved in different physiological functions and interact in part with a specific subset of effectors. Although they are expressed in different tissues, it is unclear how their further functional specificity is achieved. While Rac1 is ubiquitously expressed, Rac2 is largely restricted to hematopoietic tissues and Rac3 to neural tissues. To isolate novel Rac interacting proteins, we screened a human HeLa cDNA library using the yeast two-hybrid method with the constitutive active isoforms Q61ERac1 and Q61ERac3 as baits. Among a variety of putative interaction partners a yet uncharacterized protein containing three SH3-domains was identified. The open reading frame shows about 42% homology to the Rac-specific effector Plenty of SH3 (POSH). POSH acts as a scaffold protein and contributes to Rac-induced signal transduction pathways. Overexpression of POSH induces apoptosis and its specific GTPase binding domain prevents Rac from degradation. In contrast to POSH the newly identified protein does not induce apoptosis, instead we observed effects on the reorganization of the actin cytoskeleton. The cellular distribution was studied with EGFP fusion proteins in transiently transfected HEK293 and HeLa cells. The overexpressed SH3 domain containing protein localizes in the cytoplasm of HEK293 and HeLa cells. To verify the result of the YTH screen, we are performing *in vitro* binding assays using bacterially expressed glutathione-S-transferase (GST) fusion proteins.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, Albertstr. 25, 79104 Freiburg, Germany

148

SUBSTRATE SPECIFICITY OF YOPT CLEAVAGE

F. Füller, K. Aktories and G. Schmidt

The *Yersinia enterocolitica* outer protein YopT is a 35-kDa cysteine protease, which removes the C-terminal isoprene anchor of small GTPases RhoA, Rac and Cdc42. This modification leads to the inactivation of the GTPases, disruption of the actin cytoskeleton and finally cell rounding. YopT cleavage of the GTPases is depended on the isoprenylation of the *CAAX*-box cysteine catalysed by geranylgeranyltransferaseI or farnesyltransferase (Shao et al. 2003). The two additional steps of posttranslational modification of proteins with *CAAX*-box are removal of the *AAX*-tripeptide by the ras converting enzyme 1 (Rce1) and methylation of the carboxyl group of the isoprenylcysteine by the isoprenylcysteine carboxyl methyltransferase (IcMT) (Otto et al. 1999). Whereas endoproteolytic removal of the *–AAX*-tripeptide is necessary for YopT activity; the methylation of the isoprenylcysteine is not required for YopT cleavage (Fueller et al. 2006).

In order to analyse the differences in substrate recognition and cleavage of RhoA, Rac and Cdc42 by YopT we established the posttranslational modification *in vitro*. Depending on the polybasic sequence at the C-terminus of RhoA; cleavage of RhoA by YopT is faster in comparison to Rac and Cdc42. The posttranslational modifications of the GTPases *in vitro* enable us to study the involvement of nucleotide binding or the kind of isoprenylation of the GTPases in substrate recognition and cleavage by YopT. Furthermore interaction and cleavage of RhoA in the GDI-complex by YopT can be characterised in more detail.

Shao, F., Vacratsis, P. O., Bao, Z., Bowers, K. E., Fierke, C. A., and Dixon, J. E. (2003). Biochemical characterization of the Yersinia YopT protease: Cleavage site and recognition elements in Rho GTPases. Proc.Natl.Acad.Sci.USA 100, 904-909.

Otto, J. C., Kim, E., Young, S.G., Casey, P. J. (1999). Cloning and characterization of a mammalian prenyl protein-specific protease. J Biol Chem. 26;274(13):8379-82.

Fueller, F., Bergo, MO., Young, S.G., Aktories, K., Schmidt, G. (2006). Endoproteolytic processing of RhoA by Rce1 is required for the cleavage of RhoA by Yersinia enterocolitica outer protein T. Infect Immun. 74(3):1712-7.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Alberstraße 25, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg i. Brsg.

149

P38 MAP KINASE AND THE CYTOTOXIC EFFECT OF *CLOSTRIDIUM DIFFICILE* TOXIN B

F. Schulz, J. Huelsenbeck, R. Gerhard, A. Kotlyarov, G. Fritz, I. Just, H. Genth

The small monomeric RhoB GTPase is reportedly involved in the regulation of apoptosis. RhoB is upregulated in response to inactivation of Rho and Ras proteins by e.g. farnesyl-/geranylgeranyltransferase inhibitors or Rho/Ras-inactivating toxins such as the clostridial glucosylating toxins. *Clostridium difficile* toxin B (TcdB) glucosylates and thereby inactivates RhoA, Rac1, Cdc42 and up-regulates RhoB. Here we studied the involvement of p38 MAP kinase in up-regulation of RhoB and in the cytotoxic effect of TcdB. *RhoB* promoter studies in mouse embryonic fibroblasts (MEFs) revealed that the basal activity of the promoter in $p38_{\alpha}$ -/- MEFs was elevated by a factor of 4 compared to wild-type MEF. In wildtype MEFs, TcdB induced a strong increase in promoter activity. TcdB only very faintly activated the *rhoB* promoter in $p38_{\alpha}$ -/- MEFs, indicating a role of p38 MAP kinase in the regulation of *rhoB*. In contrast, the kinetics of the actin cytoskeleton re-organisation by TcdB ("cytopathic effect") was comparable in wild-type and p38(-/-) MEFs, suggesting that p38 MAP kinase did not contribute to the cytopathic effect of TcdB. The cytotoxic effect of TcdB (induction of apoptosis) was analysed in synchronized MEFs in terms of phosphatidylserine exposure by annexin V staining. TcdB-induced apoptosis was inhibited by caspase inhibitors and by SB203580, an inhibitor of p38 MAP kinase. Accordingly, apoptosis induced by TcdB was reduced in p38(-/-) MEFs in which TcdB failed to activate the *rhoB* promoter. In conclusion, our data suggest that p38 MAP kinase - likely in a RhoB-dependent manner - is involved in the cytotoxic effect of TcdB.

Institute für Toxikologie und Biochemie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany; Institut für Toxikologie der Universität Mainz, D-55131 Mainz, Germany.

150

GLUCOSYLATION OF RHO G CONTRIBUTES TO THE CYTOPATHIC EFFECT OF CLOSTRIDIAL GLUCOSYLATING TOXINS

I. Halabi-Cabezon, J. Huelsenbeck, F. Hofmann, I. Just, and H. Genth

Glucosylation of Rho proteins by clostridial toxins results in re-modelling of the actin cytoskeleton („cytopathic effect"). The Rho family proteins RhoG and TC10 exhibit a similarity at amino acid level to Rac1 of 70 %. Rac1 is glucosylated by all members of the toxin family. In this study, the substrate specificity of the glucosylating toxins is re-analysed using a subset of recombinant Rho proteins including RhoG and TC10. *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB) glucosylate Rho(A,B,C), Rac1, Cdc42, RhoG, and TC10, as determined by [^{14}C]glucosylation of recombinant Rho proteins. Toxin B from the variant *C. difficile* strain 1470 serotype F (TcdB-F) and lethal toxin from *C. sordellii* strain 6018 (TcsL) share these substrates except for Rho(A,B,C). The glucosylation of RhoG and TC10 was analysed in a toxin concentration-dependent manner. Rac1 and RhoG were modified by TcdB and TcdB-F with comparable kinetics, while TC10 turned out to be a minor substrate. TcsL glucosylated Rac1, RhoG, and TC10 with comparable kinetics. To show that RhoG and TC10 were involved in the cytopathic effect of the glucosylating toxins, a "protection assay" exploiting the inhibition of RhoGAP proteins by ectopic expression of constitutively active Rho proteins was applied. The inhibition of the GAP proteins results in elevated levels of the active, GTP-bound form of the respective endogenous Rho protein which are less efficiently glucosylated than the GDP-bound forms. Cells with elevated levels of either active endogenous Rac1, RhoG, or Cdc42 were protected from the cytopathic effect of TcdB, suggesting that glucosylation of these Rho proteins contributed to the cytopathic effect. Cells with an elevated level of active TC10 were only partially protected whereas an elevated level of active RhoA did not protect cells from the cytopathic effect at all. In conclusion, glucosylation of RhoG and TC10 (to lower extent) contributes to the cytopathic effect of clostridial glucosylating toxins.

Institut für Toxikologie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany.

151

UP-REGULATION OF RHOB BY THE CYTOTOXIC NECROTIZING FACTOR 1 FROM *ESCHERICHIA COLI*

H.-J. Meyer, S. Dreger, G. Fritz, K. Aktories, G. Schmidt, and H. Genth

The immediate-early gene product RhoB is up-regulated in response to genotoxic stress (UV light, alkylating agents) or to inactivation of Rho and Ras proteins by farnesyl-/geranylgeranyltransferase inhibitors, HMG-CoA reductase inhibitors („statins"), or Rho/Ras-inactivating toxins among them the clostridial glucosylating and C3-like ADP-ribosylating toxins. The activity of the *rhoB* promoter was shown to be positively regulated by Rac1 (Fritz et al. JBC 272, 30637–30644). The cytotoxic necrotizing factor-1 (CNF-1) toxin is a virulence factor produced by uro-pathogenic *Escherichia coli* strains, responsible for urinary tract infections. CNF1 mediates specific cellular responses such as actin remodeling, macropinocytosis, and nuclear responses by activation of Rho proteins. The activation of Rho proteins is based on the de-amidation of a pivotal glutamine residue residing in the switch-2 region into glutamic acid, thereby blocking the intrinsic as well as the GAP-stimulated GTPase activity. We show that activation of Rho proteins by CNF1 caused up-regulation of RhoB. Interestingly, CNFγ, an isoform from *Yersinia pseudotuberculosis* that specifically activates RhoA but neither Rac1 nor Cdc42, failed to up-regulate RhoB. The *rhoB* response was further analyzed on the *rhoB* promoter as well as on the *rhoB* mRNA level, showing that up-regulation of RhoB was based on transcriptional activation. This is the first study reporting on up-regulation of RhoB in response to activation of Rho proteins.

Institut für Toxikologie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany; Institut für Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg/Brsg., D-79104 Freiburg, Germany; Institut für Toxikologie der Universität Mainz, D-55131 Mainz, Germany.

152

SYSTEMATIC DESIGN OF FRET-BASED cGMP INDICATORS

M. Russwurm, D. Koesling

The second messenger cGMP is involved in diverse physiological processes such as smooth muscle relaxation and synaptic transmission. Indicators that allow monitoring of cGMP signals in living cells will substantially improve our knowledge about cyclic nucleotide signalling. Here, we report on the systematic design of FRET-based cGMP indicators from the two known types of cGMP-binding domains, cNMP-BD and GAF, found in the cGMP-dependent protein kinases and phosphodiesterase 5, respectively. More than 50 constructs differing in the cGMP-binding domains were created and tested in vitro for their biochemical features, i.e. affinity and selectivity, association and dissociation kinetics. Interestingly, only cGMP-binding domains arranged in a tandem configuration as in their parent proteins were cGMP-responsive; constructs composed of a single cNMP-BD or GAF were insensitive to cGMP. cGMP-responsive sensors designed from the GAF domains displayed extremely slow binding and dissociation kinetics preventing their application as indicators for fast intracellular cGMP signals. The tandem cNMP-BDs from the cGMP-dependent protein kinase yielded a range of cGMP-responsive constructs. Based on dynamic range and cGMP affinity, three indicators were selected covering a range of cGMP affinities (EC_{50} 500 nM - 6 µM) that allow detection of cGMP concentrations between 100 nM and 35 µM. These indicators are highly selective for cGMP, respond to changes in the cGMP concentration in a fast and reversible manner and feature twice the dynamic range of existing cGMP sensors. The *in* vivo performance is demonstrated by measuring cGMP signals in living cells with the new indicators and validated by comparison with cGMP concentrations detected by radioimmunoassays.

Institut für Pharmakologie und Toxikologie, Ruhr-Universität Bochum, Medizinische Fakultät MA N1, 44780 Bochum, Germany

153

ROLE OF TYROSINE 143 FOR THE ADENOSINE AND cAMP BINDING TO S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE

M. Hermes, H. Osswald, R. Riehle, and D. Kloor

S-adenosyl-L-homocysteine (AdoHcy) hydrolase (AdoHcyase) catalyzes the hydrolysis of AdoHcy, which is a product inhibitor of S-adenosyl-L-methionine-dependent transmethylations. Gene analysis in a human case of AdoHcyase deficiency revealed a maternally derived stop codon and a paternally derived missense mutation, where tyrosine 143 is mutated to cysteine (Y143C), affecting the oxidation state of enzyme-bound NAD^+. In the present study we examined adenosine and cAMP binding characteristics and enzymatic activity of recombinant wild-type and mutant Y143C AdoHcyase. Oligonucleotide-directed mutagenesis was used to prepare mutated AdoHcyase. Wild-type and mutated human AdoHcyase have been overexpressed and purified from *E. coli*. Quantification of enzyme-bound NAD^+ and NADH was performed using HPLC-analysis of the released nucleosides. AdoHcy hydrolysis was determined photometrically at 292 nm, AdoHcy synthesis was measured by HPLC. Saturation binding experiments were performed with purified enzyme and [^{3}H]-adenosine or [^{3}H]-cAMP at a final ligand concentration of 0.01-50 µM and 1-250 nM, respectively. The recombinant wild-type AdoHcyase is characterized by a K_M of 14.9±1.0 µM and a V_{max} of 0.33 U for the hydrolytic direction, and by a K_M of 4.5±0.3 µM for adenosine and a V_{max} of 0.8 U for the synthetic direction. Y143C mutation did not change the K_M values. However, mutation reduced the V_{max} in the hydrolytic and synthetic direction, by 50% and 80%, respectively, which might be the result of an increased NADH content of 30% in Y143C AdoHcyase. Binding of adenosine to wild-type and mutated AdoHcyase was saturable with one apparent affinity of K_D 12.6±0.8 µM and K_D 6.93±0.75 µM, respectively. In contrast, cAMP exhibited one high affinity binding site with a of K_D 29.4±1.0 nM (wild-type) and K_D of 56.6±2.6 nM (Y143C). Moreover, Y143C mutation reduced adenosine and cAMP binding capacity by 65-75%. The interesting finding of our study is the reduced enzymatic activity and binding capacity of the mutant Y143C AdoHcyase. We suggest that this tyrosine residue is crucial for AdoHcyase conformation.

Department of Pharmacology and Toxicology, Faculty of Medicine, Eberhard-Karls University of Tübingen, Germany

154

FRET-BASED ANALYSIS OF CYCLIC AMP SIGNALS IN PRIMARY CELLS OF THE VASCULAR SYSTEM

R. C. Werthmann*, K. von Hayn*, M. J. Lohse, M. Bünemann

Cyclic nucleotides play a major role in the physiology of the vascular system including regulation of blood pressure and maintaining endothelial barrier function. These functions are regulated via a variety of different signalling pathways, most prominently G protein coupled pathways. The spatiotemporal integration of these signals will eventually generate physiological effects. We utilized Epac1-based FRET sensors (Nikolaev et al., JBC, 2004) in primary endothelial (HUVEC) and aortic smooth muscle cells (mouse) in order to measure cAMP gradients in individual cells. Electroporation of these cells yielded in transfection efficiencies >50%. On the day after transfection single-cell fluorescence was recorded while cells were challenged with different agonists in order to evaluate which receptors influence cAMP levels in these cells. Isoprenaline in both cell types stimulated cAMP levels with an EC_{50} of 7.1nM and 16.6nM for HUVECs and smooth muscle cells, respectively. Norepinephrine even at saturating concentrations was slightly less potent to induce cAMP elevations compared to isoprenaline. We studied kinetics of β-adrenergic receptor mediated cAMP production and hydrolysis after agonist withdrawal. This initial characterization of temporal changes in cAMP within single living primary cells proves that FRET-based sensors are well suited to study dynamics of cAMP/cGMP in vascular tissues.

University of Würzburg, Department of Pharmacology and Toxicology, Würzburg, Germany. *: These authors contributed equally to this work.

155

INHIBITORY ACTION OF CLONIDINE ON HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED (HCN) CHANNELS

Xiangang Zong[1], Anne Knaus[2], Lutz Hein[2], Martin Biel[1]

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to a wide range of physiological functions including cardiac and neuronal pacemaker activity. Recently we found that the α2 adrenoceptor agonist clonidine is an efficient blocker of both native and cloned HCN channels. This inhibitory action substantially contributes to the negative chronotropic effect of clonidine. Here, we have carried out patch-clamp recordings to investigate the molecular mechanisms underlying the inhibitory action of clonidine. In whole cell recodings extracellular clonidine reversibly blocked HCN1 and HCN2 currents. In contrast, clonidine did not reduce the HCN current recorded in excited inside-out patches when present in the bath solution suggesting that it binds to a channel domain that is accessible from the extracellular face of the plasma membrane. Binding was voltage-dependent being more profound at less hyperpolarized voltages. The block by clonidine differed in several aspects between HCN1 and HCN2. Firstly, the IC_{50} value of clonidine was about 5 times higher in HCN1 than in HCN2 (40 µM vs. 8 µM). Moreover, clonidine blocked HCN2 more easily when present during repetitive hyerpolarizing pulses (0.5 Hz) as compared to double pulse protocols with long interpulse durations (80 s) while the HCN1 block was comparable in both stimulation protocols. However, when channels were activated using a long-lasting hyperpolarization (for 80 s at –100 mV) clonidine blocked the same fraction of HCN1 and HCN2 currents. This finding suggests that in HCN2 access to the binding site requires longer times than in HCN1 and, hence, is only complete upon long hyperpolarizing steps or series of shorter pulses. In agreement with a kinetic model to explain the different efficacy of clonidine the unblocking of drug bound to the channel occured profoundly faster in HCN1 than in HCN2. Taken together our results suggest that based on the clonidine structure it may be a feasible goal to develop blockers with even more pronounced isoform specificity among the HCN channel family.

[1]Department Pharmazie, Pharmakologie für Naturwissenschaften, Ludwig-Maximilians Universität München, Butenandtstr. 7, 81377 München
[2]Institute für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, Albertstr. 25, 79104 Freiburg

156

MOLECULAR ANALYSIS OF THE INTERACTION OF *BORDETELLA PERTUSSIS* ADENYLYL CYCLASE WITH FLUORESCENT NUCLEOTIDES

M. Göttle[1], J. Geduhn[2], B. König[2], W.-J. Tang[3], R. Seifert[1]

Bordetella pertussis bacteria cause whooping cough, one of the top five child killers. Adenylyl cyclase toxin (CyaA) from *Bordetella pertussis* exhibits anti-inflammatory effects, weakening immune response and increases infection severity. Thus, CyaA inhibitors with high potency and selectivity with respect to mammalian adenylyl cyclase isoforms are needed. We examined the inhibitory potencies of 2´,3´-*N*-methylanthraniloyl-, anthraniloyl- and trinitrophenyl-substituted nucleotides on CyaA catalytic activity. Fluorescence resonance energy transfer (FRET) and direct fluorescence were used to monitor nucleotide binding, to estimate inhibitor potencies and to investigate kinetics of toxin activation and inactivation and the hydrophobic properties of the catalytic site. Compared to mammalian adenylyl cyclase isoforms and *Bacillus anthracis* adenylyl cyclase toxin edema factor, CyaA displayed lower sensitivity to inhibitors. Introduction of the MANT substituent at 2´,3´-ribosyl position of NTPs resulted in 4 to 170-fold increased potency. K_i-values of 3'MANT-2'd-ATP and 2'MANT-3'd-ATP in the AC activity assay using Mn^{2+} were 220 nM and 340 nM, respectively. However, other purine and even pyrimidine nucleotides inhibited CyaA as well. MANT-nucleotide binding to CyaA generated a FRET signal from W69, yielding K_d-values of 300 nM for 3´MANT-2´d-ATP and 400 nM for 2´MANT-3´d-ATP. Kinetics of CyaA activation by calmodulin and inactivation by {9-[2-(phosphonomethoxy)ethyl]adenine diphosphate} (PMEApp) occurred within seconds. As a result from direct fluorescence experiments, the MANT group is supposed to interact with F306. In conclusion, the catalytic site of CyaA possesses high conformational flexibility and we provide fluorescence-based approaches and structure/activity relationship data for high-throughput screening of novel inhibitors.

[1] Department of Pharmacology and Toxicology, University of Regensburg
[2] Department of Organic Chemistry, University of Regensburg
[3] Ben-May Institute for Cancer Research, University of Chicago, IL, USA

157

GINKGO BILOBA EXTRACT (EGb 761) INHIBITS ENDOTHELIAL CELL PROLIFERATION IN VITRO

A. Koltermann, A. M. Vollmar, S. Zahler

Proliferation of endothelial cells is crucial for tumor growth and vascularisation. Therefore, antiproliferative molecules are under intense investigation and promising new approaches in cancer therapy. The standardised Ginkgo biloba extract (EGb 761) has attracted much attention due to its human health benefits. However, its anti-proliferative properties have as yet been widely unknown. Therefore, the aim of the present study was to examine its effects on endothelial proliferation and the underlying molecular mechanisms as well as signalling pathways. Long-term treatment of human microvascular endothelial cells (HMEC-1) with EGb 761 significantly suppressed cell proliferation and enhanced apoptosis after 72 and 48 hours, respectively. Furthermore, the preincubation with EGb 761 (500 mg/L, 30 min) before growth factor stimulation (FGF, 5 µg/L, 30 min) significantly reduced FGF induced ERK phosphorylation. In addition to the influence of EGb 761 on upstream kinases Raf-1 and MEK1/2, we assessed its effects on phosphatases. As demonstrated by protein tyrosine as well as serine/threonine phosphatase assays and western blot analysis using phosphatase inhibitors (NaF, Na_3VO_4) EGb 761 inhibits ERK phosphorylation via induction of protein tyrosin phosphatases. Furthermore, we were able to identify MKP-1 as one phosphatase which is induced by EGb 761. Moreover, treatment with EGb 761 enhanced endothelial nitric oxide production. Preincubation with the NOS inhibitor L-NAME and subsequent exposure to EGb 761 and FGF however, did not effect the reduction of FGF induced ERK phosphorylation by EGb 761. Additionally, Ginkgo significantly increased cAMP levels (ELISA). Preincubation with PKA inhibitors cAMPS-Rp or PKI did not influence the reduction of ERK phosphorylation by EGb 761. In conclusion, EGb 761 has an anti-proliferative effect on endothelial cells via inhibition of ERK phosphorylation. The examination of the underlying molecular mechanism showed an involvement of protein tyrosine phosphatases like MKP-1. Furthermore, we demonstrated that the second messengers, NO and cAMP, did not influence the suppressed ERK phosphorylation by EGb 761. Ginkgo biloba extract, thus, is an interesting candidate for further investigations on anti-angiogenic properties.

(supported by DFG – GRK438)

Department of Pharmacy, Ludwig-Maximilians-University, Munich, Germany.

158

ANTI-ANGIOGENIC EFFECTS OF NEW CDK INHIBITORS LGR 561, LGR 848, AND LGR 849 ON ENDOTHELIAL CELLS

J. Liebl[1], V. Krystof[2], P. Pechan[3], A. M. Vollmar[1], and S. Zahler[1]

LGR 561, LGR 848 and LGR 849 are new derivatives of roscovitine, a cyclin dependent kinase (Cdk) inhibitor that competes for the ATP binding site on the kinase. Cdk inhibiting drugs have shown preclinical and clinical anticancer activity, but are not well-investigated in endothelial cells. Since proliferating endothelial cells are interesting targets for anti-angiogenic therapy, aim of our study was to elucidate a potential anti-angiogenic effect of LGR 561, LGR 848 and LGR 849 in human microvascular endothelial cells (HMEC-1) and human macrovascular umbilical vein endothelial cells (HUVEC). To this end, effects on proliferation, adhesion, migration and tube formation were examined. First, we determined effects on cell growth, cell cycle progression and apoptosis in HMEC-1. 72 hours treatment with LGR 561, LGR 848 and LGR 849 dose dependently decreased cell proliferation (EC 50 ~ 10 µM). To understand the underlying mechanisms, we investigated their effects on cell cycle and apoptosis by flow cytometry. Concomitant with a G2/M cell cycle arrest after 48 hours, we observed increased apoptosis in proliferating HMEC-1. Whilst cell adhesion was not influenced, migration and tube formation of HUVECs clearly were inhibited under treatment with the three substances. Excitingly, for the first time, we demonstrated a difference between the S- and R-isomers concerning their ability for inhibiting cell migration. LGR 849 and LGR 848 represent the S- and R- isomer of one compound, respectively. LGR 561 shows S-configuration. The two S-isomers LGR 561 and LGR 849 decreased migration to 35%, whereas the R-isomer LGR 848 showed a reduction only to 80%. In conclusion, LGR 561, LGR 848 and LGR 849 inhibit angiogenesis much more potently than the mother substance roscovitine. Therefore, further studies concerning the signalling leading to the mentioned effects are in progress.

(supported by the EU project "PROKINASE" No. 503467)

[1]Department of Pharmacy, University of Munich, Germany.
[2]Palacky University & Institute of Experimental Botany, Czech Republic.
[3]C3 Bio, Munich, Germany.

159

DOWN-REGULATION OF THE AKT PATHWAY BY NOVEL TETRACYCLIC TRITERPENOIDS INDUCES CELL CYCLE ARREST AND TRIGGERS APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

A.C. Estrada, T. Syrovets, B. Büchele, T. Schmidt, Th. Simmet

Akt is a family of serine/threonine kinases, which control pathways involved in cell metabolism, proliferation and apoptosis. Akt is thought to play an important role in progression and chemoresistance of several human cancer types, including prostate cancer. Indeed, loss of the 'phosphatase and tensin homolog deleted on chromosome ten' (PTEN) expression, a phosphatase inhibiting Akt, is associated with increased biologic potential of aggressive behaviour in prostate cancer. We analyzed the expression and function of Akt isoenzymes in androgen-dependent LNCaP and androgen-independent PC-3 and DU 145 prostate cancer cells. Akt1 and Akt2, but not the Akt3 isoform are expressed and constitutively active in all three cell lines. Three structurally different Akt inhibitors exerted cytotoxic effect on LNCaP and PC-3 cell lines indicating that the Akt pathway is indispensable for the proliferation of PTEN-deficient prostate cancer cells. The oleogum resins from various *Boswellia* species contain a complex mixture of mono- and triterpenoids that possess biological activities including antitumor properties. In search for well-tolerated and stable Akt inhibitors, we have isolated several tetracyclic triterpenoids from the oleogum resin of *Boswellia carterii* and purified them to chemical homogeneity by reversed phase high-performance liquid chromatography; we confirmed their chemical structures by mass spectrometry. 3-Keto–tirucallic acid, α-acetyl-

tirucallic acid and β-acetyl-tirucallic acid potently inhibited the activities of human recombinant Akt1 and Akt2 in *in vitro* kinase assays. Similarly, the triterpenoids inhibited Akt activity immunoprecipitated from PC-3 cells, but did not affect the activity of immunoprecipitated IKK. The triterpenoids also inhibited the phosphorylation of cellular Akt, β-catenin and glycogen synthase kinase (GSK)-3β, whereas extracellular signal-regulated kinase (ERK)1/2 phosphorylation, on the contrary, remained unaffected. In addition, the compounds down-regulated the expression of the crucial cell cycle regulators cyclin D1, cyclin E, cyclin-dependent kinase (cdk)4, followed by reduction of phosphorylation of retinoblastoma protein were observed. These events culminated in cell cycle arrest of the prostate cancer cells. A rapid induction of cytotoxicity and apoptosis was detected by XTT assays, the analysis of phosphatidylserine expression on the cell surface and the formation of DNA laddering. These results suggest that the inhibition of Akt activity is sufficient to trigger apoptosis in prostate cancer cells. Therefore, tetracyclic triterpenoids inhibiting Akt might provide a novel approach for the treatment of chemoresistant human prostate cancer.

Institute of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, D-89081 Ulm, Germany

160

AKT1 FUNCTIONS IN INSULIN-DRIVEN VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) BIOSYNTHESIS IN KERATINOCYTES

E. Müller, I. Goren, P. Gutwein, J. Pfeilschifter, and S. Frank

Wound healing of the skin represents the highly coordinated response of an organism to deal with external injuries. Injured cutaneous tissue initiates a scenario of different tissue movements which finally aims to reconstruct and close the wounded area. Activation of the protein kinase B (Akt) changes cellular metabolism, growth and differentiation which represent central processes in active skin repair. Here, we investigated the role of Akt in a mouse model of cutaneous wound healing. Interestingly, Akt expression and phosphorylation increased upon skin wounding of mice. Akt1 and Akt2 mRNA and phosphorylated Akt (Thr308, Ser473) protein was observed in proliferating keratinocytes located at the margins of the wound. Importantly, Akt1 was the predominant isoform in skin tissue and in cultured keratinocytes (HaCaT). As wound margin keratinocytes represent the predominant source of wound-derived vascular endothelial growth factor (VEGF), we determined the role of Akt1 activation for VEGF biosynthesis from the cells. Epidermal growth factor (EGF), cytokines and insulin potently induced keratinocyte Akt phosphorylation and a marked release of VEGF from the cells. Inhibition of the phosphatidyl-inositol-3 kinase (PI3K)/Akt pathway by wortmannin restrictively blunted only insulin-, but not EGF- or cytokine-induced VEGF production from keratinocytes. Remarkably, insulin-stimulated VEGF production in keratinocytes was mediated by Akt at the post-transcriptional level through a downstream phosphorylation of eucaryotic initiation factor 4E binding protein (4E-BP1) via activation of the mammalian target of rapamycin (mTOR) kinase. In accordance, overexpression of a myristoylated and thus constitutively active Akt1 protein in keratinocytes provided a rapamycin-sensitive phosphorylation of 4E-BP1 in functional connection to a marked VEGF release from the cells. In summary, these data constitute evidence that insulin contributes to VEGF release in skin wounds through an Akt1-mediated post-transcriptional mechanism in keratinocytes.

pharmazentrum frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

161

SPHINGOSINE-1-PHOSPHATE INDUCED MTOR ACTIVATION IS MEDIATED BY PAM AND RHEB INDEPENDENTLY OF TSC2

C. Maeurer, S.C. Pierre, W. Potstada, G. Geisslinger, K. Scholich

Mammalian target of rapamycin controls cell growth and protein synthesis-dependent changes by regulating protein synthesis and ribosome biogenesis. The clinical importance of mTOR signaling has been underlined by the use of its inhibitor Rapamycin in a variety of pathological settings including allograft rejection, restenosis, rheumatoid arthritis or cancer. mTOR activity is stimulated by the small GTPase Rheb, that is under tight regulation by the GAP (GTPase activating protein) activity of TSC2 (tuberous sclerosis complex 2). The mammalian Protein PAM (protein associated with Myc) interacts physically and genetically with TSC2. PAM is a proposed ubiquitin ligase and can be activated by the phospholipid sphingosine-1-phosphate (S1P). Therefore we speculated, that PAM modulates mTOR activity through the regulation of TSC2 protein levels. Accordingly we found that S1P induced a PAM- but also Rheb-mediated stimulation of mTOR resulting in the phosphorylation of its substrates p70-S6 kinase and 4E-BP1. Although we could show that PAM can specifically ubiquitinate TSC2, the S1P induced mTOR activation did not involve TSC2 or other known regulators of the pathway such as AKT, ERK and PI3K. Taken together, our results introduce PAM as a new regulatory protein into the mTOR signaling-pathway independent of TSC2.

pharmazentrum frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Institut für Klinische Pharmakologie, Theodor-Stern-Kai7, 60590 Frankfurt/Main Germany

162

cGMP/cGKI-SIGNALING PROMOTES THE GROWTH OF PRIMARY VSMCs ISOLATED FROM MOUSE AORTA

P. Weinmeister[1], R. Lukowski[1], C. Traidl-Hoffmann[2], S. Linder[3], F. Hofmann[1], R. Feil[4]

cGMP-dependent protein kinase type I (cGKI) is a major mediator of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway. Recent results of the analysis of atherosclerosis *in vivo* in transgenic mice strongly suggest that activation of cGKI in vascular smooth muscle cells (VSMCs) promotes the phenotypic modulation of medial VSMCs and, thus, vascular lesion formation. In contrast, numerous *in vitro* studies suggested an anti-proliferative effect for cGKI. The analysis of primary wild-type and cGKI-deficient VSMCs that were treated with the membrane-permeable cyclic nucleotide analogue 8-Br-cGMP revealed that activation of cGKI in primary VSMCs strongly promotes growth. The analysis of proliferation, apoptosis, cytoskeletal dynamics and various signaling pathways indicated that an increase in cell adhesion is a major mechanism for cGKI-mediated "growth" in primary VSMCs. The pro-adhesive effect of cGKI might be mediated via an inhibition of Rho kinase (ROCK) and enhanced integrin signaling.

[1] Institut für Pharmakologie und Toxikologie, TU München, Germany
[2] Zentrum für Allergie und Umwelt, TU München, Germany
[3] Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, LMU München, Germany
[4] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany

163

ANALYSIS OF SMOOTH MUSCLE FUNCTION IN IRAG KNOCKOUT MICE

K. Sigl[1], D. Bernhard[1], A. Jochim[1], J. Wegener[1], F. Hofmann[1], J. Schlossmann[1, 2]

Signaling by NO/cGMP relaxes various smooth muscles, modulating thereby vascular tone and gastrointestinal motility. An important signaling pathway of cGMP-dependent protein kinase type I (cGKI)-dependent relaxation is mediated by IRAG (IP_3 Receptor Associated cGKI substrate). In order to elucidate the physiological role of IRAG we generated IRAG knockout mice. To obtain this mouse strain, the modified IRAG gene was integrated into ES cells by homologous recombination. Correctly targeted ES cells were injected into mouse blastocysts to generate chimeric mice that transmitted the modified allele to the germline. The generation of the mouse strain and its initial analysis will be presented. The analysis of smooth muscle function indicates that IRAG is essential for NO/cGMP signaling. Furthermore, the effect of IRAG deletion on blood pressure regulation will be shown.

[1]Institut für Pharmakologie und Toxikologie, TU München, Germany
[2]Institut für Pharmakologie und Toxikologie, Universität Regensburg, Germany

164

IRON ANAEMIA OF CONVENTIONAL cGKI-KNOCKOUT MICE IS NOT RESCUED BY A SMOOTH MUSCLE-SPECIFIC RECONSTITUTION OF THE KINASE

R. Lukowski[1], P. Weinmeister[1], J. Schlossmann[1], P. Luppa[2], F. Fend[3], F. Hofmann[1]

Nitric oxide (NO) is of crucial importance for smooth muscle cell (SMC) function and exerts numerous, sometimes opposing, effects on the vasculature. In SMCs, many effects of NO are mediated via cGMP-dependent protein kinase type I (cGKI). Two isozymes, the cGKIα and cGKIβ that differ only in their individual amino-terminal sequences are expressed in SMCs. Both are products of the same gene, *prkg1*. Recently, two mouse lines were generated that express either the Iα or Iβ isozyme specifically in SM22α positive SMCs. As compared to conventional cGKI-knockouts, which are deficient for both isozymes in every cell, Iα and Iβ reconstituted mice have a significantly prolonged life expectancy. The present work focuses on a previously unidentified disorder of the iron metabolism that was detected in the reconstituted and conventional cGKI-knockout mouse lines. Preliminary results indicate that "older" Iα or Iβ reconstituted and cGKI-deficient animals display a severe anaemia. They develop a marked splenomegaly, which is associated with a disordered splenic iron uptake and storage as well as loss of iron through the gastrointestinal tract. Supporting this view, blood counts of Iα or Iβ reconstituted and cGKI-knockout animals demonstrated that the number and maturity of blood cells from the myeloid lineage (e.g. erythrocytes and platelets) was changed in comparison to controls. Although, these abnormalities are most likely not due to a defect of vascular or intestinal SMCs, these findings may contribute to the reduced life expectancy of reconstituted animals.

[1] Institut für Pharmakologie und Toxikologie, TU München, Germany
[2] Institut für Klinische Chemie und Pathobiochemie, TU München, Germany
[3] Institut für Allgemeine Pathologie und Pathologische Anatomie, TU München, Germany

165

PHENOTYPIC ANALYSIS OF THE SMOOTH MUSCLE-SPECIFIC cGKIα OR Iβ ISOFORM RECONSTITUTED MICE

F. Hofmann[1], R. Lukowski[1], D. Bernhard[1], P. Weinmeister[1], S. Weber[1], S. Feil[2], J. Schlossmann[1], J. W. Wegener[1], R. Feil[2]

The signaling molecule nitric oxide (NO) exerts both beneficial and deleterious effects on the vasculature, however, the molecular signaling pathways are incompletely understood. In smooth muscle cells (SMCs), the second messenger cyclic guanosine monophosphate (cGMP) mediates many effects of NO via the activation of cGMP-dependent protein kinase type I (cGKI). The cGKI gene, *prkg1*, encodes two isoforms, cGKIα and cGKIβ, both of which are expressed in SMCs. The isoforms differ only in their individual amino-terminal ends, which mediate their interaction with partner proteins and target the kinases to different subcellular locations. Conventional knockouts carrying a cGKI null mutation that affects both isoforms ($cGKI^{-/-}$) have multiple phenotypes and a reduced life expectancy. These animals are difficult to analyse, since 50% die before adulthood. Recently, two mouse lines were generated that express either the Iα or Iβ isoform specifically in SMCs of $cGKI^{-/-}$ mice. These mouse lines allowed to examine individual smooth muscle functions of the cGKI isoforms in adult "healthy" animals. As compared to conventional knockouts, which lack cGKI in all cells, the smooth muscle reconstituted Iα and Iβ mice have a prolonged life expectancy. 8-Br-cGMP treatment reduced norepinephrine- and depolarization-induced Ca^{2+}-increases to wild type levels in aortic SMCs of Iα or Iβ reconstituted mice. Furthermore and in contrast to the results with $cGKI^{-/-}$ aorta and jejunum, 8-Br-cGMP-induced relaxation of hormone contracted smooth muscle was intact in reconstituted animals. Finally, telemetric blood pressure recordings in awake, freely moving animals revealed no differences between reconstituted and control (wild type) mice. Taken together, these results suggest that both isoforms can rescue a major part of the cGMP/cGKI signaling in SMCs. Interestingly, expression of either the Iα or Iβ isoform can compensate the other isoform in the modulation of vascular tone.

[1] Institut für Pharmakologie und Toxikologie, TU München, Germany
[2] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany

166

TWO cGMP ANALOGS THAT ARE COMMONLY USED AS cGKI INHIBITORS SHOW UNEXPECTED EFFECTS

N. Valtcheva[1], P. Weinmeister[2], F. Hofmann[2], S. Feil[1], R. Feil[1]

Signalling pathways mediated by the second messenger cGMP play an important role in many physiological and pathophysiological processes. In contrast to the components upstream of cGMP, relatively little is known about its downstream effectors. An attractive cGMP receptor is the cGMP-dependent protein kinase type I (cGKI). Indeed, this protein kinase has been implicated in many cGMP effects based on the use of pharmacological "cGK inhibitors". However, it is increasingly recognized that many of these inhibitors may be less specific than previously thought or may not at all inhibit cGKI activity in intact cells. This concern was especially propagated for the active site inhibitor KT5823, one of the most frequently used cGK inhibitors. Consequently, more and more studies are being performed with another class of cGK inhibitors, the so-called Rp-cGMP-Isomers,

which are thought to block cGK activation by binding to the regulatory cGMP-binding sites. In this study, we have analysed the effects of two increasingly used Rp-cGMP-Isomers, Rp-8-Br-PET-cGMPS and Rp-8-pCPT-cGMPS, on the growth of wild-type and cGKI-deficient cells as well as on the kinase activity of cellular and purified cGKI. Surprisingly, the results indicate that neither compound efficiently inhibits cGMP-stimulated cGKI activity in intact cells and that, at least in vitro, both drugs are partial agonists for cGKI rather than antagonists. Thus, it appears difficult to interpret data obtained from experiments with Rp-8-Br-PET-cGMPS and Rp-8-pCPT-cGMPS. Both compounds should be used with caution as "cGK inhibitors".

[1] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
[2] Institut für Pharmakologie und Toxikologie, TU München, Germany

167

cGMP-DEPENDENT KINASE I SUPPORTS FORMATION OF ASSOCIATIVE FEAR MEMORY AND LONG-TERM POTENTIATION IN THE LATERAL AMYGDALA

C. Paul[1], P. Weinmeister[1], C. Christel[1], S. Moosmang[1], R. Feil[2], F. Hofmann[1], T. Kleppisch[1]

Nitric oxide (NO) and cGMP have been reported to modulate memory consolidation of Pavlovian fear conditioning and long-term potentiation (LTP) of synaptic transmission in the amygdala. The cGMP-dependent protein kinases, cGKI and cGKII, representing universal effectors in the NO/cGMP signalling cascade are both expressed in the amygdala. We sought to elucidate the function of cGKI for amygdala-dependent fear conditioning and synaptic plasticity in the amygdala. Behavioural analysis in cGKI null mutants ($cGKI^{-/-}$) is obstructed by their premature death resulting from severe smooth muscle dysfunction. cGK-deficient mice live to adulthood when the expression of the cGKI is rescued selectively in smooth muscle cells. We used this mouse model (cGKI rescue) to examine amygdala-dependent cued fear and contextual fear behaviour. Freezing to a tone previously conditioned with a foot shock (cued fear) was significantly reduced in cGKI rescue mice compared with control suggesting an impairment of the tone-shock association. Freezing in the conditioning environment was not different in control and cGKI rescue mice indicating that contextual fear memory was normal. Associative fear learning is thought to rely on synaptic plasticity in various inputs to the lateral amygdala. We examined LTP in the amygdala of wild type and $cGKI^{-/-}$ mice using extracellular recording. Fitting with the behavioural defect observed in cGKI rescue mice, $cGKI^{-/-}$ animals showed reduced LTP in the cortical input to the lateral amygdala. These findings demonstrate a critical role of the cGKI isoform for associative learning likely related to mechanisms supporting memory consolidation. Our findings also show that synaptic plasticity in the amygdala is supported by cGKI. Similar findings in the hippocampus have been linked to cGKI-dependent control of transcription in pyramidal neurons. The role of cGKII remains to be elucidated.

[1] Institut für Pharmakologie und Toxikologie, TU München, München, Germany
[2] Interfakultäres Institut für Biochemie, Eberhard Karls Universität Tübingen, Tübingen, Germany

168

THE MEMBRANE DEFORMING AGENTS TRINITROPHENOL AND CHLORPROMAZINE ARE NOVEL MODULATORS OF TRPA1

K.Hill, M.Schaefer

TRPA1 is a member of the transient receptor potential (TRP) family of ion channels and is expressed in peripheral sensory neurones where it is considered to contribute to a variety of sensory processes such as the detection of painful stimuli. Moreover, TRPA1 can be found in inner hair cells of the ear but its involvement in sensing mechanical forces is still being controversially discussed. Amphiphatic molecules such as trinitrophenol and chlorpromazine have been shown to provide a useful tool to study mechanosensitive ion channels. Depending on their charge, they partition and accumulate in the inner or outer leaflet of the lipid bilayer causing a curvature of the membrane, which has been demonstrated to activate or inhibit mechanosensitive ion channels. We analysed the effect of two of these compounds on gating characteristics of TRPA1 in calcium imaging and electrophysiological recordings. Both molecules modulate TRPA1 gating but do so in different ways: Applicaton of the anionic amphiphatic molecule trinitrophenol at concentrations from 200 – 1000 µM led to a robust and reversible activation of TRPA1 channels both in the whole-cell and cell-attached configuration. The properties of the evoked currents resemble those previously described for TRPA1. In contrast, exposure of allylisothiocyanate-activated TRPA1 channels to 50 µM chlorpromazine, a cationic amphipath, led to a voltage-dependent block at positive potentials but markedly increased the open probability at negative holding potentials. This overall effect on the open probability is caused by a shortened closed time of the single channels at both positive and negative holding potentials together with a reduced open time only at positive holding potentials.
The presented data give a first indication that TRPA1 gating may be sensitive to membrane deformation. Furthermore, the variability in the shape of the I-V curve suggests that native mechanically activated TRPA1 currents must not necessarily exhibit the same biophysical properties as ligand-activated TRPA1 currents.

Institut für Pharmakologie, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Thielallee 67-73, 14195 Berlin, Germany

169

TRPC3 contributes to PLC-mediated Ca^{2+} signalling in somatic endothelial progenitor cells

*M. Poteser, §A. E. Yates, §H. Maechler, *A. Graziani, *M. Krenn, *P. Eder and *K. Groschner

Recent evidence suggests that human adipose tissues host a significant fraction of somatic endothelial progenitor cells (EPCs). In culture, these cells generate colony forming units (CFUs) that respond to vascular endothelial growth factor (VEGF) with further proliferation and are able to organize in tube-like structures. We observed abundant expression of TRPC3 channel protein in freshly isolated EPCs as well as in early EPC culture (24h) by flow-cytometry and immuno-histochemical imaging. The population of TRPC3 expressing cells derived from the fresh adipose tissue preparation overlapped with the fraction expressing the selective stem-cell surface marker CD133. After 24h in culture, $TRPC3^{+}/CD133^{+}$ CFUs were identified, which showed a heterogenous distribution of $TRPC3^{+}$ cells within the cell clusters, with TRPC3 expressing cells preferentially located in the outer rim of the colonies. Culture of CD133-immunoselected cells in VEGF supplemented matrigel and led to the formation of typical tube-like structures within 48 hours.Fura-2 Ca^{2+}-imaging experiments of CD133+ cell colonies revealed that VEGF-induced Ca^{2+} entry was significantly larger in TRPC3 expressing "outer rim" cells as compared to cells in the center of the clusters. Transfection of these cells with a N-terminal fragment of TRPC3 that exerts a dominant negative effect on the channel function reduced the VEGF triggerd Ca^{2+} signals. Thus, TRPC3 is suggested as an element of the VEGF signalling pathway in EPCs, contributing to stem cell proliferation and may be essential for vasculogenesis. TRPC3 may be considered as a possible target that allows for selective interference with vasculogenesis and angiogenesis.

*Karl-Franzens University Graz, Inst. of Pharmacological Sciences, Graz, Austria
§Medical University Graz, Dept. of Cardiac Surgery, Graz, Austria

170

TRPC4 COUPLES MUSCARINIC RECEPTORS TO MEMBRANE DEPOLARIZATION AND SMOOTH MUSCLE CONTRACTION

V.V. Tsvilovskyy, T. Aberle, A.V. Zholos*, M. Freichel & V. Flockerzi

The two full length TRPC4 cDNAs were cloned from isolated ileal smooth muscle cells and the two proteins are readily detectable in cell lysates but are absent in TRPC4-deficient cells. The excitatory effect of acetylcholine in ileal smooth muscle is mediated by muscarinic receptor gated ion channels which underlie a cation current baptized mI_{cat}. This current has been characterized above all in guinea pig smooth muscle cells. Like guinea pig mI_{cat} the mouse mI_{cat} is activated by carbachol or by GTPγS and suppressed after preincubation cells in the presence of pertussis toxin or the PLC-inhibitor U73122. Addition of diC_8-PIP_2 (10 µM) to the pipette solution strongly inhibited mI_{cat} (-2.4 ± 0.4 pA/pF in the presence of diC_8-PIP_2, n = 11, vs -10.0 ± 0.5 pA/pF without diC_8-PIP_2, n = 12, at -50 mV) suggesting that local membrane PIP_2 depletion is required for mI_{cat} activation. In TRPC4-deficient cells ($TRPC4^{-/-}$) mI_{cat} was greatly reduced (WT: -11.8 ± 0.4 pA/pF, n = 53 vs $TRPC4^{-/-}$ -1.9 ± 0.3 pA/pF, n = 18, at -50 mV) which concurs with the assumption that TRPC4 channels do underlie mI_{cat}. Outside-out single channel current measurements revealed that a 55 pS conductance typical for the muscarinic receptor gated ion channels was not observed in $TRPC4^{-/-}$cells. Perforated patch current clamp recordings showed that carbachol induced a depolarization in wild type cells which was significantly attenuated in $TRPC4^{-/-}$cells, suggesting that TRPC4 mediates depolarization which then triggers activation of the high voltage-activated $Ca_V1.2$ channels. Finally, atropine sensitive neurogenic contraction elicited by electrical field stimulation (30 Hz, 1 ms) was greatly reduced in $TRPC4^{-/-}$ ileum smooth muscle strips (WT: 77 ± 4 %, n = 16 vs $TRPC4^{-/-}$ 28 ± 2 %, n = 16) demonstrating the decisive role of TRPC4 in coupling agonist stimulation of G-protein coupled receptors to $Ca_V1.2$ activation and smooth muscle contraction.

Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, 66421 Homburg, Germany and * Physiology, Queens's University Belfast BT9 7BL, UK

171

REGULATION OF CLASSICAL TRANSIENT RECEPTOR POTENTIAL CHANNEL 6 (TRPC6) BY DIACYLGLYCEROL (DAG) KINASES IN PULMONARY SMOOTH MUSCLE CELLS

A. Dietrich[1],H. Kalwa[1], B. Fuchs[2], N. Weissmann[2] and T. Gudermann[1]

We recently demonstrated that the non-selective, DAG-sensitive cation channel TRPC6 is essential for hypoxic pulmonary vasoconstriction (HPV), a regulatory mechanism which adapts the regional blood flow in the lung to the local alveolar ventilation (Weissmann et al. Proc. Natl. Acad. Sci. USA 103: 190093-190098 (2006)). After initial priming with endothelin (4 nM) hypoxia induced DAG-accumulation at the plasma membrane of precapillary pulmonary smooth muscle cells (PASMC). DAG appears to exert its action via TRPC6, as DAG kinase inhibition provoked HPV in WT mice. In TRPC6-deficient mice however, regional hypoventilation resulted in severe arterial hypoxemia. To further elucidate the signal transduction cascade of this vital mechanism, we show that H_2O_2 is able to induce vasoconstriction in WT but not in TRPC6-/- PASMC, pointing to a mechanistically important role of hypoxia induced superoxide anion production in these cells. As DAG is degraded into phosphatidic acid (PA) by DAG kinases, we isolated total RNA from murine PASMC and identified DAG kinase □ and □ as the predominantly expressed DAG kinases in these cells. Most interestingly, the enzymatic activity of both DAG kinase isoforms □,Iand □,□is inhibited by H_2O_2 (100 µM). Furthermore DAG kinase □□ heterologously expressed in HEK 293 cells is translocated to cytoplasmic vesicles upon application of H_2O_2 to these cells. These data favour a model of basal PLC dependent DAG production and hypoxia-induced DAG accumulation by superoxide anion inhibition of DAG kinase activity for the regulation of TRPC6 activity.

[1]Institute for Pharmacology and Toxicology, University of Marburg, Germany; [2]University of Giessen Lung Center (UGLC), Department of Internal Medicine II/V, Germany

172

INHIBITION OF STEROID ACTIVATED CALCIUM ENTRY PATHWAYS IN ENDOCRINE CELLS BY INTRODUCTION OF TRPM1

S. Mannebach, S. Lambert, T.F.J. Wagner, V. Flockerzi, J. Oberwinkler, S.E. Philipp

TRPM3 form ionotropic steroid receptors which are activated by the neuroactive steroid pregnenolone sulphate (Wagner et al. submitted). The TRPM3 gene encodes a number of different splice variants which arise by alternative splicing of their transcripts. They differ by the presence or absence of five short stretches of 10 to 25 amino acid residues encoded by exons 8, 13, 15, 17, 24 and by their aminotermini encoded by exon 1 or exon 2. Coimmunoprecipitation experiments and measurements of the fluorescence resonance energy transfer of different TRPM fusion proteins indicated homomeric interaction of TRPM1 and TRPM3 channel subunits. Furthermore we had strong evidence for heteromeric interaction of TRPM3 proteins with TRPM1. In contrast to TRPM3, HEK-293 cells overexpressing TRPM1 did not show any additional channel activity compared to untransfected control cells. Conceivably heteromeric channels

consisting of TRPM1 and TRPM3α2 should display reduced Ca^{2+} entry compared to homomeric TRPM3α2 complexes. We tested this hypothesis by introducing both channels in HEK-293 cells. We found that the pregnenolone sulphate induced Ca^{2+} entry through TRPM3α2 is significantly reduced in the presence of TRPM1. Similar results were obtained after cotransfection of TRPM3α2 and TRPM3α1, a splice variant that was recently shown to have much reduced divalent cation permeability. Since we found TRPM3 to be expressed in pituitary GH3 cells and pancreatic INS-1 cells we analyzed the consequences of TRPM1 overexpression in these cells. Similar to HEK-293 cells overexpressing TRPM3α2, introduction of TRPM1 exerted strong dominant negative effects on endogenous steroid activated Ca^{2+} entry whereas introduction of TRPM3α2 further increased pregnenolone induced Ca^{2+} entry. Our data strongly suggest that introduced TRPM1 (or TRPM3α1) subunits assemble with endogenous channel proteins, which are very similar to TRPM3α2, thereby severely disturbing steroid activated Ca^{2+} entry. We conclude that TRPM3 channels build pregnenolone sulphate inducible Ca^{2+} entry pathways in pancreatic and pituitary gland cells.

Oberwinkler,J., et al. (2005) Alternative Splicing Switches the Divalent Cation Selectivity of TRPM3 Channels. *J. Biol. Chem.* 280, 22540-22548

Experimentelle Pharmakologie und Toxikologie, Universität des Saarlandes, D- 66421 Homburg

173

TRPM3α2 CHANNELS ARE IONOTROPIC STEROID RECEPTORS IN ENDOCRINE CELLS

T.F.J. Wagner, S. Loch, I. Straub, S. Lambert, A. Lis, S. Mannebach, V. Flockerzi, S.E. Philipp, J. Oberwinkler

TRPM3α2 is a highly Ca^{2+} permeable ion channel when heterologously overexpressed in HEK 293 cells. We employed Fura-2 based Ca^{2+} imaging on a cell line stably overexpressing TRPM3α2 in order to screen for substances that influence TRPM3α2 channel activity. Given the predominant expression of TRPM3 in the central nervous system, we initially focused on substances with known neuroactive properties. We established that TRPM3α2 is strongly activated by extracellular application of pregnenolone sulfate, a neuroactive steroid, but not by most other steroids tested. Only pregnenolone, DHEA and DHEA-S also showed agonistic effects on TRPM3α2. Pregnenolone sulfate, however, was by far the most potent agonist. Pregnenolone sulfate induced currents through recombinant TRPM3α2 channels have distinct biophysical features: The channels are highly permeable to divalent cations, but are inhibited by extracellular monovalent cations, especially Na^{+}. In another series of screening experiments we tried to identify substances that inhibit TRPM3α2 channel activity. Surprisingly, we found that nifedipine, the prototypical dihydropyridine antagonist of L-type Ca^{2+} channels, also activated TRPM3α2. Using pregnenolone sulfate and nifedipine as pharmacological tools, we tested a variety of cell lines for the presence of pregnenolone sulfate induced Ca^{2+} signals. With this approach we identified GH3 and INS1 cells to contain ion channels with pharmacological and biophysical properties very similar to recombinant TRPM3α2 channels. Both, GH3 and INS1 cells were derived from rat endocrine tissues, the anterior pituitary and pancreatic β-cells, respectively. Consequently, we were able to demonstrate pregnenolone sulphate induced Ca2+ signals in mouse pancreatic β-cells and in a subset of mouse pituitary cells in primary culture. Again, electrophysiological analysis revealed that in both systems, the channels underlying the steroid-induced Ca^{2+} signal were very similar to recombinant TRPM3α2 channels. Also, we demonstrated the presence of TRPM3 mRNA in mouse pituitary glands and pancreatic islets. Our data indicate that TRPM3α2 channels form ionotropic steroid receptor in cells from the endocrine system.

Experimentelle und klinische Pharmakologie und Toxikologie, Uniklinikum des Saarlands, Homburg

174

NEW AGONISTS FOR THE COLD RECEPTOR TRPM8

M. Bödding, U. Wissenbach and V. Flockerzi

Proteins of the transient receptor potential (TRP) family are not only involved in a variety of physiological functions but may also participate in the development of diseases such as most common cancers. One member of the melastatin (M) group, namely TRPM8 is up-regulated in prostate cancer and other malignancies. This cation channel is activated by cold and cooling compounds such as menthol and icilin. The effects of the carboxamides WS-12, CPS-113, CPS-369, the carboxylic acid WS-30 and the phosphine oxide WS-148 on TRPM8 expressing HEK-293 cells was tested by Ca^{2+} imaging experiments and whole-cell patch-clamp recordings. All compounds induce a cooling effect and show a dose-dependent and reversible activation of TRPM8 with EC_{50} values in the nM to low μM range. The carboxamide WS-12 is most potent in activating TRPM8. Like menthol it induces a leftward shift of the voltage dependence of TRPM8 activation towards a more physiological voltage range. The cooling compounds described here represent new tools to dissect TRPM8 functions and may serve as chemical leads for the development of additional TRPM8 agonists and novel antagonists. Such compounds may be beneficial for preventing noxious cold perception. They could also be useful in diagnosis and treatment of most common cancers in which the TRPM8 gene is up-regulated in comparison to the corresponding normal tissue.

Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, D-66421 Homburg, Germany

175

SURFACE EXPRESSION AND ASSEMBLY OF THE COLD-SENSITIVE TRPM8 CHANNEL

I Erler, D. M.M. Al-Ansary, U. Wissenbach, V. Flockerzi and B. A. Niemeyer

TRPM channels are distinct from many other members of the TRP family in regard to their overall size (over 1000 amino acids), the lack of N-terminal Ankyrin like repeats and hydrophobicity predicitions that may allow for more than six transmembrane regions. Common to each TRPM member is a prominent C-terminal coiled-coil region. Here we show that TRPM8 channels assemble as multimers using the putative coiled-coil region within the intracellular C-terminus and that this assembly can be disturbed by a single point mutation within the coiled-coil region. This mutant neither gives rise to functional channels nor do its subunits interact or form protein complexes that correspond to a multimer. However, they are still transported to the plasma membrane. Furthermore, wild-type currents can be suppressed by expressing the membrane attached C-terminal region of TRPM8. To separate assembly from trafficking we investigated maturation of TRPM8 protein by identifying and mutating the relevant N-linked glycosylation site and show that glycosylation is neither essential for multimerization nor for transport to the plasma membrane per se, but appears to fascilitate efficient multimerization and transport. We are currently investigating dynamic mechanisms affecting ion channel transport of TRPM8.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, Geb. 46, 66421 Homburg, Germany

176

CALMODULIN INTERACTS WITH TRPV4 BY ITS C-TERMINAL LOBE

R. Strotmann, T. Schöneberg

We have recently shown that the activity of the osmo- and 4α-phorbol ester sensitive cation channel TRPV4 is modulated by the intracellular Ca^{2+} concentration in that elevated Ca^{2+} concentrations potentiate channel activation. The potentiation mechanism is still not resolved, but Ca^{2+}-dependent CaM binding to a carboxy-terminal domain has been shown to be a crucial prerequisite. The present study aimed to elucidate the molecular mode of CaM binding, as a first step towards the understanding of the potentiation mechanism. CaM is involved in many intracellular regulatory processes in Ca^{2+}-dependent and independent ways. In most cases, the highly symmetrical CaM molecule wraps around the helical target structure, thus forming a complex that involves both functional lobes of the CaM molecule. The CaM binding domains of the target often share a common structure with hydrophobic amino acid residues at key positions. The C-terminal CaM interaction domain in TRPV4 resembles classical CaM binding motifs, but lacks aromatic side chains that are known to interact with the CaM N-lobe. A predominant role of the CaM C-lobe in the TRPV4 interaction was thus expected. Protein interaction experiments with CaM fragments, Ca^{2+}-binding deficient CaM mutants and a C-terminal fragment of TRPV4 confirmed this. Purified proteins were subjected to an AlphaScreen-based binding assay or fluorescence-anisotropy measurements. At 100 μM Ca^{2+}, the affinity of the N-lobe for TRPV4 was more than 50fold lower than that of the C-lobe. Using intramolecular helix chimaeras and point mutants, the structural features of the CaM molecule that contribute to V4 interaction were further characterized. Binding experiments revealed that helices E and H of the CaM molecule are crucial for the interaction and define the specificity of the CaM C-lobe over the N-lobe. Under physiological conditions, CaM can thus be expected to bind to the CaM binding site in TRPV4 with its C-lobe while the N-lobe is available for other interactions. CaM may serve as a scaffolding structure that brings other signaling components into contact with the C terminus of TRPV4.

Institute of Biochemistry, Dpt. of Molecular Biochemistry, Medical Faculty, Universität Leipzig

177

CONDITIONAL INACTIVATION OF THE TRPV6 CHANNEL IN MICE

P. Weißgerber*, J. Olausson*, U. Kriebs, V. Flockerzi and M. Freichel

TRPV6 is a member of the superfamily of transient receptor potential (TRP) channels which are involved in a variety of important physiological functions ranging from phototransduction, olfaction, nociception, and heat and cold sensation to epithelial calcium transport. The mouse TRPV6 gene is localized on chromosome 6 flanked by EPH-6 gene and the TRPV5 gene and extends over 15.66 kb. The encoded TRPV6 protein comprises 727 amino acid residues with a calculated relative molecular mass of 83,210 Da. Mouse TRPV6 transcripts are found in pancreas, kidney and placenta. Recently, it could be shown that overexpression of TRPV6 cDNAs in eukaryotic cell lines induces formation of constitutively active channels highly selective for Ca^{2+}. However, such currents have not been detected in primary cell types so far. To investigate the special role of the TRPV6 gene in its native environment, we used the Cre/loxP recombination system to inactivate the mouse TRPV6 gene both ubiquitously and in a time dependent or tissue-specific manner. Accordingly, we used homologous recombination in embryonic stem cells (R1) to generate mice carrying an allele in which exon 13 to 15 were flanked by loxP sequences (L3F2). These exons contain the sequence contributing to the selectivity filter of the channel and upon removal of these exons the ion conducting pore of the TRPV6 channel will be deleted. Chimeric males were obtained that transmitted the mutant allele to their progeny. These heterozygous F1 offspring ($TRPV6^{+/L3F2}$) were crossed to Cre deleter mice (CMV-Cre) to generate $TRPV6^{+/-}$ mice. These $TRPV6^{+/-}$ mice were intercrossed to produce F2 homozygous mice, as confirmed by Southern blot analysis. The mutation was transmitted at Mendelian ratio, suggesting normal fetal and embryonic development of homozygous mutant mice. The inactivation of the TRPV6 gene was confirmed by Northern blot analysis. No transcripts could be detected in poly(A)$^{+}$ RNA prepared from $TRPV6^{-/-}$ placenta (embryonic day 15.5 and 16.5) using a probe covering the targeted exons 13 to 15. Intercrosses of $TRPV6^{-/-}$ animals exhibit reduced fertility which might be due to impaired transplacental Ca^{2+} transport.

* contributed equally

Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, D-66421 Homburg.

178

MURINE ORAI2 SPLICE VARIANTS FORM FUNCTIONAL CRAC CHANNELS

SA Gross, U Wissenbach, V Flockerzi, A Cavalié

The stimulation of membrane receptors coupled to the phopholipase C pathway leads to release of Ca^{2+} from intracellular stores and the resulting depletion of Ca^{2+} stores triggers in turn the activation of the Ca^{2+} release activated Ca^{2+} (CRAC) channels. Recent evidence indicates that ORAI1 is an essential pore subunit of CRAC channels activated by STIM1. Here, we show the genomic organization, tissue expression pattern and functional properties of murine ORAI2 variants in comparison to ORA1I and ORAI3. The ORAI2 loci were localized in the mice chromosomes 5 and 16 and a long and a short splice variant of ORAI2 (respectively ORAI2L and ORAI2S) was identified and cloned from mice brain. Northern blots revealed a prominent expression of the ORAI2 variants in the brain, lung, thymus and intestine, whilst ORAI1 and ORAI3 appear to be near ubiquitously expressed in mice tissues. The functional properties of ORAI2L and ORAI2S were described and compared to those of ORAI1 in HEK 293 and RBL 2H3 cells that were co-transfected with identical amounts of STIM1 and either cDNA. Our data indicates that ORAI2L and ORAI2S are capable of forming functional CRAC channels in combination with the ubiquitously expressed STIM1.

Uniklinikum Homburg, Pharmakologie und Toxikologie, Gebäude 46, 66421 Homburg, Germany

179

EXPRESSION OF BETA-SUBUNITS EXPLAINS HEART-FAILURE PHENOTYPE OF HUMAN AND MURINE CARDIAC L-TYPE CALCIUM-CHANNELS

J. Matthes[1], S. von Vietinghoff[2], I.F.Y. Khan[1], I. Bodie[3], R. Gilsbach[4], E. Schmitteckert[5], M. Bünemann[5], A. Schwartz[3], R. Hullin[6], L. Hein[4], S. Herzig[1]

L-type calcium-channel (LTCC) activity is differentially modulated by auxiliary β-subunit isoforms, with β_2-subunits being strong stimulators (Hullin et al., JBC 2003;278:21623-30). In human failing ventricles we find enhanced β_2-subunit expression (mRNA and protein). Hence, increased LTCC activity previously observed in human heart-failure might be explained by expression-pattern and composition of the channel complex. In young (4 months) mice with cardiac-specific overexpression of the human pore-forming subunit $Ca_v1.2$ (*tg* $Ca_v1.2$) single-channel activity of ventricular LTCC was significantly decreased, in line with reduced β_2-subunit expression and increased expression of "weakly stimulating" β_1-subunits as detected in Western-blots (Groener et al., BBRC 2004;314;878-84). We now examine older *tg* $Ca_v1.2$ when developing severe heart-failure (>9 months). Here, single LTCC activity is significantly enhanced (I_{peak}: *old tg* $Ca_v1.2$ -56±14fA, n=11; age-matched *wildtype* -40±9 fA, n=11; *young tg* $Ca_v1.2$ -23±7fA, n=13), accompanied by increased β_2-subunit protein expression. Interestingly, while single LTCC from *young tg* $Ca_v1.2$ are not activated by 8Br-cAMP and okadaic acid, stimulation is restored in *old tg* $Ca_v1.2$ (+47±5%, n=3; $p<0.05$). To test for a causal role of β-subunit up-regulation on LTCC activity in heart we crossbred *tg* $Ca_v1.2$ mice and a novel mouse with inducible cardiac β_2-subunit overexpression. Transgene induction by tebufenocide significantly enhanced ventricular single-channel activity in young double-transgenics (I_{peak}: *tg* $Ca_v1.2/tg_{ind}$ β_2 -72±10fA, n=10), exceeding the level observed in either age-matched mock-induced *wildtype* (-44±11fA, n=6) or *old tg* $Ca_v1.2$ *(see above)*. Our study provides direct evidence for a cause-effect relationship between β-subunit expression and single LTCC activity. This may explain the phenotype of ventricular LTCC in human heart-failure.

[1]Department of Pharmacology, University of Cologne, Germany; [2]Franz Volhard Clinic, Medical Faculty, Charité, Berlin, Germany; [3]Institute of Molecular Pharmacology and Biophysics, College of Medicine, University of Cincinnati, OH, USA; [4]Department of Exp. and Clin. Pharmacology and Toxicology, University of Freiburg, Germany; [5]Department of Pharmacology, University of Wuerzburg, Germany;[6]Department of Cardiology, Swiss Heart Center Bern, University Hospital, Switzerland.

180

REDUCED NEURONAL EXCITOTOXICITY AND INCREASED HIPPOCAMPAL SEIZURE RESISTANCE IN $CA_V2.3$ DEFICIENT MICE

M Weiergräber *, N Matar, M Henry, T Schneider*

Voltage-gated calcium channels are key components in the etiology and pathogenesis of epilepsies, and recent findings point to an important role of the $Ca_v2.3$ E/R-type voltage-gated Ca^{2+} channels in ictogenesis and seizure propagation. Based on the observation that $Ca_v2.3$ is thought to induce plateau potentials in CA1 pyramidal cells, which can trigger epileptiform activity, our recent investigation revealed reduced PTZ-seizure susceptibility and altered seizure architecture in $Ca_v2.3^{-/-}$ mice compared to controls (Weiergräber et al., 2006). In the present study we tested hippocampal seizure susceptibility in $Ca_v2.3$ deficient mice using surface and deep intrahippocampal telemetric EEG recordings as well as phenotypic seizure video analysis. Administration of kainic acid (30 mg/kg i.p.) revealed clear alteration in behavioral seizure architecture and dramatic resistance to limbic seizures in $Ca_v2.3^{-/-}$ mice compared to controls. The same tendency was observed for NMDA seizure susceptibility (150 mg/kg i.p.). In addition, histochemical analysis within the hippocampus revealed that excitotoxic effects following kainic acid administration are absent in $Ca_v2.3^{-/-}$ mice, whereas $Ca_v2.3^{+/+}$ animals exhibited clear and typical signs of excitotoxic cell death. These findings clearly indicate that the $Ca_v2.3$ voltage-gated calcium channel plays a crucial role in both hippocampal ictogenesis and seizure generalization and is of central importance in neuronal degeneration following excitotoxic events.

Weiergräber M, Henry M, Krieger A, Kamp MA, Radhakrishnan K, Hescheler J, Schneider T (2006) Altered seizure susceptibility in mice lacking the Cav2.3 E-type Ca^{2+} channel. Epilepsia 47: 839-850.

Neurophysiology & *Center for Molecular Medicine Cologne (CMMC), Universität zu Köln, Robert-Koch-Str. 39, 50931 Köln.

181

THE L-TYP CALCIUM CHANNEL $Ca_V1.2$ MODULATES *MPER1* GENE INDUCTION AND INFLUENCES PHASE SHIFTS OF THE CIRCADIAN CLOCK

N. Langwieser[1], I. Schmutz[2], U. Albrecht[2], F. Hofmann[1] and S. Moosmang[1]

Biological rhythms are driven in mammals by a central circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Generation and maintenance of circadian rhythmicity rely on complex transcriptional/translational feedback loops involving a set of clock genes. Among the molecular components responsible for the mammalian clock are the period genes *mPer1* and *mPer2*. Because the periodicity of the clock is not exactly 24 hr, it has to be adjusted frequently. The major stimulus for adjustment (resetting) of the clock is nocturnal light. It evokes activation of signaling pathways in the SCN that lead to expression of *mPer1* and *mPer2* genes conveying adjustment of the clock. Here, we show that brain-specific $Ca_v1.2$ knockout mice are defective in resetting the circadian clock, as assessed by changes in the onset of wheel running activity after a light pulse at circadian time CT22. At the molecular level, light induction of *mPer1* is strongly reduced at CT22, whereas mPer2 induction is unchanged in the knockout mice. Additionally, we show that light induction of *cfos* at several time points is not affected in these animals. The L-type calcium channel $Ca_v1.2$ plays a role in the clock-resetting mechanism. In particular, the ability to advance the clock phase is affected in brain-specific $Ca_v1.2$ knockout mice.

[1]Institut für Pharmakologie und Toxikologie der Technischen Universität München, 80802 München
[2]Department der Medizin, Abteilung Biochemie, Universität Fribourg, 1700 Fribourg, Schweiz

182

TRPC4 EXPRESSION DETERMINES SENSITIVITY OF THE PLATELET-TYPE CAPACITATIVE CA^{2+} ENTRY CHANNEL TO INTRACELLULAR ALKALOSIS

I. Wakabayashi*, M. Marumo*, A. Graziani†, M. Poteser† and K. Groschner†

[Background] The present study was designed to analyze the molecular basis of the intracellular pH-dependent capacitative Ca^{2+} entry (CCE) of human platelets and megakaryocytic cells, specifically to test the hypothesis that members of the classical transient receptor potential (TRPC) protein family are involved in the CCE pathway that is promoted by intracellular alkalosis.

[Methods] Intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular pH (pHi) of washed human platelets and cultured CMK and MEG-01 cell lines were measured using a fluorescent Ca^{2+} indicator, fura-2 and a fluorescent pH indicator, 2'7'-bis-(2-carboxyethyl)-5(6)-carboxy-fluorescein (BCECF), respectively. CCE was induced by an extracellular addition of Ca^{2+} to platelets in which Ca^{2+} stores had been depleted by stimulation with thapsigargin in nominally Ca^{2+}-free medium. Monensin was used to induce intracellular alkalosis. Expression of TRP (transient receptor potential) channels was detected by Western blotting. HEK293 cells transiently transfected with TRPC4α were used to test involvement of TRPC4α in pH sensitivity of CCE.

[Results] Human platelets as well as the tested megakaryocytic cell lines (CMK cells, MEG-01 cells) and the fibroblast-like HEK293 cells displayed thapsigargin-induced CCE and responded to monensin with comparable elevation in intracellular pH. Promotion of CCE by monensin-induced intracellular alkalosis, however, was profound in mature platelets, moderate in CMK cells and lacking in MEG-01 cells as well as in HEK293 cells. Analysis of the TRPC expression pattern by immunoblotting revealed that mature platelets and CMK cell express TRPC4 along with TRPC1 and TRPC3, while TRPC4 is lacking in MEG-01 cells. MEG-01 cells displayed CCE characteristics as well as lack of TRPC4 expression similar to HEK293 cells. Overexpression of TRPC4 in HEK293 cells was found to result in a gain of pH-sensitivity of CCE with clearly detectable promotion of CCE in response to monensin.

[Conclusion] Platelet CCE channel complexes is suggested to contain TRPC4 as a molecular component that determines sensitivity of CCE to intracellular alkalosis.

*Department of Environmental and Preventive Medicine, Hyogo College of Medicine, Hyogo 663-8501, Japan
†Department of Pharmaceutical Sciences, Pharmacology & Toxicology, University of Graz, Graz 8010, Austria

183

PROTONS ACTIVATE AND POTENTIATE CURRENTS THROUGH TRPC5

M. Semtner, O. Pinkenburg, M. Schaefer*, T.D. Plant

TRPC5 is a Ca^{2+} permeable nonselective cation channel activated in a PLC-dependent manner by an as yet unidentified messenger. It has previously been shown that, unlike most other Ca^{2+} permeable channels, TRPC5 can be potentiated by micromolar concentrations of La^{3+} and Gd^{3+}. This effect results from an action of the cations at two extracellular glutamate residues flanking the putative pore loop. Analogous residues in TRPV1 are involved in activation and potentiation of this channel by protons. Since pH could also be an important modulator of TRPC5 channel activity, we studied the effect of pH changes on TRPC5. Currents were recorded from HEK293 cells transiently transfected with TRPC5 using the whole cell configuration of the patch clamp technique. Decreasing the pH led to increases in both spontaneous and GTP-γ-S-activated currents. Potentiation was already observed with small reductions in pH (from 7.4 to 7.0) and increased up to pH 6.5 ($pEC_{50} \approx 6.8$). Below this pH value, potentiation was weaker or currents inhibited. In contrast to TRPC5, TRPC6 showed no potentiation, only inhibition with an pIC_{50} of 5.7. Mutation of each of the key glutamate residues in TRPC5 to a neutral glutamine led to a loss of proton potentiation in E543Q, and a decreased activation by protons in E595Q. In the outside-out configuration, reducing the pH to 6.5 led to TRPC5 single channel currents with longer open times and smaller amplitudes; an effect very similar to that of micromolar La^{3+} or Gd^{3+}. In conclusion, our data show that, like TRPV1, TRPC5 can be activated and potentiated by protons acting at glutamate residues close to the channel pore. Thus, TRPC5 may act as a low threshold pH sensor linking changes in extracellular pH to cellular signalling.

Institut für Pharmakologie und Toxikologie, Fachbereich Medizin, Philipps-Universität Marburg
*Institut für Pharmakologie, Charíte-Universitätsmedizin Berlin, CBF

184

A ROLE FOR PLCε IN THE REGULATION OF TRPC6 MEDIATED CATION INFLUX IN GLOMERULAR PODOCYTES?

H. Kalwa[1], U. Storch[1], M. Schmidt[2], F. Hildebrandt[3], T. Gudermann[1] and A. Dietrich[1]

The classical transient receptor potential channel 6 (TRPC6) is activated by diacylglycerol (DAG) produced by isoforms of the phospholipase (PLC) family. Mutations in TRPC6 identified in patients with hereditary kidney disorders link this channel to structural components of the glomerular slit diaphragm in podocytes. Recent work (Hinkes et al., Nat. Genet. 38: 1397-1405 (2006)) identified mutations in PLCε in patients with similar kidney disorders. Therefore, we investigated possible interactions between these two important mediators of cell signalling cascades in podocytes. Transient overexpression of PLCε in a HEK293 cell line stably expressing TRPC6 resulted in a significantly increased Mn^{2+} influx after activation of the endogenous M3 muscarinic acetylcholine receptor. Along these lines, siRNA-mediated downregulation of endogenous PLCε in HEK 293 cells decreasd receptor-mediated cation entry and reduced currents of PLCε-mediated TRPC6 conductance. The pathophysiological relevance of this mechanism was further emphasized by the fact that PLCε mutations identified in patients showed similar biophysical characteristics. The activation of TRPC6 by PLCε was independent of ras, because deletion of one or both ras binding domains of PLCε did not influence TRPC6-mediated cation influx. These data suggests an important role of PLCε in the regulation of TRPC6 activity in glomerular podocytes.

[1]Institute for Pharmacology and Toxicology, University of Marburg, Marburg, Germany; [2]Department of Molecular Pharmacology, University of Groningen, The Netherlands; [3]Department of Pediatrics, University of Michigan, Ann Arbor, USA.

185

A NEGATIVELY CHARGED AMINO ACID RESIDUE IN THE PORE REGION OF TRPM7 IS CRITICALLY INVOLVED IN THE PERMEATION OF DIVALENT CATIONS

J. Wäring, M. Mederos y Schnitzler, V. Chubanov and T. Gudermann

TRPM7, a melastatin-related, *transient receptor potential* kinase-linked cation channel has been found to be a housekeeping protein essential for Mg^{2+} homeostasis. It is tightly regulated by intracellular Mg^{2+} as well as Mg^{2+}–ATP and is highly permeable to a broad range of divalent cations. In divalent free bath solutions, TRPM7 is able to conduct monovalent cations, thus exhibiting anomalous mole fraction behaviour. To gain insight into the molecular permeation mechanisms of TRPM7, we performed systematic site-directed mutagenesis of the amino acid sequence of the putative pore-forming region and examined the biophysical consequences using conventional whole-cell patch-clamp measurements. We found that the substitution of a single glutamate residue by glutamine specifically affected the permeation of divalent cations through TRPM7. Unlike wild type TRPM7, this mutant showed large inward currents carried by monovalent cations in a physiological bath solution. By analyzing the influence of different extracellular divalent solutions on reversal potentials, we found that, in comparison to wild type TRPM7, the mutant displayed strong shifts towards negative potentials, indicating the loss of divalent cation permeation. Furthermore, the extracellular divalent block for monovalent outward currents intrinsic to wild type TRPM7 was almost completely abolished in the mutated channel. In contrast, exchanging the glutamate for an aspartate resulted in the restitution of this extracellular block. Interestingly, this glutamate is not involved in regulation by $[Mg^{2+}]_i$. Therefore, it is likely that another mechanism is responsible for the regulation by $[Mg^{2+}]_i$. Our observations indicate that the negatively charged residue of the glutamate may directly interact with extracellular Mg^{2+} or Ca^{2+}, and that this interaction might be essential for the conductance of divalent cations through TRPM7. In conclusion, we defined an amino acid residue involved in the formation of the TRPM7 pore which is critically important for the divalent permeation characteristics of the channel.

Institut für Pharmakologie und Toxikologie, Philipps-Universität Marburg, 35043 Marburg, Deutschland

186

FUNCTIONAL ANALYSIS OF THE VOLTAGE SENSOR OF EXPRESSED $CA_V3.1$ T-TYPE CALCIUM CHANNEL

M. Eschbach[1], M. Kurejova[2], L. Lacinova[2] and N. Klugbauer[1]

Voltage-gated calcium channels play an important role in mediating calcium ion influx in electrically excitable cells. They comprise voltage-sensing domains that undergo conformational changes in response to alterations of the membrane potential leading to pore opening. Crucial to a detailed understanding of this voltage dependent gating is the analysis of the location and movement of the S4 transmembrane helix as an important part of the voltage sensor. In our studies we focus on the four domain containing T-type calcium channel $Ca_V3.1$ since this model enables us to establish a structure-function analysis across domain-spanning regions. First we investigated the effects of redox agents on the $Ca_V3.1$ calcium channel stably expressed in HEK293 cells using the whole-cell configuration of the patch-clamp technique. The oxidizing agent dithio-bis-(2-nitrobenzoic acid), DTNB, decreased T-type current amplitude in a concentration-dependent manner. This effect of DTNB was accompanied by deceleration of channel activation and acceleration of channel inactivation. In contrast, low concentrations of the reducing agent dithiothreitol (DTT) did not significantly alter the peak current of $Ca_V3.1$ channel. High concentrations of DTT (> 10 mM) decreased T-type current amplitude. After establishing this basic characterization of $Ca_V3.1$ by redox agents we were able to use this tool to manipulate the voltage sensing parts of the channel. We addressed the question of the protein environment of all four S4 segments by replacement of positively charged arginines by neutral cysteins. We employed a disulfide formation approach to determine the location and movement of the S4 segment. In some of the double mutants disulfide bond formation, mediated by DTNB, between S4 and the pore domain immobilized the S4 segment indicating a close proximity of the corresponding amino acids.

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg; [2]Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovak Republic

187

DIFFERENCES IN SINGLE-CHANNEL ACTIVTY OF T-TYPE CALCIUM CHANNEL SUBTYPES ARE DETERMINED BY THE C-TERMINAL DOMAINS

P. Bartels[1], K. Behnke[1], J.-H. Lee[2], P. Barrett[3], E. Perez-Reyes[3], J. Matthes[1], S. Herzig[1]

The family of low-voltage activated Ca^{2+}-channels consists of three T-type Ca^{2+}-channel isoforms, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$, respectively. At the whole-cell level kinetic (Klöckner et al., Eur J Neurosci 1999;11:4171-8) and pharmacological (Todorovic et al., Mol Pharmacol 2001;60:603-10) differences have been described for $Ca_v3.1$ and $Ca_v3.2$, suggesting individual biophysical fingerprints. To examine the underlying structure-function relationship in more detail, single-channel measurements (charge carrier: 110 mM Ba^{2+}) were performed using HEK293-cells transiently transfected with the pore-forming subunits $Ca_v3.1$ and $Ca_v3.2$, respectively. Single-channel activity of $Ca_v3.1$ was considerably lower compared to single $Ca_v3.2$ (e.g. at a test potential of -20 mV: peak ensemble average current I_{peak}: -8.2±2.1 fA vs. -19.8±3.6 fA, $p < 0.05$; open probability in active sweeps P_{open}: 2.2±0.2% vs. 4.5±0.5%, $p < 0.05$). To localize the structural parts of the channel molecule mediating this difference, two chimeric constructs were examined. Construct I, composed of the N-terminal domains D1 and D2 of $Ca_v3.1$ and the C-terminal domains D3 and D4 of $Ca_v3.2$ was significantly more active than construct II consisting of D1 and D2 from $Ca_v3.2$ and D3 and D4 from $Ca_v3.1$, respectively (e.g. I_{peak}: -15.2±2.5 fA vs. -6.8±1.5 fA, $p < 0.05$; P_{open}: 4.3±0.3% vs. 2.3±0.2%, $p < 0.05$). An ANOVA of the data obtained with $Ca_v3.1$, $Ca_v3.2$, construct I and construct II confirmed these significant differences. However, construct II was biophysically similar to $Ca_v3.1$, and construct I could not be distinguished from $Ca_v3.2$. Thus our data suggest that the C-terminal domains D3 and D4 determine the differences in single-channel activity between $Ca_v3.1$ and $Ca_v3.2$. Future cloning studies will enable us to further discriminate whether D3 or D4 or both are necessary for this difference.

[1]Department of Pharmacology, University of Cologne, Cologne, Germany; [2]Department of Life Science, Sogang University, Seoul, Korea; [3]Department of Pharmacology, University of Virginia, VA, USA.

188

MUTATION OF A HUMAN CARDIAC L-TYPE Ca^{2+} CHANNEL β_1- SUBUNIT BY TRUNCATING THE N-TERMINUS TO UNDERSTAND ITS FUNCTIONAL PROPERTIES

W. Jangsangthong[1], R. Akhavan-Malyer[1], R. Hullin[2], I.F.Y. Khan[1], J. Matthes[1], S. Herzig[1]

L-type Ca^{2+}-channels are composed of a pore α_1 and auxiliary β- and $\alpha_2\delta$-subunits. Coexpression of several β-subunit isoforms with α_1-subunits in HEK293 cells differentially modulates the channel activity (Hullin et al., JBC 2003;278:21623-30). Natural alternative splicing of β_2-subunits yields proteins differing only at their N-termini. Interestingly, the extent of channel activity is inversely correlated with the length of N-terminal domain, but not with any particular sequence therein (Khan et al., Biophys J 2004;86:273a). To test whether this is a common phenomenon among β-subunit genes, we next examined the β_1-subunit that contains a long (57 aa) N-terminal domain D1 that is not subject to natural splicing. β_{1a} was subcloned in pIRES2-EGFP. Thus, three mutants with shortened N-terminal domain D1 of 18, 27 and 48 aa length, respectively, were created by means of restriction and ligation methods. Then, the full-length β_{1a}-subunit and the three mutants were transiently coexpressed with a $\alpha_2\delta$-2 subunit in HEK293 cells that stably express a cardiac α_1 ($Ca_v1.2$) subunit of the L-type calcium channel. Single-channel activity was measured as described (Hullin et al., JBC 2003;278:21623-30) and analysed by Markov modelling. All β_{1a} constructs fulfil the expectation with respect to the length-dependent modulation. Peak ensemble current, open probability, latency to first opening, and time-dependent inactivation follow the same rank order of activity: $\beta_{1aN57} < \beta_{1aN48} < \beta_{1aN27} < \beta_{1aN18}$. Open times and closed time distributions were not obviously influenced by the structure of the N-terminus. We conclude that a mass effect of the N-terminus of a β_{1a}-subunit controls channel function, confirming our findings obtained with β_2-subunits in a previous work.

[1]Department of Pharmacology, University of Cologne, Germany; [2]Department of Cardiology, Swiss Heart Centre Bern, University Hospital, Switzerland.

189

INVOLVEMENT OF $Ca_V1.2$ IN THE PROCESS OF FEAR MEMORY FORMATION IN THE MOUSE

C. Christel, N. Langwieser, C. Wotjak, T. Kleppisch, F. Hofmann and S. Moosmang

Calcium signaling is an important prerequisite for memory formation in fear dependent learning tasks. This finding is paralleled by a calcium dependence of long-term potentiation (LTP) in the amygdala. Calcium channels potentially involved in fear memory formation are NMDA-receptors e.g. NR2B as well as voltage-gated L-type Ca^{2+}-channels. It is heavily discussed to what degree these ion channels contribute to different aspects of learning such as memory consolidation, extinction or reconsolidation. Here, we show that brain-specific $Ca_V1.2$-Knockout mice exhibit a reduction of fear dependent learning performance compared to the control groups after 1 day of conditioning. Accordingly, intracerebroventricular injections of 100µM of the $Ca_V1.2$ antagonist isradipine in wild-type mice provoked similar results. The differences in freezing levels achieved 1 day after conditioning were lost after re-exposure to the conditioned stimulus at day 7. The same procedures with selective NR2B antagonist ifenprodil produced similar results on day 1 but retained significant differences in freezing levels on day 7. These findings suggests that NR2B and $Ca_V1.2$ are both involved in fear memory development and indicate that only $Ca_V1.2$-dependent components of this process are suggestible to extinction. We also performed electrophysiological experiments paralleling our behavioural studies. Presynaptic stimulation via the thalamic input into the lateral amygdala (LA) and paired postsynaptic evocation of action potentials in single cells of the LA produced LTP in these cells which was similarly reduced by either administration of isradipine or ifenprodil. Combination of both drugs resulted in a complete loss of potentiation and produced effects similar to long-term depression.

Institut für Pharmakologie und Toxikologie der Technischen Universität München, 80802 München

190

THE PLATIN COMPOUNDS CARBO-, CIS- AND OXALI-PLATIN CAUSE DIFFERENT EFFECTS ON $[Ca^{2+}]_i$ IN MALIGNANT AND NON-MALIGNANT CELLS

I. Kunz and D. Büsselberg

Introduction: $[Ca^{2+}]_i$ is an intracellular messenger which is very sensitive for the effects of chemotherapeutics on cells. In a normal cell cycle $[Ca^{2+}]_i$ is regulated in many different ways to keep the cell functional ($[Ca^{2+}]_i$ is involved in cell-cell-communication, reproduction and apoptosis). Platin compounds, which are widely used as chemotherapeutics in modern anti-cancer-therapy, affect some of these mechanisms in certain ways. Therefore we use the changes of $[Ca^{2+}]_i$ to explore whether these substances influence cellular pathways or not. We tested the chemotherapeutics carbo-platin (cbPt), cis-platin (CDDP) and oxali-platin (oPt) on malignant neuronal cells (human neuroblastoma cells = SY-5Y) and on non-malignant neuronal cells (dorsal root ganglion cells of the rat = DRG).

Methods: The cells were loaded with calcium sensitive dye Fluo-4 which fluorescence is an indicator for $[Ca^{2+}]_i$. All substances (10µM) were applied by flow-system for at least 60min. $[Ca^{2+}]_i$ was measured by a confocal laser-scanning-microscope (Zeiss 510) with a 40x water immersion lens at room-temperature.

Results: The increase of $[Ca^{2+}]_i$ in malignant cells is higher than in non-malignant cells (cbPt: 76.37 $\pm$ 16.27% vs. –30.79 $\pm$ 11.87%; CDDP: 98.8 $\pm$ 54.38% vs. –3.49 $\pm$ 23.77%) only oPt causes a reverse reaction (oPt: 16.06 $\pm$ 25.72% vs. 29.56 $\pm$ 34.26%). The effects triggered by cbPt and CDDP are higher than the effects caused by oPt and the effects occur to be independent of each other.

Conclusion: The platin compounds seem to be differentially efficient in the modulation of $[Ca^{2+}]_i$ in malignant as well as in non-malignant cells.

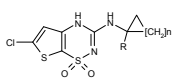
Open Questions: Is oPt able to elevate $[Ca^{2+}]_i$ under different circumstances (other cell line, concentration…) or are the effects caused by different mechanisms? Is the $[Ca^{2+}]_i$ increase sufficient to trigger apoptosis?

Institute of Physiology, University Hospital Essen, Hufelandstr.55, 45122 Essen

191

DEMETHYLATION OF 6-CHLORO-3-ALKYLAMINO-4H-THIENO[3,2-e]-1,2,4-THIADIAZINE 1,1-DIOXIDE DERIVATIVES RESULTS IN THE TRANSITION OF AN AGONISTIC TO AN ANTAGONISTIC BEHAVIOUR OF COMPOUNDS IN SUR2B- BUT NOT IN SUR1-TYPE K_{ATP} CHANNELS

H. Lemoine, C. Teschner, L. Kästner, J. Küper, D. Grittner, A. Rood



Nielsen et al. (J Med Chem 45: 4171, 2002) reported on new K_{ATP}-channel openers (KCO) derived from diazoxide characterized by nanomolar affinities for SUR1-type K_{ATP} channels. To elucidate the selectivity of compounds for SUR1- and SUR2B-type K_{ATP} channels we chose CHO cells stably transfected with SUR1/ Kir6.2 and smooth muscle cells derived from rat aorta (RASMC), respectively. As test compounds we chose the cyclobutyl- (n=2), cyclopentyl- (n=3) and cycloheptyl- (n=4) derivative termed C54, C55 and C56 with R=CH_3 and termed C50, C51 and C52 in their demethylated (R=H) form, respectively. KCO-effects were tested in measuring membrane potentials using $DiBAC_4(3)$ (1.5 µM; excitation 588 nm, emission > 515 nm) as a fluorescent dye. Cells were cultivated in 12-well strips (96-well format) up to confluency; membrane potentials were assayed in a HEPES (20 mM, pH 7.4) buffered salt solution composed of 120 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 5 mM glucose. Concentration-effect curves were performed resulting in a significant hyperpolarisation in SUR1/Kir6.2-transfected cells. In RASMC, only C54, C55 and C56 caused hyperpolarizing effects with different intrinsic activities (ISA), whereas the demethylated compounds C50, C51 and C52 were inactive. Unexpectedly, however, after prestimulation with 0.2 mM diazoxide C50, C51 and C52 caused a nearly complete repolarization, thereby characterized as antagonists at SUR2B-type K_{ATP} channels.

			SUR1			SUR2B		
	R	*n*	*pEC₅₀±ASD*	ISA	*(n)*	*pEC₅₀±ASD*	ISA	*(n)*
C54	CH_3	2	8.09±0.02	95±2	(27)	6.95±0.04	65±2	(31)
C55	CH_3	3	6.75±0.04	97±3	(17)	7.15±0.02	80±2	(14)
C56	CH_3	4	6.35±0.03	89±3	(18)	6.78±0.02	87±1	(17)
						antagonistic activity		
C50	H	2	6.34±0.04	94±3	(10)	7.06±0.04	86±3	(12)
C51	H	3	6.63±0.03	87±2	(11)	7.00±0.02	98±2	(15)
C52	H	4	5.31±0.03	67±3	(8)	6.27±0.03	79±2	(13)

Molekulare Wirkstoff-Forschung und Institut für Lasermedizin, Heinrich-Heine-Universität, D-40225 Düsseldorf

192

ROLE OF THE NBFs OF SUR2B FOR THE OPENER-INDUCED ACTIVATION OF THE VASCULAR K_{ATP}-CHANNEL, $K_{ir}6.1$ / SUR2B

U. Russ[1], T. Amann[1], M. Schwanstecher[2] and U. Quast[1]

K_{ATP} channels are complexes of pore-forming and regulatory subunits, $K_{ir}6.x$ and sulphonylurea receptors, SURs. The vascular subtype of K_{ATP}-channels is composed of $K_{ir}6.1$ and SUR2B, and pharmacological activation of this channel by K_{ATP}-channel openers has beneficial cardiovascular effects. Openers activate the channel by binding to SUR2B with high affinity and binding and channel activation require the presence of hydrolysable Mg-nucleotides. There is also an alternative way of opener action which occurs at ~ 200-fold higher concentrations and is independent of the presence of hydrolysable nucleotides. Activity of the vascular channel is known to strongly depend on the presence of nucleoside diphosphates. In intact HEK-cells, the $K_{ir}6.1$/SUR2B channel was inactive, but it activated upon dialysis of the cell with an MgATP/MgGDP-containing solution in the whole-cell patch-clamp configuration. Additional activation of the channel is achieved by openers like P1075 in the nM concentration range. In contrast, channels containing a SUR2B mutant with inactivated nucleotide binding folds (NBFs; SUR2B(K707R,K1348R)) did not show any spontaneous activation after dialysis. P1075, was still able to activate the channel and an EC_{50} value of ~3 µM was determined for this process. This value is 10-100x higher than that determined for wild-type channel. A precise determination of EC_{50} values is hampered by the strong and rapid tachyphylaxis of wild-type and mutant channels in the presence of high opener concentrations, probably leading to an underestimation of the EC_{50} values. The results show that the channel with inactivated NBFs shows no spontaneous activity but can be activated by the opener, P1075. They also suggest that the actions of P1075 on the mutated channel and on the wild-type channel in the absence of hydrolysable nucleotides are based on the same mechanism.

[1] Department of Pharmacology and Toxicology, Medical Faculty, University of Tübingen, Wilhelmstr. 56, D-72074 Tübingen
[2] Molecular Pharmacology and Toxikology, Technical University of Braunschweig, Beethovenstraße 55, D-38106 Braunschweig

193

EFFECT OF THE $K_{IR}6.2$(V59G) MUTATION ON THE ATP- AND GLIBENCLAMIDE-SENSITIVITY OF PANCREATIC K_{ATP} CHANNELS

M. Winkler[1], U. Russ[1], J. Bryan[2] and U. Quast[1]

ATP-sensitive potassium channels consist of pore-forming subunits ($K_{IR}6.x$) and regulatory subunits (sulfonylurea receptors or SURs). Channels are closed by ATP binding to $K_{IR}6.2$ and opened by interactions of MgATP/MgADP with SUR. In pancreatic β-cells, $K_{IR}6.2$/SUR1 channels couple insulin release to the blood glucose level. Mutations in $K_{IR}6.2$ and SUR1 can produce disorders in insulin secretion. The exchange of valine 59 for glycine in $K_{IR}6.2$ causes a severe neonatal diabetic phenotype with developmental delay, epilepsy or muscle weakness (DEND syndrome).$K_{IR}6.2$(V59G)/SUR1 channels exhibit an increased open probability and greatly decreased sensitivity to inhibitory ATP. We report here that the mutated channel is not affected by 1 µM glibenclamide whereas wild-type channels are inhibited by nanomolar concentrations. However, glibenclamide (1 µM) greatly increases the sensitivity of the mutant channel for ATP block. Phosphatidylinositol-(4,5)-bisphosphate (PIP_2) and oleoyl-coenzyme A activate wild-type channels, but fail to stimulate $K_{IR}6.2$(V59G)/SUR1 channels. Neomycin and poly-L-lysine (wt 500-2000) which inhibit the wild-type channel by shielding the activating lipids are only weakly active on the mutant channel. Current models of $K_{IR}6.2$ predict valine 59 to be part of the "slide helix" proposed to lie at the cytosol/membrane interface and control gating. Secondary structure prediction algorithms (nnPredict and PREDATOR) calculate a disruption of the slide helix in the Val59Gly mutant. This apparently stabilizes the channel in an open conformation thus reducing the apparent activation by phospholipids and impairing the ability of ATP and glibenclamide to stabilise the closed state. It is remarkable that glibenclamide (1 µM) alone is ineffective, but can increase the sensitivity for ATP block implying that some positive cooperativity between glibenclamide and ATP in closing the channel is preserved in the mutant channel.

[1] Department of Pharmacology and Toxicology, Medical Faculty, University of Tübingen, Wilhelmstr. 56, D-72074 Tübingen
[2] Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston Texas, 77030

194

B-CELLS FROM SUR1-KO MICE ARE LESS PRONE TO OXIDATIVE STRESS THAN WT B-CELLS

B. Gier*, P. Krippeit-Drews*, L. Aguilar-Bryan, J. Bryan, G. Drews*, M. Düfer*

We have shown previously that H_2O_2 inhibits insulin secretion by hyperpolarising the B-cell membrane potential (Vm) due to opening of K_{ATP} channels which in turn decreases cytosolic Ca^{2+} ($[Ca^{2+}]_c$). The present study was aimed to evaluate whether loss of functional K_{ATP} channels renders B-cells less sensitive to oxidative stress. In isolated islets from WT mice insulin secretion measured in the presence of 15 mM glucose (G15) was inhibited by addition of H_2O_2 down to concentrations as low as 0.025 mM. By contrast, hormone secretion from islets of SUR1-KO mice was not affected by low concentrations of H_2O_2 (0.025 and 0.1 mM), but started to decrease at concentrations of 0.25 mM. To elucidate the mechanism underlying the different sensitivity to H_2O_2, we examined the effects of H_2O_2 on V_m and $[Ca^{2+}]_c$. In WT B-cells the addition of 0.01 mM and 0.1mM H_2O_2 in the presence of G15 resulted in a reversible hyperpolarisation of Vm consistent with the opening of K_{ATP} channels. Vm dropped from -44 ±1 mV to -60±1 mV (0.01 mM H_2O_2, n=11, P<0.01) and -61±3 mV (0.1 mM H_2O_2, n=4, P<0.01), respectively. Accordingly, action potential frequency was decreased or even abolished 5 min after addition of 0.01 mM or 0.1mM H_2O_2 (spikes per minute 113±9 vs. 32±13 and 121±13 vs. 23 ±23, respectively). In B-cells from SUR1-KO mice application of 0.01 mM (n=6) or 0.1 mM (n=6) H_2O_2 neither caused membrane hyperpolarisation nor significantly decreased spike activity. $[Ca^{2+}]_c$ was decreased to basal levels in WT B-cells after exposure to 0.01 mM H_2O_2 in the presence of G15. In SUR1-KO B-cells treated with H_2O_2 $[Ca^{2+}]_c$ remained elevated due to Ca^{2+}-influx through L-type Ca^{2+}-channels. Evidently, as long as Ca^{2+} action potentials and Ca^{2+} influx are preserved, insulin secretion is not impaired despite the treatment with H_2O_2. The data show that B-cells without functional K_{ATP} channels are less sensitive to H_2O_2-induced oxidative stress. Thus, K_{ATP} channels may be an interesting drug target to protect B-cells again oxidative stress which is known to induce B-cell destruction in type-1 diabetes mellitus.

*Pharmazeutisches Institut, Auf der Morgenstelle 8, D-72076 Tübingen

195

MECHANISM OF BLOCKER/OPENER INTERACTION AT SULFONYLUREA RECEPTORS

M. Schwanstecher, A. Toman, I. Uhde and C. Schwanstecher

Blockers (e. g. glibenclamide) and openers (e. g. diazoxide) of ATP-sensitive potassium channels (K_{ATP}-channels) have been shown to exert their specific pharmacologic effects by interaction with distinct receptor sites on sulfonylurea receptors (SURs). To further analyze the complex interaction of these sites we constructed a series of SUR mutations, which were transiently expressed in COS-cells and analyzed by means of binding studies. These experiments showed that strict negative allosterism could be attenuated and finally completely abolished via substitution of one single amino acid. Further, they demonstrated for the first time that the type of interaction can be turned from negative to positive allosterism. This conclusion was confirmed by synthetic structural studies. Starting from a com-pound inducing complete glibenclamide displacement by binding to the opener site, here we finally found a structure mediating a positive allosteric effect on glibenclamide binding. The results confirm a model of SUR with strictly separated receptor sites exerting allosteric interaction through additional linking regions of the protein. They suggest positive allosterism as additional mode of interaction of assumed endogenous ligands thus adding a further aspect to models of physiologic channel control.

TU Braunschweig, Molekulare Pharmakologie und Toxikologie, Beethovenstraße 55, 38106 Braunschweig

196

β-SUBUNITS KCHIP2, KCNE2 AND DPP6 MODULATE EFFECTS OF FLECAINIDE ON TRANSIENT OUTWARD CURRENT I_{TO}

S. Radicke[1], M. Vaquero[2], L. Nunez[2], R. Caballero[2], E. Delpon[2], J. Tamargo[2], U. Ravens[1], E. Wettwer[1]

In addition to blocking cardiac Na^+ channels, the antiarrhythmic drug flecainide (F) also impairs I_{to} by acting as an open-channel blocker. In native myocytes Kv4.3 channels exist as multiprotein complexes, however, it is not known whether the various subunits modulate drug sensitivity of the channel complex. We have therefore examined the effects of flecainide on I_{to} measured in a CHO cells co-expressing the pore-forming α-subunit Kv4.3 and β-subunits KChIP2, KCNE2 or DPP6.

Channel complex composition	**IC_{50}, µM**	**IC_{50}, µM**	**τ_{ACT} (ms)**		**τ_{INACT} (ms)**	
	AUC	**Peak**	**Control**	**F (20µM)**	**Control**	**F (20µM)**
A: Kv4.3+KChIP2	9.2±1.0	23.7±3.9	0.8±0.1	0.9±0.1	29.8±4.1	13.3±2.3*
B: Kv4.3+KChIP2+KCNE2	6.9±0.9	9.5±1.3	3.5±1.0[δ]	4.2±1.8	68.4±13.1[δ]	19.7±3.6*
C: Kv4.3+KChIP2+KCNE2+DPP6	2.0±0.8	41.4±6.3	0.6±0.1	0.4±0.1*	26.7± 3.4	5.6±1.0*

* p<0.05 *vs.* control, [δ] p<0.01 *vs.* A and B

The fleacainide concentrations of half maximum inhibition (IC_{50}) were either calculated from drug effects on charge carried by I_{to} (area under the curve, AUC) or on peak I_{to}. The IC_{50} values for I_{to}(AUC) did not differ in the 3 channel complexes, whereas the IC_{50} value for peak I_{to} was lower in channel complex B compared with A and C. In addition, the effects of flecainide on the time constants of I_{to} activation and inactivation (τ_{ACT} and τ_{INACT}, respectively, obtained by exponential curve fitting) varied between the different channel complexes. Flecainide (20µM) did not affect τ_{ACT} in channel complex A and B, but significantly reduced τ_{ACT} in channel complex C. However, flecainide shortened τ_{INACT} in all channel subtype compositions investigated.

We speculate that the larger potency of flecainide in reducing peak I_{to} of complex B compared to A and C reflects increased block development due to larger τ_{ACT} and hence longer availability of

open channels for block. We conclude that the β-subunits KChIP2, KCNE2 and DPP6 modulate the effects of the open-channel blocker flecainide on I_{to} via an influence on time course of activation.

[1]Dept. Pharmacology, Dresden University of Technology, Dresden, Germany, [2]School of Medicine, Universidad Complutense/CSIC, Madrid, Spain

197

INFLUENCE OF DIFFERENT STAGES OF ELECTRICAL REMODELING ON THE ATRIAL EFFECTS OF AMIODARONE IN COMPARISON WITH I_{KR}- AND I_{TO}/I_{KUR}-BLOCKERS IN VIVO

D. Linz, H. Rütten, A. Afkham, G. Itter, KJ. Wirth

Introduction: Amiodarone is the gold standard in the prevention of the recurrence of atrial fibrillation but the reasons for its clinical superiority are neither understood nor reflected in pharmacological models. Atrial electrical remodeling changes the atrial efficacy of antiarrhythmic drugs. While blockade of later activating potassium current I_{Kr} showed a loss in atrial efficacy after 48h of electrical remodeling in goats, combined blockade of the early activating currents I_{to} and I_{Kur} significantly gained efficacy. The aim of the study was to investigate the effect of electrical remodeling in different stages on the atrial efficacy of amiodarone in awake instrumented goats and to compare it with the selective I_{Kr}-blocker dofetilide and two I_{to}/I_{Kur}-blockers, AVE1231 and AVE0118. Method: Atrial refractory periods were measured in sinus rhythm as well as after 5, 24 and 72h of atrial tachypacing in five awake instrumented goats. Amiodarone was additionally investigated after 3.5h of atrial tachypacing in six anesthetised pigs. Results: Electrical remodeling at 5h and 24h strongly increased the efficacy of amiodarone (about 2-fold, $p<0.01$ after 24h), but at 72h the early gain was lost and the efficacy was similar to sinus rhythm again. This early gain in atrial efficacy in the goat could be confirmed in the pig with 3.5h of atrial tachypacing (about 2.4 fold, $p<0.01$). Electrical remodeling progressively increased the atrial efficacy of AVE1231 an AVE0118 (about 2-fold increase in atrial refractory periods at 72h versus sinus rhythm, $p<0.01$) but drastically decreased the efficacy of dofetilide (<0.5-fold, $p<0.05$ at 24h). Conclusion: Atrial electrical remodeling changed the atrial efficacy of all agents tested. The preservation of the atrial efficacy of amiodarone in electrical remodeling and its transient gain in efficacy in early atrial remodeling help to understand its superiority over other existing antiarrhythmic drugs. Despite the initial transient gain in efficacy of amiodarone in early electrical remodeling the I_{to}/I_{Kur}-blockers AVE1231 and AVE0118 were always superior to amiodarone.

Sanofi-Aventis Deutschland GmbH, S&MA Cardiovascular, Frankfurt

198

ELECTROPHYSIOLOGICAL AND FLUORESCENCE MICROSCOPY STUDIES WITH HERG CHANNEL/EGFP FUSION PROTEINS

S. Claassen[1], J. Ludwig[2] and B.J. Zünkler[1]

HERG (human ether-a-go-go-related gene) encodes the Kv11.1 protein α-subunit that underlies the rapidly activating delayed rectifier K^+ current (I_{Kr}) in the heart. Mutations in HERG result in the proarrhythmic congenital type 2 long-QT-syndrome (LQT2). Most mutations in HERG produce abnormalities in the intracellular transport to the cell surface membrane (protein trafficking) of Kv11.1 channels. Trafficking defects are often studied using enhanced green fluorescent protein (EGFP) tagged either to the C- or N-terminus of HERG channels. The aim of the present study was to characterize the electrophysiological and pharmacological properties and the plasma membrane localization of HERG channels tagged with EGFP either to the C-terminal domain (C-EGFP) or to the N-terminal domain (N-EGFP) which had been transiently expressed in human embryonic kidney (HEK) 293 cells. The whole-cell configuration of the patch-clamp technique was used to study K^+ currents through the fusion proteins, and immunocytochemical experiments were performed employing a confocal laser scanning microscope, primary anti-HERG antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. K^+ currents with tail currents characteristic for HERG channels were observed for both expressed N- and C-EGFP channels. For N-EGFP channels the deactivation kinetics were faster and the peak tail current density was reduced when compared to both wild-type HERG channels and C-EGFP channels. Laser scanning microscopic studies showed that both fusion proteins were localized in the cytoplasm and on discrete microdomains in the plasma membrane. The extent of labelling with anti-HERG antibodies of HEK 293 cells expressing N-EGFP channels was less when compared to HEK 293 cells expressing C-EGFP channels. The sensitivity towards domperidone-induced block was similar between wild-type HERG, C-EGFP and N-EGFP channels. In conclusion, both electrophysiological and immunocytochemical studies showed that the number of N-EGFP channels in the plasma membrane is lower when compared to C-EGFP channels, which might suggest that the N-EGFP channels themselves have a protein trafficking defect.

[1] Federal Institute for Drugs and Medical Devices, Kurt-Georg-Kiesinger-Allee 3, 53175 Bonn, Germany
[2] Molecular Bioenergetics, IZMB, University of Bonn, Kirschallee 1, 53115 Bonn, Germany

199

FUNCTIONAL EXPRESSION OF ANGIOTENSINOGEN DEPENDS ON A SPLICING ENHANCER IN EXON 2

[1]C.C. Cardoso, [1,2]D. Cabrini, [1]C.S. Wilhelm, [1,3]N.E. Balboa, [1]C. Cayla, [1,4]T. Walther, [1]M. Bader

Angiotensinogen is the natural substrate for renin required for the production of all active peptides of the renin-angiotensin system. The gene coding for angiotensinogen is composed of 5 exons. Exon 2 (Ex2) is with 854 nucleotides (nt) the longest exon and contains the translational start codon, the signal peptide, and the angiotensin I encoding sequence. However, the length of Ex2 by far exceeds the normal length of internal exons in mammalian genes (150-250 nt). Thus, we hypothesized that a splicing enhancer may be necessary to include this exon into mature mRNA. Indeed, by studying the expression of the mouse angiotensinogen (mAOG) gene by RT-PCR and RNase protection assay (RPA) in different tissues, we detected a small portion (<10%) of mAOG-mRNA which lacked Ex2 confirming alternative splicing of this gene. By expressing a full-length mAOG-gene with a viral promoter in COS7 cells followed by RPA analysis, we could detect the appearance of both mRNA isoforms in these cells (70% full-length, 30% truncated). This cell culture system allowed us to localize the putative splicing enhancer within Ex2 responsible for exon inclusion. To this purpose, the mAOG gene was linearized with *Sfi* I and the exo-endonuclease *Bal-31* was used to generate internal deletions of different lengths in Ex2. These truncated mAOG gene clones were again transfected into COS7 cells and expression analysis was made by RPA. Clones containing an Ex2 of less than 200 nt included it into the mature mRNA probably due to the small size. Clones with Ex2 longer than 340 nt excluded it (30% full-length, 70% truncated) as long as nt 102 to 174 were missing in the construct. We conclude that this region contains an exonic splicing enhancer necessary to avoid skipping of the Ex2. Since Ex2 is essential for angiotensinogen function, blocking this splicing process might be a novel method to pharmacologically interfere with the renin-angiotensin system.

[1]Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Germany; [2]Universidade de Santa Catarina, Curitiba, Brazil; [3]Universidade de Salamanca, Spain; [4]Erasmus Medical School, Rotterdam, The Netherlands

200

THE VASOACTIVE EFFECT OF FIBRINOGEN ON PORCINE ARTERIES. A STUDY TO THE ANGIOTENSIN-CONVERTING-ENZYME-INHIBITOR (ACEI)–INDUCED ANGIOOEDEMA

N. Kirchhartz[2], J. Hochfeld[2], C. Tüllmann[2], M. Bas[1], T.K. Hoffmann[1], H. Bier[1], G. Kojda[2]

During acute interval patiens with angiotensin-converting-enzyme-inhibitor (ACEi)–induced angiooedema have an elevated plasma-concentration of fibrinogen (14± 0.91 µM). The vascular activity of fibrinogen and its effect on bradykinin-induced vasodilation and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) were investigated in small porcine coronary (diameter: 0.8-1.4 mm, length: 4-5 mm). Therefor pieces of the distal first side branch of right coronary arterie were dissected. With those were series of tests in special organ-bathes as well as for further clarification of the results VASP western blots done. Fibrinogen (1-15µM) induces a concentrations-depending vasodilation in preconstricted arteries. This effect was in concentrations higher than physiological plainest and was endothelium dependent (E+ 75± 21 %, vs. E- 86± 9 %, n=9, $p<0.0001$, Two-Way-Anova). Blocking NO with L-NAME showed no significant change. Preincubation with potessium channel blocker glibenclamide (100µM) significantly inhibited fibrinogen-induced vasodilatation over the full cocentration range (max: 11.3± 4.6 %, $P<0.0001$). The concentration-dependent bradykinin-effect was done after one hour preincubation with 15 µM fibrinogen. Here arised a significant increase of the vasodilator potency of bradykinin (Fib+ 66± 36 %, vs. Fib- 76 ± 30 %). Fibrinogen increased the bradykinin induced, NO dependent phosphorylation of Ser239. We could verify this effect via VASP western blots (Fib- 100 %, Fib+ 183± 31 %). Synopsis: Fibrinogen dilatates small porcine coronary arteries concentrations-dependent. This effect is partly dependent of endothelium. The endothelial NO-release has just a smal influence. Fibrinogen-dependent vasodilation dependents on endothelial, ATP-controlled potassium channels. Fibrinogen increases the vasodilative effect of bradykinins in small arteries and increases bradykinin induced VASP phosphorylation.

[1]Hals-, Nasen- und Ohrenklinik, [2]Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany

201

ANGIOTENSIN II SUBTYPE 2 RECEPTOR-DEFICIENCY IS ASSOCIATED WITH INCREASED MORBIDITY AND MORTALITY IN A MOUSE MODEL OF CHRONIC RENAL FAILURE

[1] Ralf A. Benndorf, [2] Saskia Schröder, [1] Andreas Heilmann, [1] Rainer H. Böger, [2] Ulrich O. Wenzel

Background: Angiotensin II, the main effector peptide of the Renin-Angiotensin-System, activates at least two G protein-coupled receptors, the AT1 and AT2 receptor (AT1-R; AT2-R) and plays an important role in the pathogenesis of renal diseases. In contrast to the AT1 receptor, the role of the AT2 receptor in chronic renal failure remains less well defined.

Methods: To identify the influence of AT2 receptors on the development and progression of chronic renal failure, we performed 5/6 nephrectomies in wild type und AT2 receptor *knock out* (KO) mice (FVB/N background) a procedure that induces chronic renal failure in treated mice. Subsequently, we investigated postoperative mortality during a two month observation period. Moreover, blood pressure was determined non-invasively using the tail-cuff method and urinary albumin concentrations were measured to indicate renal damage.

Results: In our model of chronic renal failure we observed a significantly higher mortality rate in KO mice as compared to wild type mice (66,7% vs. 28,6% mortality, p=0.008 (Log-Rank-Test); n=21/21). Furthermore, renal damage as measured by its surrogate albuminuria was significantly higher in KO mice than in wild type mice (densitometric analysis of urinary SDS page gels normalized to urinary creatinin concentration; KO vs. wild type: 314±277% vs. 100±134 %, $p<0.05$ (n=10/12); three weeks post-procedural). Higher mortality and renal morbidity were not due to differences in post-procedural blood pressure values (KO vs. wild type: 115±17 vs. 116±14 mmHg (n=15/13); three weeks post-surgical).

Conclusion: In our model AT2 receptor-deficiency aggravates chronic renal failure in a blood pressure independent manner. Upcoming investigations will clarify the mechanisms responsible for this effect.

[1] Institut für Experimentelle und Klinische Pharmakologie, Arbeitsbereich Klinische Pharmakologie, [2] Zentrum für Innere Medizin, III. Medizinische Klinik und Poliklinik, UKE

202

ENHANCED ALOSTERONE AND BLOOD PRESSURE RESPONSE TO ANGIOTENSIN II IN OBESE ZUCKER RATS.

H. Müller, O. Jöhren, P. Dominiak, W. Raasch

Obesity is frequently accompanied by hypertension and is positively correlated with plasma aldosterone, suggesting a link between obesity hypertension and increased mineraclocorticoid levels. Angiotensin II (ANG) is known to stimulate aldosterone release and angiotensinogen levels and ACE activity have been shown to be heightened in obesity. Thus, we investigated, whether an ANG-induced elevation in blood pressure will be selectively increased in obesity as a result of an enhancement of circulating aldosterone. Lean (LZR) and obese (OZR) leptin resistant Zucker rats were treated with ANG (0 or 9 µg/h; 4 weeks) via subcutaneously located osmotic minipumps. Blood pressure was determined via catheter. ANG, aldosterone and leptin were measured in serum by RIA. Adrenal mRNA levels of AT_{1B}-receptor and aldosterone synthase were quantified by qPCR. Leptin resistance of OZR was documented by the dramatic increase of bodyweight and plasma leptin. Baseline blood pressure did not differ between saline treated LZR and OZR. In response to ANG, blood pressure was increased in LZR (32 mmHg), but substantially more in OZR (83 mmHg). As a result to

the pronounced blood pressure response to ANG, the left ventricular weight of OZR was enhanced. Due to ANG treatment, circulating ANG levels similarly in LZR and OZR compared to baseline levels. Plasma aldosterone as well as the adrenal mRNA levels of the aldosterone synthase and the AT_{1B}-receptor were selectively increased in ANG treated OZR compared to LZR.We conclude, that the ANG-induced blood pressure response is enhanced in obesity due to a specific raise in circulating aldosterone. Selectively in obesity, the adrenal AT_{1B}-receptors is upregulated under concomitant ANG stimulation, leading to an enhanced adrenal synthesis of aldosterone and its secretion. Thus, our results may help to explain, that aldosterone-induced obesity hypertension is probably a result of an overactive adrenal response to ANG. Whether factors originating from adipose tissue triggers the upregulation of AT_{1B}-receptor in the adrenal glands has to be clarified.

Institute of Experimental and Clinical Pharmacology and Toxicology, University Clinic of Schleswig-Holstein, Campus Lübeck

203

THE STRESS SENSITIVY IS INCREASED IN THE BRAIN SPECIFIC ANGIOTENSINOGEN DEFICIENT TRANSGENIC RAT

W. Raasch, J. Kröger, H. Müller, O. Jöhren, M. Bader*), P. Dominiak

Recently we have shown that antihypertensive treatment by inhibition of AT_1-receptors attenuates HPA-axis reactivity independently of blood pressure reduction. Both, a reduced pituitary may have the potential to reduce HPA-axis activity during chronic AT_1-blockade. This study aimed to approve these central mechanisms by using the genetic rat model of the brain specific angiotensinogen deficient rats (TGR(ASrAOGEN)). HPA-reactivity was determined in TGR(ASrAOGEN) and appropriate controls by CRH-Test, forced swim test (FST) and ACTH/dexamethasone test. Blood pressure was determined via catheters. ANG, aldosterone, ACTH and corticosterone were measured in plasma by RIA. Adrenal mRNA levels of AT_{1A}-, AT_{1B}- and MC2-receptors were quantified by qPCR. Blood pressure was not reduced in TGR(ASrAOGEN), even though circulating ANG and aldosterone were diminished. Baseline levels of stress hormones did not differ between TGR(ASrAOGEN) and controls. During CRH-test and FST, the plasma concentrations of corticosterone but not of ACTH were increased. During ACTH/dexamethasone test the plasma concentrations of corticosterone did not differ between TGR(ASrAOGEN) and controls. No differences could be detected regarding the adrenal mRNA levels of AT_{1A}-, AT_{1B}- and MC2-receptors. Since in contrast to our expectations the FST- and the CRH-induced stress response was rather increased than attenuated in the TGR(ASrAOGEN), we conclude, that the reduced HPA-reactivity during AT_1-blockade could not be mimicked in a specific transgenic rat model that features a central inactivated RAAS. Which probable counterregulations lead to the increased stress sensitivity in the TGR(ASrAOGEN) are unknown. The independency of corticosterone release on ACTH during CRH-test indeed indicated an adrenal mechanism, but this could not be approved in the ACTH/dexamethasone test. Alterations of the expression of AT_{1A}- and MC2-receptors seems not to be involved into the facilitation of the adrenal corticosterone release.

Institute of Experimental and Clinical Pharmacology and Toxicology, University Clinic of Schleswig-Holstein, Campus Lübeck; *)Max-Delbruck-Center for Molecular Medicine (MDC), Berlin-Buch

204

THE ANGIOTENSIN GENERATING SYSTEM IN THE INSULINOMA CELL LINE INS-1

M. Siebelmann, G. Miesner, J. Fabian, E.J. Verspohl

ctivation of a local pancreatic islet RAS (Renin Angiotensin System) is associated with a regulation of hormone secretion. Several components of RAS have been found in the endocrine pancreas. Angiotensin IV receptors (AT_4-receptor/insulin regulated aminopeptidase (IRAP)) are expressed in INS-1 cells. Degradation were investigated in INS-1 cells by incubation with angiotensin peptides (angiotensin I to IV) at 37 °C for 2 or 4 h in KRH-buffer in 24well plates. Supernatants were analyzed by HPLC-ESI-MS (Hydro-RP 80 column, linear gradient) and degradation and forming of angiotensin peptides (angiotensin I to IV) was monitored. A polyclonal anti-IRAP antibody was used for Western Blot analysis. INS-1 cells were stably transfected with an IRAP-GFP plasmid using fugene6® and controlled by confocal laser scanning microscopy. IRAP activity was monitored by the hydrolysis of l-leucyl ß-naphthylamide (Leu-Na) in a fluorescence assay. AngI was cleaved by INS-1 cells to the bioactive compounds AngII and Ang(1-7), but not to AngIII and AngIV. Several other degradation products could be identified. AngII was metabolised to AngIII. AngIII was fastly cleaved to AngIV and this is rapidly catabolized to Ang(4-8) and Ang(5-8). Western Blot analysis showed that INS-1 cells express the AT_4-receptor/IRAP. The cellular localisation of the receptor could be demonstrated by confocal laser scanning microscopy: A high amount of IRAP is predominantly located in vesicles near the nucleus and a minor fraction at the plasma membrane. IRAP fluorescence assay showed that AngIV (IC_{50} 0.5 µM) and AngIII (IC_{50} 0.3 µM) inhibit the maximal IRAP activity up to 65 % at a concentration of 100 µM, whereas AngI (IC_{50} > 10 µM) and AngII (IC_{50} > 1 µM) have no effect on IRAP activity. Thus various still bioactive angiotensin peptides are enzymatically generated in INS-1 cells. The rapid metabolism and the severe control of AngIV and AngIII concentration in the environment of INS-1 cells as well as control of IRAP activity indicate their importance for INS-1 cell function.

Department of Pharmacology, Institute of Medicinal Chemistry, Münster, Germany

205

POTENCY OF AT_1 RECEPTOR ANTAGONISTS TO BLOCK ANGIOTENSIN II-EVOKED INCREASE IN cGMP IN A MOUSE NEUROBLASTOMA CELL LINE

H. Pinheiro[1,2] D. Moura[1] , O. Viegas[3], C. Calhau[4], S. Guimarães[1]

The rank order of potency of several antagonists to block vascular responses to angiotensin II is different prejunctionally and postjunctionally (Guimarães and Pinheiro, 2005, Cardiovasc Res 67: 208). Chaki and Inagami (1992, Eur J Pharmacol 225: 355) had described that angiotensin II increases cGMP levels in a mouse neuroblastoma cell line (Neuro-2A) and that either losartan or AT_2 antagonists fail to antagonize this response to angiotensin II. We aimed at further characterizing the angiotensin II receptor in Neuro-2A cells. Cells were differentiated in Minimal Essential Medium supplemented with 10 % fetal bovine serum in the presence of prostaglandin E_1 (100 nM) and exposed to angiotensin II in HEPES buffered saline containing IBMX (100 µM), in the absence or in the presence of antagonists. cGMP levels were determined by direct enzimatic immunoassay. The potency of the antagonists was expressed as pA_2 values ou pD'_2 values according to the type of antagonist, competitive (surmountable) or non-competitive (insurmountable), respectively. Angiotensin II (1 nM – 1 µM) produced an increase in cGMP levels in a concentration dependent manner. The AT_2 selective antagonist PD123319 (1 µM) had no effect on the angiotensin II response, while the non-selective antagonist, saralasin (1 µM) abolished the action of 100 nM of angiotensin II. The most potent antagonist was candesartan (pD'_2 = 9.18) and the less potent was losartan (pA_2 = 6.63). The rank order of potencies for the selective AT_1 antagonists was the same as that observed for the receptor involved in the prejunctional action of angiotensin II in the sympathetic nerve terminals: candesartan > ZD 7155 > eprosartan > losartan. In conclusion, we propose that the angiotensin receptor which is involved in the increase of cGMP levels in Neuro-2A cells, as well as the angiotensin prejuncional receptor, is also AT_{1B}.

[1]Institute of Pharmacology and Therapeutics and [4] Laboratory of Biochemistry, Faculty of Medicine; [2]Laboratory of Pharmacology, Faculty of Pharmacy; [3]Faculty of Nutrition and Food Sciences. University of Porto, Portugal

206

TELMISARTAN MEDIATES BLOOD PRESSURE-INDEPENDENT RECOVERING OF END-ORGAN DAMAGE IN MALE SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

A. Grundt, C. Grundt, M. Schwaninger, B. Lemmer

Background Spontaneously hypertensive rats (SHR) develop severe cardiovascular and renal damage ascribed to increased blood pressure. It is known that blood pressure reduction on its own is not sufficient to regenerate organ damages. In this study we demonstrate that an AT_1-receptor blockade in SHR using Telmisartan has a long lasting effect on end-organ recovering independently from blood pressure.

Methods Telmisartan in a dosage of 3 mg/kg was applied intraperitoneally in 48-hours intervals to 11 weeks old WKY (WKY(+Tel)) and SHR (SHR(+Tel)) for six weeks and discontinued for further six weeks in SHR (SHR(-Tel)). Vehicle-treated WKY and SHR served as control. Blood pressure and heart rate were recorded by radiotelemetry. Urinary nitrate/nitrite (NOx) and creatinine were measured colorimetrically. Heart weight/body weight was determined as cardiac hypertrophy index.

Results Blood pressure as well as cardiac mass of SHR(+Tel) could be significantly reduced to values of WKY. Neither urinary NOx nor protein excretion, representing markers of arterial vessel and renal function, were significantly restored in SHR(+Tel). After six weeks of drug discontinuation blood pressure of SHR(-Tel) increased to SHR values without treatment. The cardiac weight of SHR(-Tel) increased significantly in comparison to SHR(+Tel). Urinary NOx in SHR(-Tel) was increased and protein excretion was significantly reduced versus untreated SHR and SHR(+Tel).

Conclusion Our data demonstrate that chronic antihypertensive treatment of SHR using Telmisartan results in cardiac mass reduction correlating to blood pressure decrease. In contrast, recovering of arterial vessel and renal function by Telmisartan is a long lasting effect and occurs independently from blood pressure decrease.

Institute of Pharmacology & Toxicology, University of Heidelberg, Maybachstraße 14, 68169 Mannheim

207

ANTAGONISM AT ANGIOTENSIN II TYPE 1 RECEPTORS CAN REDUCE FOOD INTAKE IN RATS

J.-P. Voigt[1,2], P. Bramlage[3]and H. Fink[1]

Recent experimental and clinical studies report beneficial metabolic effects of antihypertensive drugs interfering with angiotensin. Antagonists at the angiotensin 1 (AT1) receptor can reduce blood glucose and triglyceride levels. So far, there is little evidence, however, that AT 1 receptor antagonists can also affect food intake. Particularly unknown is if drugs of this class can have acute effects on short term feeding. To address this issue, the AT 1 receptor antagonist irbesartan was studied in a one-hour feeding paradigm in rats. In this study, irbesartan was investigated in comparison with fenfluramine, an established satiating drug, and the angiotensin converting enzyme (ACE) inhibitor captopril. We found a significant reduction of one-hour food intake following 100 – 200 mg/kg (IP) irbesartan. The ACE inhibitor captopril (25 – 100 mg/kg IP) remained without effect on food intake and fenfluramine showed the expected hypophagic action starting at 1 mg/kg (IP). The hypophagic effect of irbesartan could not be attributed to sedation or any gross effect on motor activity as determined both upon feeding and in independent activity experiments. Fenfluramine (1mg/kg) and irbesartan (100 mg/kg) did not reduce the latency to feed, but similarly reduced the eating rate at the beginning of the test meal. In conclusion, the present study demonstrates a hypophagic effect of the AT 1 receptor antagonist irbesartan that cannot be attributed to sedation or antidipsic effects of the drug.

[1]Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, Koserstr. 20, D-14195 Berlin, Germany
[2] University of Nottingham, School of Veterinary Medicine and Science, Sutton Bonington Campus, Sutton Bonington, Leicestershire, LE12 5RD, UK
[3] Institute for Clinical Pharmacology, Technical University Dresden, Medical Faculty Carl Gustav Carus, Fiedlerstr. 27, D-01307 Dresden, Germany

208

CHRONIC TREATMENT OF MALE TGR(mREN2)27 WITH THE AT1 RECEPTOR ANTAGONIST EPROSARTAN: EFFECTS ON CARDIOVASCULAR AND RENAL PARAMETERS

C. Grundt, S. Klimm, A. Grundt, B. Lemmer

Background - Mere blood pressure reduction has been shown not to be sufficient in hypertension to restore cardiovascular health. Therefore, the aim of this study was to evaluate the effects of chronic AT1 receptor antagonism with eprosartan on blood pressure, urinary protein as well as on structural changes of end-organs in aged male TGR(mREN2)27 (TGR).

Methods - Eprosartan was administered orally for 6 weeks starting at the age of six months (TGR-E) in a dosage of 20 mg/kg/d. TGR and Sprague-Dawley rats (SPRD) receiving vehicle were used as controls. Blood pressure and heart rate were monitored in conscious rats by radiotelemetry. Heart and kidney histopathology as well as vascular structure of thoracic aortae were assessed. Urinary protein was measured colorimetrically.
Results - Blood pressure was reduced in TGR-E reaching normotensive values of SPRD whereas heart rate was not affected. Due to a pronounced blood pressure reduction during day by eprosartan a physiological profile with higher values during night could be restored in TGR-E. Cardiac hypertrophy and collagen content as well as aortal wall thickness were reduced in TGR-E. Eprosartan reduced proteinuria as well as glomerulosclerosis both observed in TGR.
Conclusion - This data demonstrate that eprosartan administration in TGR results in significant blood pressure reduction to normotensive values and restoration of the pathological 24h-blood pressure profile both contributing to protection of end-organs such as heart, aorta and kidney.
Institute of Pharmacology & Toxicology, University of Heidelberg, Maybachstr. 14, 68169 Mannheim

209

EFFECT OF THE COX-2 INHIBITOR SC-58236 ON RENIN SECRETION AND RENIN SYNTHESIS IN THE RAT

K. Höcherl[1], C. Matzdorf[2], A. Kurtz[1]

The role of COX-2 activity for the physiologic control of renin is still a matter of debate, since studies with COX-2 deficient mice or with COX-2 inhibitors produced conflicting findings. Therefore, we studied time-dependent effects of the COX-2 inhibitor SC-58236 on the regulation of renin gene expression and renin secretion in adult rat kidneys. We found that renocortical tissue levels of prostaglandin E_2 (PGE_2) and renal urinary excretion of PGE_2 were decreased to about 70 and 50% of control values already after one day of treatment with SC58236 (10 mg / kg x d) and did not further decrease upon prolonged treatment. Basal plasma renin activity (PRA) and renin mRNA levels began to decrease three days after start of treatment with SC-58236 and reached a constant level corresponding to about 60% of the respective values of vehicle-treated rats after five days of treatment. Infusion of isoproterenol (160 µg / kg x h) or left renal artery clipping increased PRA and renin mRNA to similar absolute levels both in vehicle and SC-58236-treated rats after two days. Pre-treatment with SC-58236 for five days, however, lowered PRA and renin mRNA levels in animals treated with isoproterenol or in animals with left renal artery clipping for two days. Notably, the relative increases were not different between vehicle and SC-58236-treated rats. Similar findings were observed for the stimulation of the renin system by angiotensin II-inhibition and for the regulation of the renin system by salt intake. The findings indicate that COX-2 inhibition attenuates renin secretion and renin gene expression stimulated by a variety of parameters in proportion to the lowering of basal renin activity, whilst it does not interfere with the different stimulatory mechanism per se. As a consequence, it appears as if COX-2 activity essentially determines the set point of the activity of the renin system, that can be acutely stimulated by isoproterenol, low salt diet or angiotensin II antagonists.
Institutes of Physiology[1] and Pharmacology[2], Universität Regensburg, Universitätsstr. 31, D-93040 Regensburg, Germany

210

Ca^{2+} SIGNALING OF KININS IN CELLS EXPRESSING RAT, MOUSE AND HUMAN BRADYKININ B_1/B_2 RECEPTOR

R. Zubakova, A. Gille, A. Faussner and U. Hilgenfeldt

Kinins are endogenous paracrine-autocrine vasodilators that exert their biological effects *via* the B_1 and B_2 bradykinin receptors (B_1R and B_2R). The B_2R is expressed ubiquitously. Its physiological ligands in humans are bradykinin (BK) and kallidin (KAL). In contrast, BK was believed to be the only kinin acting on the B_2R in rat and mouse. However, a kallidin-like peptide (KLP) was recently isolated from plasma and urine of rats (Hilgenfeldt *et al.*, 2005, Br J Pharmacol). B_1R is expressed under inflammation condition. The naturally occurring metabolites of KAL and BK, des-Arg^{10}-KAL and des-Arg^9-BK are binding with high affinity to the B_1R. Both bradykinin receptors are coupled to $G_{q/11}$ proteins that activate the phosphoinositide pathway and increase the intracellular Ca^{2+}-concentration. We systematically compared the effects of BK, KAL, KLP, des-Arg^9-BK, des-Arg^{10}-KAL and des-Arg^{10}-KLP on the intracellular calcium concentration of HEK293 cells stably expressing human, rat and mouse B_1- and B_2 receptors using the calcium-sensitive dye FURA-2. KAL, KLP, and BK expressed similar potencies and efficacies on B_2R of all three species (pM range). Des-Arg-derivatives were less efficacious and less potent (nM range) on the B_2R. In cells expressing human B_1R des-Arg^{10}-KAL mediated the strongest effect. The effect of des-Arg^{10}-BK was the smallest. In cells expressing rat and mouse B_1R des-Arg^{10}-KLP and des-Arg^9-BK caused a similar effect. Calcium signal of des-Arg^{10}-KAL was slightly lower. On The kinins (BK, KAL, KLP) were less efficacious and less potent (nM range) on the B_1R. Conclusion: Until now the ligand-receptor interaction of kinins were mainly characterized in binding studies. The data show for the first time that des-Arg^{10}-KLP mediates a similar effect on the murine B_1R as des-Arg^{10}-KAL on the human B_1R.
Department of Pharmacology, University of Heidelberg, D-69120 Heidelberg, Germany

211

OVEREXPRESSION OF THE SUPPRESSOR OF CYTOKINE SIGNALING (SOCS)-3 IN KERATINOCYTES RESULTS IN DELAYED WOUND HEALING WITH POOR EPIDERMAL REGENERATION AND PROLONGED INFLAMMATION IN MICE

A. Linke, I. Goren, J. Pfeilschifter, S. Frank

Diabetes-impaired chronic wounds represent a problem of clinical relevance. At least in mice, a severe insulin resistance of wound tissue contributes to disturbed healing conditions. The suppressor of cytokine signaling (SOCS)-3 has been shown to contribute to insulin resistance, as the protein potently blunts signaling from the insulin receptor. Here, we demonstrate that expression of the suppressor of cytokine signaling (SOCS)-3 was markedly elevated in keratinocytes of atrophied epithelia in diabetes-disturbed wounds in obese/obese (ob/ob) mice. To investigate the role of SOCS-3 in cutaneous wound healing, we established transgenic mouse lines by a SOCS-3 overexpression under control of the keratinocyte-specific keratin 5 (K5) promoter targeting the transgene restrictively to the basal epidermal compartment. As expected, immunohistological analysis of unwounded skin revealed a strong expression of SOCS-3 in basal keratinocytes and hair follicles. Interestingly, the presence of overexpressed SOCS-3 in keratinocytes was accompanied by a dramatic increase of loricrin in the cells, which represents a marker of terminally differentiated keratinocytes. In good accordance, wound healing in transgenic mice was significantly impaired and characterized by a poorly developed hyperproliferative epithelium. In contrast to the expected role of SOCS-3 to reduce inflammatory responses, surprisingly, we observed an increased and prolonged wound inflammation in the animals characterized by elevated levels of chemokines, proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β as well as cylcooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). However, our findings clearly mimick diabetes-disturbed wound conditions, which were characterized by reduced keratinocyte proliferation and an exacerbated inflammation. In summary, our data suggest SOCS-3 as a potential target to understand destructive alterations of wound responses in diabetes-impaired wound healing.
pharmazentrum frankfurt / ZAFES, Klinikum der Johann-Wolfgang-Goethe Universität, Frankfurt am Main

212

INSULIN-DRIVEN HMG-COA REDUCTASE ACTS ON KERATINOCYTE FUNCTIONS IN SKIN REPAIR

D. Schiefelbein, I. Goren, J. Pfeilschifter and S. Frank

Wound healing disorders represent one major complication associated with diabetes mellitus. At least in mice, diabetes-impaired wound tissue was characterized by a severely disturbed and malfunctioning insulin signalling machinery. To provide new insights into the role of insulin in skin repair, we identified the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase to be under control of insulin in cultured keratinocytes (HaCaT) and in murine wound tissue. Insulin mediated a marked increase in HMG-CoA reductase expression and activity in keratinocytes *in vitro*. Additionally, we could observe the epidermal growth factor (EGF) as a potent inducer of HMG-CoA reductase expression and activity in the cells. Analysis of HMG-CoA reductase regulation in wound tissue revealed a biphasic response with increased expression at day 3 and day 13 post-wounding in healthy but not in diabetic ob/ob mice. In situ hybridization demonstrated proliferating keratinocytes located at the margins of the wound to be the predominant source of HMG-CoA reductase expression at the wound site. As those cells also represent the major source of wound-derived vascular endothelial growth factor (VEGF), we determined the functional role of co-expressed HMG-CoA reductase for keratinocyte-mediated VEGF production. Interestingly, inhibition of HMG-CoA reductase enzymatic activity by simvastatin selectively blunted insulin-, but not EGF-stimulated VEGF biosynthesis from cultured keratinocytes. Inhibition of HMG-CoA reductase interfered with insulin-stimulated VEGF biosynthesis at the post-transcriptional level by a strong reduction of eukaryotic initiation factor (eIF)-4 binding protein (4E-BP1) phosphorylation. In addition, Ca2+-induced differentiation of keratinocytes could be completely blocked by simvastatin *in vitro*, suggesting a prominent role for HMG-CoA reductase in the control of keratinocyte mitogenic responses in late skin repair. In summary, our data provide evidence that insulin acts on wound keratinocyte VEGF production and differentiation by inducing HMG-CoA reductase expression and activity.
pharmazentrum frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main

213

ROLE OF SODIUM AND CALCIUM CHANNELS ON GLUCAGON SECRETION IN MICE

V. Leiss[1], V. Auer[1], S. Vignali[1], R. Lukowski[1], Q. Zang[2], P. Rorsman[2], F. Hofmann[1], A. Welling[1]

The release of glucagon by pancreatic α-cells is stimulated by hypoglycemia, whereas hormone secretion is inhibited by hyperglycemia, insulin, and somatostatin. The molecular signalling mechanisms leading to glucagon secretion are discussed controversial. However, it is suggested that sodium and high voltage activated (HVA) calcium channel subtypes expressed in α-cells might be involved in hormone secretion. In the present work, combining genetic and electrophysiological methods, $Na_V1.7$, the L-type HVA calcium channel subtypes $Ca_V1.2$ and $Ca_V1.3$, the non-L-type HVA calcium channel subtypes $Ca_V2.1$ and $Ca_V2.3$ were detected in α-cells. To further analyse the specific roles of the channels on glucagon release, α-cell $Na_V1.7$ and $Ca_V1.2$ knockout mice were generated by using the Cre/loxP approach. To examine the recombination pattern of pancreatic α-cells, the Gluc-Cre mice were crossed to Cre-reporter animals carrying the ROSA26 β-gal allele. The recombinant mice showed that the Gluc-Cre mice are a suitable tool to generate α-cell-deficient mouse lines for the two channels investigated ($Na_V1.7$ and $Ca_V1.2$). Electrophysiological measurements of islet α-cells revealed that the half-maximal inactivation ($V_{1/2}$) of Na^+-currents was shifted from -104 ± 1.2 mV in control mice to -38 ± 6.6 mV and -27 ± 2.3 mV in $Na_V1.7$ hetero- and homozygous α-cell knockout animals. Furthermore, a deletion of the $Ca_V1.2$ gene in α-cells could be verified by patch-clamp measurements, and by genomic DNA analysis of single islet cells and whole islets. However, preliminary data indicate that blood glucose does not differ between $Ca_V1.2$ knock out and control mice after insulin (1.6 mU/g) or glucagon (2.0 mg/g) injections.
[1] Institut für Pharmakologie und Toxikologie, TU München, Germany
[2] Oxford Center for Diabetes, Endocrinology & Metabolism, Oxford, Great Britain

214

TRANSFER OF THE QTL *NIDD/SJL* TO GENETICALLY DIFFERENT OBESE BACKGROUNDS RESULTS IN DIFFERENT PATTERNS OF TYPE 2-LIKE HYPERGLYCEMIA

Scherneck S[1], Nestler M[1], Kluge R[1], Neschen S[1], Jörns A[2], Rustenbeck I[3], Vogel H[1], Teichert M[1], Schmolz K[1], Schürmann A[1], and Joost HG[1]

The development of metabolic disorders is markedly dependent on genetic and environmental factors and their interaction with each other. Inbred strains of mice are well established models to investigate these complex traits. In a backcross model of NZO and SJL mice the diabetes susceptibility locus *Nidd/SJL* was identified on distal mouse chromosome 4. *Nidd/SJL* induces type 2-like hyperglycemia depending on both

early high body weight and a high dietary fat content. Here we describe the effects of *Nidd/SJL* on two obese backgrounds, the monogenic leptin-deficient *ob/ob*-mouse and the polygenic NZO mouse. First, the susceptibility locus *Nidd/SJL* was transferred to the C57BL/6 mouse strain via successive backcrossing. Secondly, reporter crosses with the two obese mouse strains were performed to generate animals with an early high body weight. Our results show that *Nidd/SJL* induces hyperglycemia when present on both backgrounds. On the *ob/ob* background, *Nidd/SJL* carriers developed significantly higher blood glucose levels than carriers of the corresponding wild-type allele in week 12 of life (225±27 mg/dl *vs*. 149±14 mg/dl, MW±SEM, p<0.05) without any significant change in insulin levels. *Nidd/SJL* carriers on the NZO background, in contrast, exhibited more severe hyperglycemia (260±24 mg/dl *vs*. 187±8 mg/dl, MW±SEM, p<0.01), associated with hypoinsulinemia and destruction of pancreatic beta cells. As a consequence of the diabetic phenotype, these mice showed a dramatic weight loss. Our results demonstrate that the characteristics of the diabetogenic allele *Nidd/SJL* are not only dependent on obesity but also on additional genetic factors. *Nidd/SJL* led to hyperglycemia on both genetic backgrounds *ob/ob* and NZO. However, islet cell failure appeared only in the presence of the NZO background, suggesting that the NZO mouse carries additional diabetogenic alleles.

[1]Department of Pharmacology, German Institute of Human Nutrition Potsdam-Rehbruecke
[2]Institute of Anatomy, Hannover Medical School
[3]Institute of Pharmacology and Toxicology, Technical University Braunschweig

215

INHIBITION OF MAFA TRANSCRIPTIONAL ACTIVITY CONTRIBUTES TO THE INHIBITION OF HUMAN INSULIN GENE TRANSCRIPTION BY MEKK1 AND INTERLEUKIN-1BETA

E. Oetjen, R. Blume, I. Cierny, A. Kutschenko, R. Krätzner, R. Stein [2], W. Knepel

Inappropriate insulin secretion and biosynthesis are hallmarks of beta cell-dysfunction and contribute to the progression from a prediabetic state to overt diabetes mellitus type 2. During the prediabetic state beta cells are exposed to elevated levels of proinflammatory cytokines. Therefore, in the present study the effect of these cytokines and of the mitogen-activated protein kinase kinase kinase 1 (MEKK1) activated by these cytokines on human insulin gene transcription was investigated. In transient transfection assays into the insulin producing pancreatic beta cell-line HIT, interleukin-1beta (IL-1beta) and over expression of the constitutively active domain of MEKK1 specifically inhibited basal and membrane depolarization plus cAMP-induced human insulin gene transcription. In the primary mature islets of mice carrying the luciferase gene under the control the human insulin promoter IL-1beta abolished glucose stimulated reporter gene expression. Promoter analysis using 5`- and 3`-deletion constructs revealed the CRE2, RIPE3b, and A1 as MEKK1-responsive elements. Mutation of CRE2 and A1 within the insulin promoter reduced its transcriptional activity by 60% but pertained the MEKK1-responsiveness. Mutation of RIPE3b decreased the promoter activity by 80% with an attenuation of MEKK1-induced inhibition. Like IL-1beta MEKK1 decreased the transcriptional activity directed by four copies of the RIPE3b element and abolished in a heterologous cell-line insulin gene transcription dependent on the RIPE3b binding protein MafA. These data demonstrate that IL-1beta/MEKK1-induced inhibition of human insulin gene transcription is conferred at least in part by decreasing MafA transcriptional activity. Considering that inappropriate insulin biosynthesis contributes to beta cell-dysfunction, inhibition of MEKK1 might prevent the progression from a prediabetic state to diabetes mellitus.

Molecular Pharmacology, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany; [2]Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, USA

216

DELETION OF THE $Na_V1.7$ SODIUM CHANNEL IN PANCREATIC A- AND B-ISLET CELLS OF MICE

V. Auer, V. Leiss, S. Vignali, F. Hofmann and A. Welling

Insulin and glucagon are stored and secreted by pancreatic B- and A-cells of the Langerhans islets. These cells are electrical excitable. Exocytosis of insulin- and glucagon-containing secretory granules is triggered by electrical signals. The mechanisms involved in insulin secretion are partially established. High blood glucose levels increase the ATP/ADP ratio leading to the closure of ATP-sensitive K^+ channels (K_{ATP} channels) and B-cell depolarization. As a consequence, voltage-gated calcium channels open and trigger exocytosis of hormone-containing secretory granules. The mechanisms involved in glucagon secretion are unknow. It has been shown that sodium channels are expressed in murine islet cells but their role still is unknown. The aim of the present study was to determine the role of sodium channels for insulin and glucagon release in murine pancreatic islet cells. By genomic analysis the expression of $Na_v1.7$ was shown. To analyse the role of this sodium channel, A- ($\alpha Na_v1.7^{-/-}$) and B-cell ($\beta Na_v1.7^{-/-}$) specific $Na_v1.7$ knockout mice were investigated using patch-clamp measurements. Single A- and B-cells were distinctly identified by RT-PCR using insulin- and glucagon specific primers, A- and B-cells in intact islets by immunostaining. So far, 92% of single A-cells and 50% of single B-cells lacking the $Na_v1.7$ sodium channel showed no sodium current versus 24% of the control A-cells and 12% of the control B-cells. In contrast, A- and B-cells in intact islets of $\beta Na_v1.7^{-/-}$- and $\alpha Na_v1.7^{-/-}$-mice showed sodium currents with an half maximal inactivation shifted to more depolarised potentials in control cells. TTX and the B-cell-selective ablation of the gene encoding the sodium channel subtype $Na_v1.7$ didn't affect spike activity in B-cells.

Institut für Pharmakologie und Toxikologie, Technische Universität München, Germany

217

THE ROLE OF ADENOSINE A_1 RECEPTORS FOR INSULIN RELEASE AND THE POSSIBLE MECHANISM INVOLVED

E.J. Versphol[1], C.E. Müller[2], M. Töpfer[1]

The role of adenosine receptor subtypes with respect to their impact on the insulin secretory system is not well investigated, probably due to a lack of specific agonists and antagonists. The presence of A_1- and A_{2B} adenosine receptors in INS-1 cells were recently demonstrated. Newly synthesized compounds were investigated thereby integrating three methods, insulin release (RIA), $^{45}Ca^{2+}$ uptake and $^{86}Rb^+$ efflux of INS-1 cells (an insulin secretory cell line). The nonselective adenosine analogue NECA (5`-*N*-ethylcarboxyamidoadenosine) had no effect on either parameter (insulin release, $^{45}Ca^{2+}$ uptake and $^{86}Rb^+$ efflux), which could be interpreted as the sum of effects of each other antagonizing adenosine receptor subtypes. A possibly hidden effect on A_1 receptors could not be revealed by using the A_1 receptor antagonist PSB-36 (1-butyl-8-(3-noradamantyl)-3-(3-hydroxypropyl)xanthine). On the other hand addition of the A_{2B} receptor antagonist PSB-1115 (1-propyl-8-(p-sulfophenyl)xanthine) to NECA increased all three parameters indicating a hidden A_{2B} receptor effect of NECA. The A_1 receptor agonist CHA (N^6-cyclohexyladenosine) increased insulin release, $^{45}Ca^{2+}$ uptake (nifedipine-dependent) and inhibited $^{86}Rb^+$ efflux which fits altogether to insulin secretion. This, however, does not fit to the cAMP-linked A_1 receptor mechanism and the effects are not antagonized by the A_1 receptor antagonist PSB-36 indicating non-selectivity of CHA. PSB-36 alone inhibits $^{86}Rb^+$ efflux, increases $^{45}Ca^{2+}$ uptake and has a concentration-dependent effect on insulin release. These non-typical effects for an antagonist can be explained by antagonizing a basal adenosine tonus of which the A_2 effect is still present and not blocked. The nonselective adenosine receptor antagonist caffeine increased insulin release. In this case CHA inhibits the caffeine effect as expected when hypothesizing that both act via A_1 receptors and are coupled to the cAMP system. Using different approaches the A_1 receptor may be linked to divergent pleiotropic effects using different 2nd messenger systems in INS-1 cells, the K^+-depolarizing and $^{45}Ca^{2+}$ uptake system as well as the cAMP system.

[1]Department of Pharmacology, Institute of Medicinal Chemistry, Münster, Germany
[2]Institute of Pharmaceutical Chemistry, Bonn, Germany

218

RX871024 BUT NOT EFAROXAN STIMULATE INSULIN SECRETION BY A K_{ATP} INDEPENDENT MECHANISM.

M. Willenborg [1], A. Wienbergen[1], L. Aguilar-Bryan[2], J. Bryan[2] ,I. Rustenbeck[1]

The imidazoline, RX871024, enhances insulin release only when the glucose concentration by itself is stimulatory (glucose-selectivity). This effect was ascribed to the activation of a K_{ATP} channel-independent pathway in addition to the direct closure of K_{ATP}-channels. Here we show that efaroxan, another glucose-selective first generation imidazoline, produces an insulinotropic effect only via the K_{ATP} channel-dependent pathway. Three parameters were measured to evaluate the effect of these imidazolines: (1) insulin release was measured in perifusion experiments on freshly isolated islets from NMRI or Sur1KO mice, (2) K_{ATP} channel activity was assessed by patch-clamp electrophysiology and (3) $[Ca^{2+}]_c$ was determined by microfluorometry. Mouse β-cell K_{ATP} channel activity was reduced by about 82% by 10 µM RX871024 or 100 µM efaroxan; complete closure was achieved with 100 µM RX871024 and 500 µM efaroxan, respectively. 10 µM RX871024 and 100 µM efaroxan stimulated insulin secretion in 10, but not 5 mM glucose. Under normoglycemic conditions (5 mM), although there was no stimulation of insulin secretion with 10 µM RX871024 or 100 µM efaroxan; there was an evident increase in $[Ca^{2+}]_i$. 100 µM RX871024 elicited a strong increase in secretion when the islets were kept at 5 mM glucose. This effect lasted for a few minutes and was only transiently changed when glucose was increased to 10 mM. Likewise, 500 µM efaroxan stimulated secretion in the presence of 5 mM glucose. In contrast to the stimulation with RX871024, this stimulatory effect of efaroxan was sustained and further augmented when the glucose concentration was increased to 10 mM. When the SUR1 KO mice islets were tested, 10 µM RX871024 was virtually without effect, whereas 100 µM had clear stimulatory effect in the presence of both glucose concentrations. Efaroxan on the other hand, at a glucose-selective (100 µM) or non-selective concentration (500 µM) was completely ineffective in stimulating insulin release in islets that lack K_{ATP} channels. Conclusion: Within a certain concentration range, both imidazoline compounds exert a glucose-selective insulinotropic effect on wild-type mouse islets. However, only RX871024 has a K_{ATP} channel-independent effect on secretion. This effect is therefore not responsible for the glucose-selectivity of these imidazolines.

[1]Institute of Pharmacology and Toxicology, Technical University of Braunschweig, Germany
[2] Baylor College of Medicine, Houston, TX, USA

219

DRUG-INDUCED DESENSITIZATION OF INSULIN SECRETION

Hatlapatka K[1], Wienbergen A[1], Jörns A[2] and Rustenbeck I[1]

Desensitization of insulin secretion is a reversible state of decreased secretory responsiveness of the endocrine pancreas after prolonged exposure to nutrient or non-nutrient stimuli. The underlying mechanisms are poorly understood inspite of their obvious relevance. Mouse islets were cultured for 20 h in RPMI medium containing tolbutamide or the imidazoline efaroxan. Secretion was measured by perifusion and ELISA. K_{ATP} channel activity was measured by patch clamping, the cytosolic calcium concentration ($[Ca^{2+}]_i$) by microfluorometry. The insulin granule content was assessed by electron microscopy or by microfluorometry of EGFP-insulin fusion proteins. 500 µM tolbutamide was about equieffective with 100 µM efaroxan to block K_{ATP} channels and to depolarize the plasma membrane. An overnight culture in RPMI 1640 (5 mM glucose) containing either 500 µM tolbutamide or 100 µM efaroxan led to a strongly diminished secretory response upon re-exposure to either drug. When the desensitization was followed by a 4 h culture in RPMI alone, an overshooting recovery of secretion resulted upon re-exposure to efaroxan, but only a partial recovery upon re-exposure to tolbutamide. A stimulatory glucose concentration (10 mM) during the 20 h culture period did not prevent the drug-induced desensitization. The pattern of $[Ca^{2+}]_i$ response upon exposure to tolbutamide or efaroxan was not altered during desensitization or recovery. Tolbutamide desensitization degranulated B-cells within islets more strongly than efaroxan desensitization. The recovery of the granule content was more extensive after tolbutamide desensitization. The use of MIN-6 cells permitted a quantitative assessment of the degranulation and regranulation process. Tolbutamide and efaroxan reduced the granule content to 46 % or 54 % of control respectively. After 4 h recovery the granule content had risen to 77 % or 72 % respectively. Conclusion: Drug-induced desensitization is not due to an impaired $[Ca^{2+}]_i$ signalling in B-cells. The responsiveness of the exocytotic machinery seems to be reduced after sulfonylurea desensitization and enhanced after imidazoline desensitization as evidenced by the discrepancies between granule content and secretion rate.

[1] Institute of Pharmacology. Technical University of Braunschweig, Germany
[2] Institute of Anatomy, Hannover Medical School, Hannover, Germany

220

IDENTIFICATION OF EIGHT HIF-3α SPLICE VARIANTS

M. Heidbreder, O. Jöhren, R. Depping, P. Dominiak and A. Dendorfer

The role of hypoxia-inducible factors (HIF) 1α and 2α in cellular response to hypoxia as well as in the progression of various neoplasms is well established, whereas little is known about the highly homologous HIF-3α. We have described the transcriptional regulation of this subtype during hypoxia and metabolic derangements, but its functional characterization is hampered by the occurrence of various splice variants which differ in their DNA binding elements and protein-protein interaction motifs. Based on predicted mRNA structures, we established a library of splice variants of HIF-3a expressed in OVCAR-3 cells by application of a 'rapid amplification of cDNA-ends polymerase chain reaction' (RACE-PCR) assay. Eight HIF-3α variants (HIF-3α V1-V8) were cloned and sequenced. Considerable variability among the variants was noted at the 5'-terminus with 3 truncated versions of the first exon and a variable inclusion of the 3rd exon. Four variants were truncated at exons 8 or 10, resulting in deletions of putative oxygen-dependent degradation and respective transactivation domains. Transient overexpression of OVCAR-3 cells with the splice variants in pcDNA3.1 expression vectors resulted in accumulation of proteins with the predicted molecular weights as observed by immunoblotting. Immunofluorescent cell labelling revealed a predominant location of all splice variants in cell nuclei with exception of HIF-3α V8 that was confined to the cytoplasm. Notably, hypoxia was sufficient to increase the protein amounts of all examined splice variants indicating an oxygen-dependent regulation. Broadening the knowledge about HIF-3α and especially its alternatively spliced variants may ultimately result in developing pharmacological strategies of fighting cancer by targeting HIF-3α.

Institute of experimental and clinical Pharmacology and Toxicology, University Hospital Schleswig-Holstein, Campus Luebeck
23538 Luebeck, Germany

221

GLUCOCORTICOIDS STIMULATE HIF-3α GENE EXPRESSION IN HIPPOCAMPAL NEURONS.

O. Jöhren, M. Heidbreder, P. Dominiak, A. Dendorfer

Adrenal glucocorticoids play an essential role in the cellular adaptation to metabolic challenges and are, as main effectors of the hypothalamic-pituitary-adrenal axis (HPA), part of the stress response assuring essential energy needs. A close connection between hypoxic pathways as represented by hypoxia-inducible factors (HIFs) and the HPA axis exists at the cellular level. Recently, we have shown the up-regulation of neuronal HIF-3α as a response to in vivo hypoglycemia and glucoprivation. Therefore, the involvement of the neuronal HIF system in metabolic disturbances as for instance caused by diabetes was suggested. To address a possible influence of glucocorticoids on the HIF system we analyzed the effect of corticosterone, aldosterone and dexamethasone on the expression of HIF-3α in immortalized mouse HT-22 hippocampal neurons. Using quantitative real-time RT-PCR (qRT-PCR), we found sufficient amounts of mRNA encoding both receptors for glucocorticoids expressed in HT-22 cells, the high affinity mineralocorticoid (MR) and the low affinity glucocorticoid receptor (GR). Treatment of HT-22 cells with corticosterone for 24 h increased HIF-3α mRNA levels dose-dependently. This effect was confirmed using the GR selective agonist dexamethasone (10 nM) while the MR selective agonist aldosterone (10 nM) showed no effects. Accordingly, siRNA knockdown of GR but not of MR abolished the effect of corticosterone on the mRNA levels of HIF-3α. Our results show a GR-mediated stimulation of HIF-3α expression at the mRNA level in hippocampal neurons and suggest a role of neuronal HIF-3α in cellular responses to stress.

Institute of Experimental and Clinical Pharmacology and Toxicology, University Clinic Schleswig-Holstein, Campus Lübeck, D-23538 Lübeck, Germany.

222

ACTIVATION OF AML DERIVED KG-1 CELLS BY IL-18 INDUCED T-BET

M. Bachmann, C. Dragoi, J. Pfeilschifter, and H. Mühl

The leukemic cell line KG-1 was isolated from a patient with acute myeloid leukemia (AML) and is regarded a cellular model of human dendritic cell (DC) progenitors. The Th1 cytokine interleukin (IL)-18 has been shown to induce maturation of these cells towards a dendritic phenotype and moreover is able to mediate interferon (IFN)-γ production in this model. Since T-bet is considered to be of paramount importance for dendritic cell function, effects of IL-18 on this transcription factor have been investigated in the current study. Here we demonstrate that activation of KG-1 cells by IL-18 induces T-bet mRNA and protein within 4h-6h of incubation. This hitherto unrecognized function of IL-18 was suppressed by inhibition of p38 MAP kinase activity and nuclear factor-κB function. Blockage of translation by cycloheximide, usage of neutralizing antibodies, and the inability of IFNγ to mediate significant p38 MAP kinase activation in KG-1 cells clearly revealed that activation of T-bet was not via autocrine IFNγ. T-bet function was evaluated by siRNA technology. Notably, specific suppression of T-bet induction impaired secretion of IFNγ by KG-1 cells under the influence of IL-18. Therapeutic application of IL-18 has the potential to profoundly impact on the biology of AML pre-dendritic cells such as KG-1 cells. Under these conditions activation of T-bet may play a key role in processes that have the potential to correct the Th1 deficiency associated with leukemia-mediated immunosuppression.

pharmazentrum frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

223

MOLECULAR MECHANISMS OF IL-8 INDUCTION BY *BORRELIA BURGDORFERI* IN HUMAN MACROPHAGE-LIKE THP-1 CELLS

C. D. Sadik[1], K. P. Hunfeld[2], P. Kraiczy[2], M. Bachmann[1], W. Eberhardt[1], V. Brade[2], J. Pfeilschifter[1], H. Mühl[1]

Lyme borreliosis is a multiorgan infection caused by spirochetes of the *Borrelia burgdorferi sensu lato* complex. In some patients *B. burgdorferi* infection can cause chronic arthritis (so-called Lyme arthritis) that is associated with elevated levels of the pro-inflammatory cytokine IL-8 as detectable in the synovial fluid of patients. Since IL-8 may play a key role in the pathogenesis of Lyme arthritis, we sought to investigate mechanisms of IL-8 induction in TPA-differentiated macrophage-like THP-1 (mTHP-1) cells under the influence of ultrasound-inactivated lysates of *B. burgdorferi* species. Non-stimulated mTHP-1 cells constitutively release IL-8 mainly due to AP-1 activation by TPA. AP-1 was not further enhanced by *Borrelia* lysates. All three isolates tested dose- and time-dependently amplified IL-8 on mRNA and protein level. *Borrelia* lysates activated the NF-κB pathway as detected by EMSA analysis. IL-8 was suppressed by inhibition of NF-κB underscoring the relevance of this transcription factor in IL-8 production mediated by *Borrelia* lysates. *Borrelia* lysates did not enhance activity of the MAP kinases JNK, ERK, and p38 in mTHP-1, though JNK and ERK activity was a prerequisite for IL-8 secretion. LPS did not induce IL-8 in this experimental setting. Therefore, IL-8 induction is unlikely to be caused by LPS contaminations of the *Borrelia* lysate preparations. Pronounced neutrophilic infiltration as observed in joints of patients with Lyme arthritis indicate that elevated levels of IL-8 may play a key role in the pathogenesis of arthritic manifestations associated with *B. burgdorferi*. Interestingly, neutralization of the functional IL-8 homologue MIP-2 ameliorated symptoms of disease in rat streptococcal cell wall-induced arthritis. Data presented herein indicate that in particular NF-κB drives IL-8 in response *Borrelia* lysates. In addition, the importance of this transcription factor as a pharmacological target for the treatment of inflammatory syndromes associated with chronic bacterial infections is strongly emphasized.

[1]pharmazentrum frankfurt and [2]Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main

224

REGULATORY POTENTIAL OF THE NADPH OXIDASE SUBUNIT P47PHOX IN TLR SIGNALLING – MOLECULAR SWITCH FOR PATHOLOGICAL IMMUNE RESPONSES?

C. Richter, J. Will, M. Herrero San Juan, R. Brandes, E. Ramos-Lopez, J. Pfeilschifter, H.H. Radeke

The innate immunity as the first defence system against invading pathogens is activated by a variety of mechanisms and recognition systems. Dendritic cells which function as "sentinels" at the interface between the innate and the adaptive immunity are predestined to capture and process antigens. The recognition of such pathogens takes place for example by Toll-like receptors (TLRs) that recognize pathogen-associated molecular pattern (PAMP) and transmit after their activation the production of pro- and anti-inflammatory cytokines. There are specific shunts in the signalling pathway downstream these TLRs that decide if an acute or a chronic inflammation will develop.

A potential role of p47phox in the regulation of TLR downstream signalling was investigated. We hypothesised a negative regulation of the TLR-mediated cytokine production by p47phox, an organizing protein of the NADPH oxidase. In spleen cells from p47phox knockout mice we found an enhanced expression of interleukin-12 after stimulation with TLR ligands compared to the wildtype mice (c57BL/6). In addition we discovered a difference between TLR4 and TLR9 signalling, since CpG ODN2216 stimulation resulted in significant higher amounts of interleukin-12 compared to LPS (TLR4 ligand) stimulation in p47phox knockout cells. In control experiments spleen cells from gp91phox deficient mice that are not able to assemble the NADPH oxidase displayed no differences in IL-12 production compared to control mice. Further we found that type I interferon (alpha) is exclusively expressed after stimulation with TLR9 ligand CpG in all three groups of mice which hints to a role of pDCs. In further experiments we want to knock down p47phox in dendritic cells via shRNA transduced by lentiviral vectors. The first results concerning transfection efficiency are very promising.

Dr. Hans Schleussner-Foundation Immune Pharmacology, pharmazentrum frankfurt, Clinic of the Goethe University, Frankfurt am Main

225

SEQUENTIAL EXPRESSION OF INTERLEUKIN-23 mRNA AND PROTEIN IN DENDRITIC CELLS MEDIATED BY CHROMATIN REMODELING AND SIGNAL TRANSDUCTION

J. Will, M. Herrero San Juan, C. Richter, J. M. Pfeilschifter and H. H. Radeke

Due to their role as antigen-presenting cells, dendritic cells have to regulate an adequate cytokine secretion dependent on different stimuli. We investigated the IL-23 expression in an immature Langerhans cell line (iLC) after stimulation with a TLR4 ligand and found contrary effects of mediators that either influence chromatin structure or MAP kinase signalling on LPS-induced IL-23 expression. On the one hand the histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and SAHA (suberoylanilid hydroxamic acid) were able to down-regulate the bioactive heterodimer, but the two IL-23 subunits, p19 and p40, were affected differentially. Both TSA and SAHA exerted a more inhibitory effect on p40. These effects were substantial on mRNA and on protein level. Interestingly, the p38 inhibitor SB202190, which exclusively inhibits the p38 alpha and beta isoform, increased LPS-induced p19 and p40 mRNA and protein monomer expression as well as IL-23 secretion in iLC. All substances used showed time- and dose dependent effects on IL-23 monomer and heterodimer expression. In summary we found a sequential regulation of IL-23 and its subunits on chromatin remodelling, mRNA, protein monomer and bioactive heterodimer expression by HDAC and p38 inhibitors. In contrast to the more general opinion which holds that HDACs are repressors of gene expression and p38 kinase takes part only in inflammatory conditions, our data propose an anti-inflammatory role for p38 MAP kinase and a pro-inflammatory function of histone deacetylation.

Dr. Hans Schleussner-Foundation Immune Pharmacology, pharmazentrum frankfurt, Clinic of the Goethe University, Frankfurt am Main

226

LISTERIA MONOCYTOGENES EXPRESSES TWO STRUCTURAL DIFFERENT LIPOTEICHOIC ACIDS (LTA) WITH UNCONVENTIONAL BIOLOGICAL PROPERTIES

C. Hermann, O. Dehus, S Meier, G. Stübs, [1]A.J. Ulmer, [2]T. Hartung, A. Wendel

Listeria monocytogenes (Lm) multiply and spread intracellularly in host cells including monocytes and liver cells. Lipoteichoic acid (LTA) is a major Gram-positive surface component of Lm which elicits an immune response. After butanol extraction of Lm two different LTAs were separated as indicated by their distinct phosphate profile. Structural analysis revealed that both LTA1 and LTA2 differ in the structure of their lipid anchor. They differ from other LTAs, e.g. the one from S. aureus (Sa) in the disaccharide moiety and the degree of lipid branching. Stimulation of cytokine release from human whole blood revealed that both LTA induce a broad cytokine spectrum and that the immunostimulatory potency of LTA1 was comparable to that of LTAs from Sa or Streptococcus pneumoniae (Sp), while the immunostimulatory potency of LTA2 was weaker. Cytokine induction by both Lm LTAs was TLR2 dependent as confirmed with cells from TLR2 knock out mice. In contrast to LTA of Sa or Sp, LTA1 and LTA2 of Lm induced the proliferation of mouse spleen cells. For Sa LTA, the induction of the lectin-binding pathway of complement activation via binding of L-ficolin followed by cleavage of C4 was shown. When LTA1 or LTA2 was incubated with L-ficolin, the L-ficolin binding activity and C4 cleavage by LTA1 was comparable to Sa LTA, while LTA2 showed only weak L-ficolin binding and failed to induce C4 cleavage. Analysis of the effect of the culture conditions reflecting either the extracellular or intracellular growth state of Lm showed that the amount of LTA1 and LTA2 of Lm varies, with LTA1 being predominantly expressed in the extracellular status.

This indicates that Lm, depending on the culture conditions, expresses two different LTAs which contain unique structures. The possible influence of the ratio of LTA1 and LTA2 on the virulence of Lm will have decisive bearings on therapeutic strategies.

Biochemical Pharmacology, University of Konstanz, Germany; [1]Research Center Borstel, Germany; [2]JRC, Ispra, Italy

227

DIFFERENTIAL SENSITIZATION OF HEPATOCYTES BY INHIBITORS OF HISTONE DEACETYLASES AGAINST DEATH RECEPTOR-INDUCED APOPTOSIS

M. Weiller, G. Dünstl, G. Künstle and A. Wendel

Background: Preclinical and clinical studies suggest that inhibition of histone/protein deacetylation by targeting histone deacetylases (HDACs) represents a novel approach in cancer therapy [1]. However, only recently it became evident that HDAC inhibitors by themselves do not only induce apoptosis but also render cells sensitive to death receptor agonists, i.e. TNF, TRAIL, and CD95 ligand (CD95L), respectively [2, 3]. For a comprehensive understanding of the pharmacodynamic properties of HDAC inhibitors (HDIs) it is madatory to elucidate and profile these sensitizing effects.

Results: Apicidin, M344 (N-hydroxy-7-(-4-dimethylaminobenzol)aminoheptanamide), CBHA (m-carboxycinnamic acid bis-hydroxamide) and valproic acid sensitized HepG2 and primary mouse liver cell cultures towards CD95L- and TRAIL-triggered apoptosis, but not towards TNFα. A similar sensitization was found in all cell types investigated with the following potency: apicidin > M344 ≥ CBHA >> VPA. Primary human hepatocytes were similarly sensitized by VPA towards CD95 as HepG2 cells. In the intact organ, i.e. in the isolated perfused mouse liver, apicidin sensitized towards CD95. Transfection of caspase-8 or addition of recombinant human caspase-8 to a cell free system resulted in an enhanced caspase-3/-7 activation for HepG2 cells treated with HDIs. In two models of the intrinsic pathway, i.e. the apoptosome model (Cyt c, dATP supplementation), or in HepG2 cells irradiated with UV light, HDIs had no significant influence. Western blot analysis showed that HDIs downregulated the anti-apoptotic proteins cFLIP and XIAP, while they upregulated the pro-apoptotic factor FADD. These findings indicate that in HepG2 cells HDIs modulate the extrinsic signaling pathway by enhancing the activation of caspase-3/-7 via caspase-8.

Conclusions: This study extends the biochemical and pharmacological basis for HDI therapy and provide an alert for clinical use in patients with a history of liver disease in which the CD95 or TRAIL system might be pre-activated.

1. McLaughlin F et al. Histone deacetylase inhibitors open new doors in cancer therapy. Biochem Pharmacol 2004, 68:1139-44

2. Chopin V et al. Synergistic induction of apoptosis in breast cancer cells by cotreatment with butyrate and TNF-alpha, TRAIL, or anti-Fas agonist antibody involves enhancement of death receptors' signaling and requires P21(waf1). Exp Cell Res. 2004, 298:560-73.

3. Nakata S et al. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene. 2004; 23:6261-71.

Biochemical Pharmacology, Faculty of Biology, University of Konstanz, Konstanz, Germany

228

SENSITIZATION OF HEPATOCYTES BY METHYLTRANSFERASE-INHIBITOR 5-AZACYTIDINE AGAINST CYTOKINE-MEDIATED CELL DEATH

Weiland T, Wendel A, Künstle G

Background: 5-Azacytidine (5-aza-CR) was developed as an antineoplastic agent long before its inhibitory activity on DNA methyltransferases and thus its ability to reverse aberrant gene silencing became evident. Today, 5-aza-CR is the first DNA hypomethylating agent approved for treatment of myelodysplastic syndromes and represents a promising drug for epigenetic cancer therapy. However, 5-aza-CR is contraindicated for patients with pre-existing liver diseases due to adverse liver effects for yet unknown reasons.

Aims: For a comprehensive understanding of the pharmacodynamic properties of 5-aza-CR it is mandatory to elucidate and profile this sensitizing effect

Results: In a time- and concentration-dependent manner, hepatocytes, i.e. primary murine and human hepatocytes and HepG2 cells, became highly sensitive towards death receptor-mediated cell death (induced either by CD95L, TRAIL or TNF) when pre-treated with sub-toxic concentrations of 5-aza-CR. 5-aza-CR-enabled hepatocyte cell death was characterized by classical hallmarks of apoptosis such as membrane blebbing, chromatin condensation and exposure of phosphatidyl serine on the outer membrane. Furthermore, we demonstrated an upregulation of pro-apoptotic regulator proteins p53 and BAX in a time- and concentration-dependent manner in 5-aza-CR treated HepG2 cells. UV radiation is a DNA-damaging stimulus that activates a p53/Bax-dependent apoptotic response in this system. We also detected an increased cytotoxicity as well as caspase-activity after irradiation with UV radiation in the presence of 5-azac-CR. Moreover, in the presence of 5-aza-CR, activation of downstream caspases was markedly enhanced when the extrinsic pathway of apoptosis was activated by heterologous and transient expression of caspase-8. In contrast, artificial activation of the intrinsic pathway of apoptosis within S-100 cytosolic fractions of HepG2 via dATP/Cytochrom C failed to induce upregulation of caspase activity.

Conclusions: This study reveals that 5-aza-CR sensitizes hepatocytes against death receptor agonists. As a mechanistic rationale, we provide evidence for a p53/Bax dependent sensitizing mechanism acting in-between the death inducing signaling complex (DISC) and above the mitochondrial level. In summary, our findings offer a mechanistic explanation for the adverse hepatotoxic properties of 5-aza-CR observed in patients with pre-existing liver disorders.

Biochemical Pharmacology, Faculty of Biology, University of Konstanz, Konstanz, Germany

229

THE SERINE PROTEASE PLASMIN TRIGGERS PROINFLAMMATORY GENE INDUCTION IN HUMAN MACROPHAGES – CHARACTERIZATION OF SIGNALING PATHWAYS

Q. Li, Y. Laumonnier, T. Syrovets, Th. Simmet

Macrophages are key regulators of the inflammatory response. Contact activation in inflamed tissues induces generation of the serine protease plasmin. We have previously demonstrated that plasmin activates human monocyte signaling via the annexin A2 heterotetramer, which is composed of annexin A2 and S100A10. Here we demonstrate that macrophages express high amounts of the annexin A2 heterotetramer as analyzed by Western immunoblot and flow cytometry. Activation of macrophages by plasmin leads to cleavage of the annexin A2 subunit of the receptor complex, followed by activation of Janus kinase JAK1/TYK2 signaling. This triggers STAT3 activation, Akt-dependent NF-κB stimulation, and phosphorylation of the mitogen-activated protein kinase p38 and the extracellular signal-regulated kinase (ERK)1/2. By activating these signaling pathways, plasmin induces nuclear translocation and DNA binding of STAT3 and p65 transcription factors as shown by DNA binding assays, followed by increased expression and release of the proinflammatory cytokines TNF-α and IL-6. Pharmacological inhibitors of JAK (AG490), p38 (SB203580) and NF-κB (AKβBA) revealed that these signaling pathways are indispensable for the plasmin-mediated induction of both, TNF-α and IL-6 genes. By contrast, the ERK1/2 activation is essential only for the expression of the IL-6 gene. At the molecular level, the activation of macrophages depends on the proteolytic activity of plasmin as shown by use of the inactivated plasmin catalytically blocked by covalent reaction with *D*-Val-Phe-Lys chloromethyl ketone. Plasmin activates macrophages via a receptor complex composed at least of annexin A2 and S100A10 because down-regulation of the expression of either the annexin A2 or S100A10 subunit by antisense oligodeoxynucleotides abolished the plasmin-induced expression of proinflammatory cytokines. In conclusion, our data show that the serine protease plasmin is a potent proinflammatory activator of human macrophages acting via the annexin A2 heterotetramer and multiple downstream signaling pathways. Thus, plasmin generated at the sites of inflammation will profoundly affect macrophage function.

Institute of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, D-89081 Ulm, Germany

230

THROMBIN-INDUCED EXPRESSION OF ENDOTHELIAL CX3CL1 AMPLIFIES MONOCYTIC/ENDOTHELIAL CELL CROSS-TALK AND SUBSEQUENT CHEMOKINE INDUCTION THROUGH ACTIVATION OF NF-κB SIGNALING

Y. Laumonnier, M. Popovic, L. Burysek, T. Syrovets, Th. Simmet

CX3CL1/fractalkine is a chemokine with chemotactic and adhesive properties. In endothelial cells, its expression is upregulated by proinflammatory cytokines such as interleukin (IL)-1 or tumor necrosis factor (TNF)-α. Here we show that the serine protease thrombin is also able to increase the expression of membrane-anchored fractalkine in human umbilical vein endothelial cells (HUVEC) in a concentration- and time-dependent manner both at the mRNA and the protein level as shown by semiquantitative reverse transcription polymerase chain reaction, Western immunoblotting, and flow cytometry. This induction is dependent on the activation of the protease-activated receptor (PAR) 1 because a PAR1-specific activating peptide (PAR1-AP), but not PAR3- or PAR4-AP, is mimicking the effect of thrombin. At the molecular level, thrombin activates nuclear factor (NF)-κB signaling as shown by the nuclear translocation of NF-κB using electromobility shift assays. Additionally, pharmacological inhibition of the NF-κB pathway by the natural compound acetyl-11-keto-β-boswellic acid (AKβBA) or transient overexpression of a transdominant negative form of IκBα (IκBα superrepressor) impairs the thrombin-induced upregulation of fractalkine confirming the central role of the NF-κB signaling pathway in this regulation. Functionally, cocultivation of the monocytic cell line Mono-Mac-6 with thrombin-pretreated HUVEC triggers an enhanced fractalkine-dependent release of the monocyte chemoattractant protein (MCP)-1; the released MCP-1 elicits monocyte migration as shown with supernatants from thrombin-prestimulated HUVEC cocultivated with Mono-Mac-6, an effect that was abolished by antibodies against MCP-1. Thus, the thrombin-induced monocytic/endothelial cell cross-talk mediated by the increased fractalkine expression leads to enhanced MCP-1 chemokine generation that will finally contribute to the recruitment of monocytes into atherosclerotic lesions.

Institute of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, Ulm, Germany

231

TARGETING NF-κB SELECTIVELY IN KUPFFER CELLS WITH NF-κB DECOY OLIGODEOXYNUCLEOTIDES IS A PROMISING TOOL FOR INFLUENCING INFLAMMATORY LIVER SIGNALING

F. Hoffmann[1], J. Zillies[1], S. Zahler[1], C. Coester[1], G. Winter[1], A. M. Vollmar[1]

The liver-resident Kupffer cells play an important role in inflammatory liver signaling. Exposure to pathogens like bacteria and viruses, as well as diverse hepatic disorders such as ischemia/reperfusion injury and alcoholic liver disease, lead to an activation of the redox-sensitive transcription factor Nuclear Factor κB (NF-κB). As a consequence, proinflammatory chemokines and cytokines like TNF-α are produced and released. Whereas exceeding NF-κB activation in Kupffer cells is associated with liver inflammation, NF-κB in hepatocytes acts in a protective manner by the transcription of antiapoptotic genes. Thus, our aim was to develop a specific carrier in order to deliver NF-κB binding decoy oligodeoxynucleotides (ODN) selectively to the resident liver macrophages. To this end, solid gelatin nanoparticles (NPs) (approximately 260 nm diameter) were loaded with 20 μM NF-κB decoy oligodeoxynucleotides.

For the proof of principle, two different rat animal models of well known NF-κB activating incidents were chosen, lipopolysaccharid (LPS) challenge and hepatic ischemia/reperfusion. By labeling the oligonucleotides with an Alexa® 488 fluorescent dye, we were able to show by confocal laser scanning microscopy that decoy ODN loaded onto gelatin nanoparticles (decoy

NPs) are exclusively taken up by Kupffer cells after intraportal application. Injection of decoy NPs 15 minutes prior to the administration of LPS completely abolished induction of NF-κB as determined by electromobility shift assay (EMSA). Moreover, real-time RT-PCR analysis revealed that TNF-α mRNA expression was diminished when the NF-κB binding ODN was used in comparison to a non-binding random (scrambled) sequence. Intraportal application of decoy NPs 15 minutes before 1h of partial warm ischemia and 2h of warm reperfusion, significantly reduced the resulting increase of NF-κB activity (EMSA) as well as the subsequent TNF-α mRNA expression (real-time RT-PCR). In summary, we demonstrated that NF-κB decoy nanoparticles are an appropriate tool for inhibiting NF-κB selectively in Kupffer cells.
[1]Department of Pharmacy, Center of Drug Research, University of Munich, Germany
supported by the DFG (FOR-440)

232

EXPRESSION OF THE CANNABINOID RECEPTOR TYPE I IN T CELLS AND ITS REGULATION BY CANNABINOIDS AND INTERLEUKIN-4

J Kraus, C Börner, A Bedini and V Höllt

The cannabinoid receptor type I (CB1), formerly designated as the "central" cannabinoid receptor, is expressed abundantly in neuronal cells. In addition, CB1 is also expressed at low levels in cells of the immune system. Although most of the effects of cannabinoids in immune cells are mediated by CB2, the formerly designated "peripheral" cannabinoid receptor, it is becoming increasingly evident that important anti-inflammatory effects of cannabinoids in immune cells are mediated by CB1. Here we demonstrate that cannabinoids themselves, and the anti-inflammatory cytokine interleukin-4 (IL-4) drastically induce expression of CB1 in Jurkat, CEM and primary T cells. Upon stimulation of the T cells, cannabinoids first induce expression and release of IL-4, as demonstrated by cannabinoid-mediated upregulation of IL-4 mRNA and by blockade of the effect by an IL-4 receptor antagonist. Downstream effects of IL-4 are typically mediated by the transcription factors STAT6 and GATA3. Using decoy oligonucleotides, which disrupt thetranscriptional activity of these factors, we show that the IL-4-mediated upregulation of CB1 is mediated by STAT6, but not GATA3. A promoter analysis of the CB1 gene revealed an IL-4-responsive sequence located between -3086/-2490 with respect to the transcriptional start site. Transfection studies and experiments using decoy oligonucleotides demonstrated interaction of STAT6 with this region. Further promoter analysis revealed various regulatory elements, which could participate in the basal regulation of the gene's expression. In particular, the rather low basal transcriptional rate of CB1 in Jurkat T cells might be explained by two elements with strong negative impact on the basal transcription, which are located upstream of nt -2490 and between nt -1653 and nt -788. Taken together, these data demonstrate fine-regulatedbasal and inducible expression of the CB1 gene in T lymphocytes, which may be of importance for anti-inflammatory actions of cannabinoids.

Inst. für Pharmakologie und Toxikologie, Universität Magdeburg, Leipzigerstr. 44, 39120 Magdeburg

233

ACTIVATION OF THE T CELL RECEPTOR INDUCES μ-OPIOID RECEPTOR TRANSCRIPTION

C. Börner, J. Kraus and V. Höllt

Several studies showed interactions of the immune and the opioid system on multiple levels. On the one hand, morphine alters a number of immune parameters, like immune cell activity and cytokine production of T cells. On the other hand, several cytokines, like tumor necrosis factor alpha and interleukin-4, induce μ-opioid receptor transcripts in various types of immune cells, which do not express this receptor under resting conditions. Here, we show that the combined activation of the T cell receptor (TCR) by CD3 and CD28, or CD3 alone, leads to an induction of μ-opioid receptor transcripts in Jurkat T cells. The induction started between 3 h and 8 h after treatment and was maximal after 24 h. The combined activation of the TCR by CD3 and CD28 activates several signaling pathways, including the MAPK pathway and the activation of Phospholipase Cγ and PKC. This leads to the activation of different transcription factors, like AP-1, NFkB and NF-AT. Using decoy oligonucleotides we investigated the role of these transcription factors in the induction of μ-opioid receptors by TCR activation showing that all three, AP-1, NFkB and NF-AT, are involved in this process. We identified AP-1 recognition elements on the promoter of the human μ-opioid receptor gene at nt-2388 and nt-1434 and NFkB recognition elements at nt-2174, nt-557 and nt-207. Promoter analysis futher revealed putative recognition elements for NF-AT at nt-2253, nt-1541, nt-1062, nt-786 and nt-487, but the physiological functions of these sites remain to be identified. Taken together, this study shows an important link between TCR activation which is a central aspect of the immune response and μ-opioid receptor expression.

Institut für Pharmakologie und Toxikologie, Universität Magdeburg, Leipzigerstr. 44, 39120 Magdeburg

234

DENDRITIC CELL MIGRATION THROUGH SKIN IS MODULATED VIA THE HISTAMIN H4 RECEPTOR. IMPLICATIONS FOR AN IMMUNOMODULATORY ROLE OF HISTAMINE.

W. Bäumer[1], S. Wendorff[4], R. Gutzmer[2], T. Werfel[2] and M. Kietzmann[1]

Dendritic cells (DC) are the major antigen presenting cells and play a key role in adaptive immunity as they are able to activate naive T cells. It was recently described, that the histamine H4 receptor (H4R) is present on human monocyte-derived DC and that chemotaxis and Th1-Th2 polarisation is mediated by this receptor (Gutzmer et al., 2005, *J.Immunol.* 174, 5224-5232). However, the distribution of histamine receptors on murine DC has not been studied, yet. Therefore, it was of interest, which histamine receptors are expressed on murine bone marrow (BM) derived DC and which DC function is modulated by histamine. The mRNA of the H1R, H2R and H4R could be detected in murine BM-derived DC, while mRNA of the H3R was found to be low or undetectable. IL-10 was elevated after LPS stimulation. Histamine enhanced the elevation of IL-10 significantly at 10^{-3} mol/l. The H1R antagonist diphenhydramine and the H4R antagonist JNJ 7777120 reduced the IL-10 secretion. IL-12 was also elevated after LPS stimulation. Histamine dose dependently reduced the IL-12 concentration in supernatants of LPS stimulated DC (significantly at 10^{-3} mol/l). However, neither diphenhydramine nor JNJ 7777120 restored the IL-12 secretion in histamine and LPS treated DC, indicating that this effect is mediated via the H2 receptor. Histamine as well as the H4R agonist clobenpropit induced enhanced chemotaxis in the skin dendritic cell migration assay. The enhanced chemotaxis was blocked by JNJ 777120. These finding was confirmed by in vitro boyden chamber experiments. Referring to DC migration, blocking the H4 receptor on inflammatory cells might be a promising anti-inflammatory, immunomodulatory strategy.

[1]Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, [2]Department of Dermatology and Allergology, Hannover Medical University, Hannover, Germany

235

IDENTIFICATION OF THE AP-1 DIMER REPERTOIRE THAT REGULATES IL-8 TRANSCRIPTION

Kettner-Buhrow D.[1], Quante T.[1], Bakiri L.[2],Wagner E.F.[2], Hoffmann E.[1], and Kracht M[1].

The transcription factor activator protein 1 (AP-1) is regulated at multiple levels. Moreover, putative AP-1 binding sites are found in a large number of genes. AP-1 is not a single protein but a homo- or heterodimer composed of members of the Jun (i.e. c-Jun, JunB, and JunD) and Fos ((i.e. c-Fos, FosB, Fos-related antigen (Fra-1, Fra-2)) protein families. Fos and Jun proteins can also dimerize to many other basic leucine zipper proteins such as ATF, C/EBP, Maf, and NF-E2, increasing the number of the potential AP-1 factors that bind to a given AP-1 site. We have previously characterized an AP-1 site in the highly inducible chemokine gene IL-8 and have shown by chromatin immunoprecipation that various AP-1 proteins can bind to this site in response to IL-1. We therefore set up experiments to identify the functional impact of AP-1 combinations on IL-8 transcription. siRNAs directed against c-Jun, JunB, JunD, c-Fos, Fra-1 and ATF-2 were all effective in inhibition of a minimal IL-8 promoter-driven reporter gene, albeit to variable degrees. Double-knock downs of c-Jun/ATF-2, c-Jun/JunB and JunB/ATF-2 showed stronger suppression of IL-8 transcription in response to IL-1 or MEKK1. To more precisely reveal the nature of the AP-1 dimers that activated IL-8 transcription we ectopically expressed 11 different AP-1 dimers as single chain proteins, whereby to subunits were fused by a flexible glycine-linker. Dimers composed of c-Jun-ATF-2, c-Jun-Fra-1, c-Jun-cFos, JunB-c-Fos activated the IL-8 promoter, whereas c-Jun-c-Jun homodimers and JunD containing dimers were largely inactive. Collectively, these loss and gain of function experiments underscore the enormous combinatorial potential of AP-1. As exemplified for IL-8, AP-1 dimers serve as signal integrators to enable activation of IL-8 by numerous proinflammatory inputs and, at the same time, provide an effector mechanism to fine-tune chemokine transcription to appropriate levels.

[1] Institute of Pharmacology, Medical School Hannover, Carl-Neuberg Strasse 1, D-30625 Hannover, Germany
[2] Research Institute of Molecular Pathology, A-1030 Vienna, Austria

236

JAM-C AND ITS INFLUENCE ON THE PATHOGENESIS OF TYPE 1 DIABETES

S. Christen, E. Hintermann, M. Bayer and U.Christen

Type 1 diabetes (T1D) results from the autoimmune destruction of insulin-producing beta-cells in the pancreas. Recruitment of inflammatory cells, such as T-cells, B-cells and dendritic cells is prerequisite to beta-cell-injury. Such a process includes several molecular interactions between circulating and endothelial cells. The junctional adhesion molecule (JAM) family proteins JAM-B and JAM–C, are expressed on endothelial cells of high endothelial vessels and appear to be involved in leukocyte rolling, firm adhesion and transmigration. It was recently demonstrated that after blocking of JAM-C cerulein-induced pancreatitis was efficiently attenuated in mice. Early intervention with a monoclonal antibody directed against JAM-C reduced cytokine production, leukocyte influx, and hence tissue damage. In order to investigate the influence of JAM-C on trafficking and transmigration of antigen-specific, autoaggressive T-cells we used the RIP-LCMV mice as a model system. These mice express the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) as a target autoantigen specifically in the beta-cells of the islets of Langerhans and turn diabetic after LCMV infection. In such diabetic RIP-LCMV mice the expression of JAM-C is detectable around the vessels within the pancreas. Interestingly, immunohistological evaluation of pancreas sections revealed that JAM-C was expressed directly in between cellular infiltrations and beta-cells. Our data suggest that JAM-C might be involved in the final steps of trafficking and transmigration of antigen-specific autoaggressive T-cells to the islets of Langerhans and might therefore play an important role in the pathogenesis of T1D.

Pharmazentrum Frankfurt / Zentrum für Arzneimittelforschung, Entwicklung und Sicherheit (ZAFES), Klinikum der Johann Wolfgang Goethe Universität, Frankfurt am Main, Germany

237

THE CYP450 MOUSE MODEL FOR AUTOIMMUNE HEPATITIS: BREAKING OF SELF-TOLERANCE IN TRANSGENIC CYP2D6 AND WILDTYPE FVB-MICE BY VIRAL INFECTION

M. Holdener[1,2], E. Hintermann[1], E.F. Johnson[3], M.P. Manns[4], M.G. von Herrath[2] and U. Christen[1]

The etiology of autoimmune hepatitis (AIH) is poorly understood although the major autoantigen, cytochrome P450 2D6 (CYP2D6), has been identified and immunodominant epitopes mapped. Therefore, we generated an animal model for human AIH using the natural autoantigen CYP2D6. We infected transgenic mice expressing human CYP2D6 in the liver (CYP-2D6 mice) with an Adenovirus-CYP2D6 vector (Ad-2D6) to break self-tolerance. Surprisingly, upon infection with Ad-2D6 not only transgenic CYP2D6 mice but also wildtype FVB mice showed several persistent features characteristic for liver damage associated with AIH. These features included massive hepatic fibrosis, ‘fused’ liver lobules, disorganized architecture, cellular infiltrations, elevated serum aminotransferase levels, and focal to confluent necrosis. Further, all Ad-2D6-infected mice (CYP2D6 and FVB) generated high titers of anti-CYP2D6 antibodies. Epitope mapping revealed that such anti-CYP2D6 antibodies predominantly recognized the core peptide sequence AQPPRD (CYP2D6 aa265-270), which is the immunodominant linear epitope recognized by LKM-1 antibodies from AIH patients. In contrast, mice infected with a control Adenovirus expressing green fluorescence protein (Ad-GFP) did neither develop liver damage nor generated anti-CYP2D6 antibodies. Interestingly the kinetics and severity of liver damage as well as antibody formation was enhanced in wildtype FVB mice compared to transgenic CYP2D6 mice. Our data indicate that the autoimmune liver damage was reduced and delayed in transgenic CYP2D6 mice due to a certain degree of tolerance towards

human CYP2D6 compared to wildtype FVBs, which do not express the human version of the 2D6 isoenzyme. In wildtype FVB mice, due to the homology of the mouse isoenzymes of the CYP superfamily to human CYP2D6, autoimmune liver damage by Ad-2D6-infection was possibly induced via true 'molecular mimicry'.
[1]Pharmazentrum Frankfurt / Zentrum für Arzneimittelforschung, Entwicklung und Sicherheit (ZAFES), Klinikum der Johann Wolfgang Goethe Universität, Frankfurt am Main, Germany, [2]La Jolla Institute for Allergy and Immunology, San Diego, CA; [3]The Scripps Research Institute, La Jolla, CA; [4]Hannover Medical School, Hannover, Germany

238

ACTIVATION OF HEPATIC STELLATE CELLS DURING FIBROSIS: COMPARISON OF THE CYP2D6 MODEL FOR AUTOIMMUNE HEPATITS AND CCL4 INJECTION

E. Hintermann, S. Christen, M. Bayer and U. Christen

Periportal fibrosis is one of the defining parameters of autoimmune hepatitis. Only little is known about the mechanisms involved in the development of fibrosis during this severe human autoimmune disease. We used the virus induced CYP2D6 model system to investigate the activation of hepatic stellate cells (HSC) and the kinetics of fibrosis in comparison with the well established CCl4-induced fibrosis model. CYP2D6 transgenic mice express the human Cytochrome P450 2D6 under its own promotor in the liver and do not develop liver damage unless infected with an Adenovirus-CYP2D6 vector. We found that in the CYP2D6 model fibrosis, as detected by Sirius Red and anti-Collagen I IgG staining, was mostly subcapsular resulting in the fusion of individual lobules. After 10-12 weeks post-infection, weak periportal fibrosis became apparent. In contrast, in CCl4-treated mice the kinetics of extracellular matrix deposition were much quicker resulting in periportal fibrosis already after 3-4 week of CCl4 administration. At later times, fibrosis was much more pronounced in CCl4-treated mice compared to virus-infected CYP2D6 mice. However, subcapsular fibrosis was not as dominat in CCl4-treated mice. Activation of HSCs could be detected by staining for α-smooth muscle actin (αSMA) in tissue sections of both CCl4-treated mice and virus-infected CYP2D6 mice. In addition, isolation of HSCs revealed an enhanced activation status (decreased amount of oil droplets, *de novo* αSMA expression) in CCl4-treated mice and virus-infected CYP2D6 mice. Our data indicate that Adenovius-CYP2D6-infected CYP2D6 mice display subcapsular and periportal fibrosis that correlates with an activation of HSCs similar to CCl4-induced fibrosis. Thus, the CYP2D6 mouse will be a good model system to further investigate the molecular mechanisms involve in fibrotic events during autoimmune hepatitis.
Pharmazentrum Frankfurt / Zentrum für Arzneimittelforschung, Entwicklung und Sicherheit (ZAFES), Klinikum der Johann Wolfgang Goethe Universität, Frankfurt am Main, Germany

239

NEW INHIBITORS OF ANGIOGENESIS AND TUMOUR PROGRESSION BY BLOCKADE OF TGF-β –INDUCED SIGNAL TRANSDUCTION

A. Serwe[1], H. Belahmer[1], G. Erkel[1] and T. Anke[1]

TGF-β is a pleiotropic cytokine that regulates a variety of cellular processes such as proliferation, differentiation, extracellular matrix elaboration, apoptosis, wound healing, fibrosis, immunity and carcinogenesis. It has been shown to induce the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and plasminogen activator inhibitor 1 (PAI-1). The ability of tumour cells to induce new blood vessel formation from pre-existing vasculature is essential for tumour progression and metastasis. Our research aims for the identification, isolation and characterization of inhibitors of TGF-β signalling in tumour cells from fungi as a novel therapeutic strategy. Because of the central role of TGF-β in angiogenesis and tumour progression, we developed a screening system in HepG2 cells in order to identify new inhibitors of TGF-β-induced signalling from fermentation of fungi. Screening of 1100 culture fluid and mycelial extracts of basidiomycetes and imperfect fungi resulted in the isolation of trichodimerol and the new fungal metabolite HB99121-4. Trichodimerol inhibited the TGF-β-mediated reporter gene expression with an IC_{50} value of 10 µg/ml (20 µM). Western blotting with phospho-specific antibodies against Smad2 showed a dose-dependent decrease in TGF-ß-mediated induction of phosphorylation, suggesting that trichodimerol acts as an inhibitor of the upstream kinases. Microarray and real time PCR experiments revealed that trichodimerol preferentially inhibited the expression of genes related to angiogenesis and metastasis in HepG2 cells. Additionally, trichodimerol inhibited the Stat1/2- Stat3- and Stat6-dependent pathways in HepG2 cells with IC_{50} values of 7.5-10 µg/ml (15-20 µM) which was due to an inhibition of the phosphorylation of the Stat transcription factors. The new fungal metabolite HB99121-4 inhibited the TGF-β-dependent promoter activity with an IC_{50} value of 80 ng/ml (216 nM) without interfering with the phosphorylation of the Smad transcription factors. In addition, the HIF-1α-mediated signalling and the Jak/Stat pathways were also inhibited with IC_{50} values of 25 ng/ml (67.5 nM) and 20-45 ng/ml (54-121.5 nM) respectively.
[1] Dept. of Biotechnology, TU Kaiserslautern, Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern

240

DISSOCIATION OF INFLAMMATION AND AIRWAY HYPERREACTIVITY IN A MOUSE MODEL OF ASTHMA BRONCHIALE

L. Chialda[1], A. Gessner[2], K. Brune[1], A. Pahl[1]

Background: IL-4 is a major inducer of Th2 responses and plays an important role in allergic pathology. However, it is still unknown, which cell population provides the early source of IL-4 required for specific Th2 responses in vivo. To address this issue and the role of IL-4 in allergic airway inflammation and airway hyper-responsiveness (AHR) we used 4 get mice and 4 get mice backcrossed to IL-4Rα$^{-/-}$ and STAT6$^{-/-}$ mice. In these mice, IL-4-expressing cells are tagged using green fluorescent protein (GFP) and allow direct visualization of IL-4 expression at the single cell level without the need of *in vitro* restimulation.
Methods: Mice were intraperitoneally sensitized and intranasally challenged with ovalbumin. Cell populations from the bronchoalveolar lavage (BAL) fluid, lung, spleen, blood and mediastinal lymph nodes were analyzed by flow cytometry based on expression of IL-4/GFP and cell surface markers. Cells were sorted and cytospin and RT-PCR analysis of sorted cell populations were performed. Sorted populations were adoptively transferred into naive mice and lung inflammation (differential cell count of BAL and lung histology) as well as AHR was determined. The effect of immunomodulatory substances on IL-4/GFP expressing cell populations was tested.
Results: Eosinophils and basophils were important and early IL-4 producing cells during the secondary immune response, whereas Th2 cells accounted for IL-4 production later in the ongoing immune response. Immunomodulatory substances differentially affected these IL-4 expressing cell populations. IL-4Rα $^{-/-}$ and STAT6 $^{-/-}$ mice had markedly reduced airway inflammation compared with wild-type mice but alternative lung pathology with a predominance of neutrophils occured. Transfer of Th0/Th1 and subsequent ovalbumin challenge led to significant AHR compared to mock transfer even though there were no significant histological changes. Surprinsingly, mice receiving Th2 cells transfer, had less AHR than mice adoptively transferred with Th0/Th1, although perivascular and peribronchial infiltrate with polymorphonuclear cells could be observed in the former case.
Conclusion: Our data indicate that CD4+ T cells can induce AHR independent of IL-4 and IL-13, whereas the presence of Th2 cells and/or eosinophils is not a prerequisite for the development of AHR in allergic asthma. Therefore inhibition of Th2 responses and/or eosinophil depletion may be not a sufficient strategy to treat asthma.
[1]Department of Experimental, [2]Department of Microbiology, University of Erlangen-Nuremberg, Germany

241

SPIEGELMER NOX-E36 FOR THE TREATMENT OF CHRONIC INFLAMMATORY DISEASES: A NEW CHEMICAL ENTITY THAT INHIBITS CCL2/MCP-1 SIGNALLING

H.-J. Anders[2], K. Buchner[1], D. Eulberg[1], S. Klussmann[1], O. Kulkarni[2], W. Purschke[1], N. Selve[1]

Spiegelmers are oligonucleotides of non-natural chirality that can bind to a target molecule like an antibody to an antigen. Spiegelmers are identified using the SELEX process which allows identification of functional species from combinatorial nucleic acid libraries of more than 10^{15} different molecules. Unlike oligonucleotides of natural chirality (e.g. aptamers or siRNA), they are not susceptible to nuclease digestion. In contrast to biologicals, Spiegelmers are chemical entities, manufactured by solid-phase synthesis from synthetic L-RNA building blocks. Moieties such as polyethyleneglycol can be employed to alter their pharmacokinetic properties. The proinflammatory chemokine monocyte chemotactic protein-1 (CCL2/MCP-1) is a protein involved in activation and recruitment of inflammatory cells into inflamed tissues, hence, CCL2 antagonism may be a promising target for the treatment of chronic inflammatory diseases. Following NOXXON's target-driven Spiegelmer selection and optimisation process, the drug development candidate NOX-E36 was created. NOX-E36 binds to human CCL2 with a subnanomolar dissociation constant. Functionally, it inhibits CCL2-induced migration of THP-1 cells *in vitro* with an IC_{50} in the similar range. The high specificity of NOX-E36 and the low homology between human and murine CCL2 requires use of the murine-specific Spiegelmer mNOX-E36 for preclinical profiling. Dose-dependent *in vivo* efficacy of mNOX-E36 was demonstrated in the autoimmune MRL$^{lpr/lpr}$ mouse model. MRL$^{lpr/lpr}$ mice spontaneously develop symptoms with striking similarity to human systemic lupus erythematosus at 8 weeks of age and die from end stage renal disease from week 20 of age. Subcutaneous treatment with PEGylated mNOX-E36 from week 14-24 three times per week significantly prolonged survival and improved lupus nephritis, peribronchial inflammation, and inflammatory skin lesions. Concomitant pharmacokinetic data do not indicate any sign of drug accumulation or metabolism. Thus, Spiegelmer-based inhibition of CCL2 represents a novel strategy for the treatment of chronic inflammation such as lupus nephritis.
[1] NOXXON Pharma AG, Berlin, Germany
[2] Nephrological Center, Medical Policlinic, Ludwig-Maximilians-University, Munich, Germany

242

D-PANTOTHENOL INDUCES HEME OXYGENASE-1 IN MACROPHAGES AND SKIN CELLS – A NOVEL ANTIOXIDANT AND ANTI-INFLAMMATORY MECHANISM

K. Erdmann, S. Schürger, S. Schulz, N. Grosser, H. Schröder

D-Pantothenol, the stable analog of pantothenic acid, is known to improve wound healing and has been reported to have anti-inflammatory and antioxidant properties. However, the underlying mechanisms remain unresolved. Emerging evidence suggests an important role of the antioxidant stress protein heme oxygenase-1 (HO-1) in wound healing. Therefore, the present study investigates the effect of D-pantothenol on the expression of HO-1 in macrophages (J774) and the contribution of HO-1 to the reduction of radical formation seen under the influence of this drug. Incubation of macrophages with NADPH markedly increased superoxide levels measured by lucigenin-enhanced chemiluminescence. A 24-h pretreatment with D-pantothenol (5-20 mM) or its active metabolite pantothenic acid (30-100 µM) followed by a complete wash-out reduced subsequently elicited NADPH-mediated radical formation. This indirect antioxidant protection was associated with a significant increase in HO-1 protein expression. A protective effect similar to D-pantothenol was observed when assessing radical formation in the presence of the antioxidant HO product, bilirubin. D-Pantothenol-dependent inhibition of free radical formation was abolished in the presence of the selective HO inhibitor ZnBG, suggesting that HO-1 and its enzymatic products are indeed of functional relevance and responsible for the observed antioxidant actions. HO-1 promoter activation preceded the onset of reactive oxygen species inhibitory actions. HO-1 promoter activity increased in a concentration-dependent manner after preincubation with D-pantothenol. Similar antioxidant and HO-1 inducing actions of D-pantothenol were observed in human skin fibroblasts (FEK4). This study demonstrates that D-pantothenol protects macrophages as well as human skin cells from oxidative damage, possibly through induction of HO-1. HO-1 induction followed by sustained antioxidant protection represents a novel mechanism of action for D-pantothenol which may have therapeutic relevance in wound healing.
Department of Pharmacology and Toxicology, School of Pharmacy, Martin Luther University, 06099 Halle (Saale), Germany

243

RADICICOL INDUCES MEMBRANE MOTILITY IN CULTURED EMBRYONAL HIPPOCAMPAL NEURONS

C. Schwan, G. Schmidt, D.K. Meyer, K. Aktories and J. Leemhuis

Regulation of neuronal morphology and extension of cell processes are required for normal synaptic connections and signaling. Only F-actin containing dendritic filopodia are precursors of dendritic spines. The Rho family of small GTPases regulates neuronal morphogenesis by controlling the stability and assembly of the actin cytoskeleton. Rac and Cdc42 facilitate the outgrowth of dendrites, dendritic branches, filopodia and spines, whereas RhoA and Rho-kinase attenuate it. Radicicol, a macrocyclic anti-fungal antibiotic is a potent inhibitor of the Src family of protein tyrosine kinases. Recent studies showed that the major intracellular target of radicicol is the heat shock protein 90 (HSP90) chaperone. Low nanomolar concentration of radicicol enhanced the survival and neurite outgrowth of the embryonic sensory and sympathetic neurons from embryonic chick dorsal root ganglia (DRGs) and sympathetic ganglia after 72 h. It is presumed that the neurotrophic effects are mediated via suppression of Hsp90 function. Inhibition of Hsp90 prevents thrombin induced RhoA activation and neurite retraction. Here we show that Radicicol (1nM) induces dendritic membrane motility in cultured embryonal hippocampal neurons within 15 min independent of the transcription/translation machinery. Radicicol activated Rac1 in a CRIB-pull-down assay and in a FRET-Time-lapse-imaging-assay and blocked *Yersinia pseudotuberculosis* cytotoxic necrotizing factor (CNFY) induced activation of RhoA. These results support that radicicol may have therapeutic potential for neurodegenerative diseases.

Institut für Klinische und Experimentelle Pharmakologie und Toxikologie Abt.I, Albertstrasse 25, Albert-Ludwigs-Universität, 79104 Freiburg im Breisgau, Deutschland

244

CLIMBING FIBER ACTIVITY TRIGGERS CALCIUM-MEDIATED 2-ARACHIDONOYLGLYCEROL PRODUCTION IN PURKINJE CELLS OF THE CEREBELLAR CORTEX

M.J. Urbanski and B. Szabo

CB_1 cannabinoid receptors are ubiquitously distributed in the brain. Their activation by exogenous cannabinoids usually leads to presynaptic inhibition of neurotransmission (Szabo and Schlicker, Handbook of Exp Pharmacol 168:327-365, 2005). Neurotransmission can also be modulated by endogenous cannabinoids (endocannabinoids). Endocannabinoids produced in postsynaptic neurons diffuse to presynaptic axon terminals and inhibit neurotransmitter release from the terminals - endocannabinoid-mediated retrograde signaling (Freund et al., Physiol Rev 83:1017-1066, 2003; Szabo et al., J Physiol 577:263-80, 2006). One known trigger of endocannabinoid production is an increase in calcium concentration in the postsynaptic neurons; however, this has been demonstrated mostly by non-physiological direct depolarization of neurons in patch-clamp experiments. The aim of our study was to find out whether the activity of the excitatory synaptic input to a neuron can lead to a calcium-mediated endocannabinoid production. Spontaneous postsynaptic currents (sPSCs) were recorded with patch-clamp techniques in Purkinje cells of the mouse cerebellar cortex. Imaging experiments showed that stimulation of excitatory climbing fibers (CFs; 5 Hz, 10 s) led to localized calcium increases in dendrites of Purkinje cells. After CF stimulation, the cumulative amplitude of sPSCs was suppressed by 35 ± 3 %. such a suppression did not occur if the calcium increase in the Purkinje cells was prevented by the chelator BAPTA (4×10^{-2} M). The CB_1 receptor antagonist rimonabant (10^{-6} M) also prevented the suppression of the sPSCs, indicating involvement of endocannabinoids and CB_1-receptors. Finally, the suppression of sPSCs was prevented by the diacylglycerol lipase inhibitor orlistat (10^{-5} M). Since diacylglycerol lipase is essential for the production of 2-arachidonoylglycerol, these latter results indicate that 2-arachidonoylglycerol played a role in the suppression of sPSCs. The experiments show that the activity of glutamatergic inputs can elicit a calcium-mediated 2-arachidonoylglycerol production in neurons. 2-arachidonoylglycerol, in turn, inhibits presynaptically the synaptic inputs to the neuron. Such a retrograde signaling is thought to play an essential role in synaptic plasticity, learning and memory.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität, Albertstraße 25, D-79104 Freiburg, Germany

245

INCREASED BRAIN INFARCTION AFTER TRANSIENT FOCAL CEREBRAL ISCHEMIA IN MICE WITH BK CHANNEL ABLATION

M. Sausbier[1], Y. Liao[1], N. Gu[2], A.-M. Kristiansen[2], E. Rundén-Pran[2], T. Sartorius[1], P. Ottersen[2], J.F. Storm[2], P. Ruth[1]

Cerebral ischemia is a severe condition, often resulting from interference with blood supply to the CNS. The results of oxygen and glucose deprivation (OGD) lead to ischemic cell death. Ca^{2+}-activated K^+ channels of BK type, which are activated by membrane depolarization and intracellular Ca^{2+}, are thought to play a key role in adaptive responses to hypoxia. It was expected that their activities play a pivotal role in negative feedback control of intracellular Ca^{2+} concentration and protect neuronal cells from excess Ca^{2+} influx through voltage-dependent Ca^{2+} channels during pathophysiological environments. Animal models of cerebral ischemia represent an important contribution to both our understanding of cerebral ischemia and identifying therapeutic strategies for minimizing the severity of ischemic damage. The minimally invasive endovascular filament model was applied to induce transient middle cerebral artery occlusion (MCAO) in BK channel-deficient ($BK^{-/-}$) mice. $BK^{-/-}$ mice showed increased infarct volume and post-ischemia mortality, and more severe neurological deficit compared with wild-type (WT) mice. There were no gross anatomy differences in the vascular pattern of the cerebral circulation. Cerebral blood flow after anesthesia, during transient MCAO and during reperfusion was not significantly different between genotypes. $BK^{-/-}$ cultures showed enhanced cell death in CA1 after OGD than WT cultures. Our results indicate that neuronal BK channels are important for protection against ischemic brain damage. Thus, BK channels may be considered as putative target for acute therapeutic intervention against stroke.

[1]Pharmakologie & Toxikologie, Pharmazeutisches Institut der Universität, D-72076 Tübingen, [2]Centre for Molecular Biology & Neuroscience, University of Oslo, N-0317 Oslo

246

BEHAVIOURAL ANALYSIS OF KO MICE LACKING EITHER ONE OF THE NO RECEPTOR GUANYLYL CYCLASE ISOFORMS (α_1-GC, α_2-GC)

E. Mergia[1], A. Becker[2], D. Koesling[1], and G. Grecksch[2]

The signalling molecule nitric oxide (NO) has been postulated to participate in long-term potentiation (LTP), the use-dependent increase of transmission efficacy at synapses implicated in learning and memory. Most of the effects of NO as a signalling molecule are mediated by the activation of the NO receptor guanylyl cyclase (GC), the enzyme which by the formation of cGMP and the resulting activation of cGMP regulated effector molecules (protein kinases, ion channels, phosphodiesterases) passes on the NO signal. Two isoforms of the NO receptor GC exist, α_1-GC, and α_2-GC, with indistinguishable regulatory properties.

The neuronal function of the GC isoforms was analysed by measuring LTP induction in cortical slices prepared from KO mice lacking either one of the GC isoforms. Whereas basal synaptic transmission was unaltered in the KO slices, LTP could not be induced neither in α_1-GC- nor in α_2-GC-deficient animals suggesting a contribution of both GC isoforms to LTP. Application of cGMP in form of a cGMP analogue resulted in restitution of LTP in both KO strains indistinguishable from that in WT mice. The successful recovery of LTP demonstrates that beside the lack of the cGMP forming enzymes all other cellular components required for potentiation of synaptic transmission are unaffected in the KO strains.

To study further the role of the GC isoforms in central nervous system, behavioural characterization of the KO strains was performed. Here, we report on behavioural responses of the KO mice concerning anxiety, locomotion, as well as spatial and conditioned learning.

[1]Institut für Pharmakologie und Toxikologie, Ruhr-Universität Bochum, 44780 Bochum, Germany [2]Institut für Pharmakologie und Toxikologie, Otto von Guericke Universität Magdeburg, 39120 Magdeburg, Germany

247

MOLECULAR MECHANISM OF BENZODIAZEPINE TOLERANCE

G. Michels[1,2], J. Walker[1], H.H. Lee[1], and S.J. Moss[1]

$GABA_A$ receptors play a key role in controlling neuronal activity and are important drug targets for benzodiazepines. Molecular biology studies have revealed that synaptic diazepam-sensitive $GABA_A$ receptor subtypes are composed of α(1-3, 5), β and γ2 subunits, while α(4,6), β and δ subunit containing - diazepam-insensitive - $GABA_A$ receptors are preferentially expressed on the extrasynaptic site. $GABA_A$ receptors are not static entities in neuronal plasma membranes but undergo rapid movement into and out of these structures. Such clathrin-mediated endocytosis is the major mechanism of receptor internalization for $GABA_A$ receptors. The most widely reported problems with benzodiazepine usage lie in the development of tolerance. Chronic benzodiazepine treatment produce alterations in the expression of individual $GABA_A$ receptor subtypes; however, the extent of these changes on mRNA level is often relatively small and might not reflect equivalent protein changes. To examine the role of classical benzodiazepines such as flurazepam on cell surface level of $GABA_A$ receptors we have used biochemical, immunostaining, live imaging and electrophysiological methods. Within 1 h flurazepam reduces α(1-3) diazepam-sensitive $GABA_A$ receptors on cell surface, while α4 diazepam-sensitive subunits are elevated proportionately. These effects are linked to benzodiazepine dosage, duration of use and rate of clathrin-dependent endocytosis. Our results demonstrate that tolerance to benzodiazepines is caused by compensatory alterations on $GABA_A$ receptor subunit level and regulated by clathrin-dependent endocytosis.

1. Department of Neuroscience, University of Pennsylvania, Philadelphia, USA; 2. Department of Internal Medicine III, University of Cologne, Cologne, Germany

248

REGULATION AND PREVENTION OF SEIZURE-INDUCED UP-REGULATION OF P-GLYCOPROTEIN IN RAT BRAIN CAPILLARIES

A. Pekcec[1], B. Bauer[2], A.M. Hartz[2], J. Winter[3], K. Töllner[3], D.S. Miller[2], and H. Potschka[1]

The blood-brain barrier's (BBB) active drug efflux transporter, P-glycoprotein (Pgp), impacts pharmacokinetics of agents acting on the CNS and impediments to pharmacotherapy of CNS disorders, like epilepsy. A seizure-induced up-regulation of Pgp has been identified in various models of epilepsy as well as human patients; however, the mechanisms of Pgp up-regulation remain unknown. As understanding of these mechanisms may yield new therapeutic strategies to improve CNS drug therapy, we studied the involvement of glutamate and cyclooxygenase on Pgp up-regulation (COX) *in vitro* and *in vivo*. After acquiring of rat brain capillaries, the impact of glutamate and COX-inhibitors (indomethacin and indomethacin-heptylester) on Pgp-expression were studied by Western Blot analysis. Pgp-expression was further substantiated by immunhistochemistry following intrahippocampal glutamate injection (5 nM) *in vivo*. One day prior pilocarpine-induced status epilepticus (SE, 10 mg/kg i.p. fractionated at 30 min intervals), adult female Sprague-Dawle rats received 2.5 mg/kg indomethacin- or vehicle-injections (i.p.) twice daily. The modulatory effect of indomethacin on Pgp-expression and neurodegeneration was investigated by immunhistochemistry two days following SE. An increase of Pgp-expression by glutamate as compared to controls was demonstrated *in vivo* and *in vitro*. This increase was counteracted by both, indomethacin and indomethacin-heptylester under *in vitro* conditions. The SE-induced up-regulation of Pgp was decreased to control levels by indomethacin. Even though SE-induced neurodegeneration was not completely suppressed by indomethacin, a significant reduction as compared to the vehicle-injected pilocarpine-treated rats was shown. Epileptic seizures have been shown to induce Pgp-expression in brain capillaries. Glutamate release, which is known to accompany epileptic seizures, has now been demonstrated to be involved in this increase. The COX-inhibitor selective drug indomethacin prevented the up-regulation of Pgp. Therefore, possible implications of COX-2 selective drugs in pharmacotherapy of epilepsy will be studied in future experiments.

[1]Inst. of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Munich, Germany [2]Laboratory of Pharmacology and Chemistry, NIH/NIEHS, NC, USA [3]Inst. of Pharmacology, Toxicology, and Pharmacy, University of Veterinary Medicine Hannover, Germany

249

LACK OF RELATIONSHIP BETWEEN RESPONSE TO ANTIEPILEPTIC TREATMENT AND P-GLYCOPROTEIN EXPRESSION IN THE PILOCARPINE MODEL OF STATUS EPILEPTICUS.

J. P. Bankstahl[1], H. Potschka[2], W. Löscher[1]

The effectiveness of antiepileptic drugs decreases with the length of a status epilepticus (SE) in humans and rat models of SE. As a reason for this loss of pharmacosensitivity two main hypotheses are discussed: (1) SE-induced alterations in drug targets like $GABA_A$ receptors, and (2) SE-induced overexpression of multidrug transporters like P-glycoprotein (P-gp). Aim of this study was to investigate possible correlations between time dependency in pharmacological sensitivity of SE and the expression of P-gp in the lithium-pilocarpine SE model, as one possible reason for pharmacoresistance. A self-sustained SE was induced by repeated administration of pilocarpine in lithium pre-treated Sprague-Dawley rats. Phenobarbital (30 – 90 mg/kg) was used to terminate the SE at different time points after onset of SE. If phenobarbital was ineffective, we used diazepam (10 mg/kg) or phenytoin/fosphenytoin (50 mg/kg) for a second injection. The dosage was considered as effective, if behavioural and electrographic seizure activity was successfully terminated during thirty minutes after injection. After termination of SE, the animals were sacrificed and brain sections were immunohistochemically stained against P-gp. The expression of P-gp was measured by computer-assisted quantification of optical density. To investigate the time-course of P-gp alterations after termination of the pilocarpine induced SE, we added animals which were sacrificed after 1, 2, or 7 days after the SE. We found that, once the SE had started, phenobarbital was ineffective to terminate SE at all time points. A subsequent injection of phenytoin and fosphenytoin was also ineffective, only diazepam terminated SE immediately. The earliest time point at which we detected increased P-gp levels was 48 h after termination of SE. So we found no correlation between P-gp expression and the lack of antiepileptic drugs to interrupt SE.

Supported by a grant of the Deutsche Forschungsgemeinschaft (Lo 274/9-3) and a scholarship of the Konrad-Adenauer-Stiftung.

[1]Department of Pharmacology, Toxicology, and Pharmacy, University of Veterinary Medicine and Centre for Systems Neuroscience, Hannover, Germany
[2]Department of Pharmacology, Toxicology, and Pharmacy, Veterinary Faculty, Ludwig-Maximilians-University, Munich, Germany

250

SEARCH FOR THE FUNCTION OF THE G PROTEIN-COUPLED ORPHAN RECEPTOR GPR55 IN THE NERVOUS SYSTEM

W. U. Baer, F. Kovacs and B. Szabo

Two $G\alpha_{i/o}$ protein-coupled receptors have been identified as targets for cannabinoids, CB_1- and CB_2 cannabinoid receptors, but there is evidence for the existence of additional cannabinoid receptors. The G protein-coupled orphan receptor GPR55, when artificially expressed in HEK cells, is activated by a series of plant-derived -, endogenous - and synthetic cannabinoids. Thus, GPR55 is potentially a new cannabinoid receptor. Our aim was to identify neuronal effects mediated by GPR55. Since mRNA for GPR55 was detected in the caudate-putamen, we searched for the function of GPR55 in this brain region. Patch-clamp recordings were carried out in slices prepared from brains of NMRI mice or of mice lacking CB_1 and CB_2 receptors (CB knockout mice; generated in the laboratory of Andreas Zimmer, Bonn). In the first series of experiments, effects of the supposed GPR55 agonist O-1602 (5-methyl-4-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-1,3-benzenediol) on the somatodendritic region of medium spiny neurons was studied. Superfusion of O-1602 (10^{-6} and 10^{-5} M) did not affect the membrane potential of the neurons. The frequency of action potential salvos, elicited by depolarising current injections, was also not changed by O-1602 (10^{-6} and 10^{-5} M). In the second series of experiments, effects of O-1602 on inhibitory neurotransmission were studied. GABAergic axons were electrically stimulated and the resulting inhibitory postsynaptic currents (IPSCs) were recorded in medium spiny neurons. In brain slices prepared from NMRI mice, O-1602 (10^{-5} M) depressed IPSCs by 39 ± 17 %; prevention of this effect by the CB_1 antagonist rimonabant (10^{-6} M) indicates that it was mediated by CB_1 receptors. In brain slices prepared from CB knockout animals, O-1602 (10^{-6} - 10^{-5} M) did not affect IPSCs, whereas O-1602 (10^{-7} M) caused a slight inhibition. The results show that activation of GPR55 does not lead to somatodendritic changes in neurons. A slight inhibition of GABAergic neurotransmission may occur, but this result needs confirmation. O-1602 is not fully selective for GPR55, but is also an agonist at CB_1-receptors.

Inst. für Pharmakologie, Albert-Ludwigs-Universität; Albertstrasse 25, 79104 Freiburg

251

GABAPENTIN AND PREGABALIN MODULATE K^+-EVOKED [^{3}H]-NEUROTRANSMITTER RELEASE FROM DISCRETE RAT CNS REGIONS AND HUMAN NEOCORTEX

B. Brawek[1], C.M. Donovan[2], M. Löffler[1], D.J. Dooley[3], A. Weyerbrock[3], T.J. Feuerstein[1]

Anticonvulsant, analgesic, and anxiolytic effects have been observed both preclinically and clinically with gabapentin and more recently with pregabalin. The site of action of these drugs appears to be the $\alpha_2\delta$ subunit of neuronal voltage-sensitive Ca^{2+} channels (VSCC) resulting in inhibition of high-threshold Ca^{2+} currents and neurotransmitter release. Both gabapentin and pregabalin have previously been shown to inhibit K^+-evoked [^{3}H]-norepinephrine release from superfused rat neocortical slices. The present study extends this finding by addressing the inhibitory effects of gabapentin and pregabalin on K^+-evoked, Ca^{2+}-dependent release of [^{3}H]-noradrenaline, [^{3}H]-dopamine, [^{3}H]-acetylcholine and [^{3}H]-serotonin from discrete rat CNS regions and from human neocortex. Gabapentin and pregabalin (100µM) produced comparable and significant inhibitions (i.e., 21%-46%) of these [^{3}H]-neurotransmitters from several rat CNS regions including neocortex, hippocampus, cerebellum, and spinal cord. However, these drugs were not active to decrease K^+-evoked [^{3}H]-dopamine, [^{3}H]-serotonin, and [^{3}H]-acetylcholine release from rat striatal slices. In human neocortical slices, gabapentin and pregabalin (100µM) significantly reduced K^+-evoked [^{3}H]-noradrenaline, [^{3}H]-serotonin, and [^{3}H]-acetylcholine release by 22%-61%, but did not affect K^+-evoked [^{3}H]-dopamine release. Gabapentin and pregabalin no longer reduced [^{3}H]-noradrenaline release when L-isoleucine, a putative $\alpha_2\delta$ antagonist, was present throughout the experiment. This finding supports the assumption that the $\alpha_2\delta$ subunit of VSCC is the main target of the two substances. Interestingly, the blocking effect of L-isoleucine was not observed with D-isoleucine. These results suggest that gabapentin and pregabalin may selectively modulate presynaptic terminal VSCC of multiple neurotransmitter systems. Release of different neurotransmitters may be affected in various ways by these two drugs.

[1] Section of Clinical Neuropharmacology, Neurozentrum, Albert-Ludwigs University, 79106 Freiburg, Germany
[2] Dept. of CNS Pharmacology, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI USA 48105
[3] Dept. of Neurosurgery, Neurozentrum, Albert-Ludwigs University, 79106 Freiburg, Germany

252

NEURONAL ELECTRICAL HIGH FREQUENCY STIMULATION ENHANCES GABA RELEASE FROM HUMAN NEOCORTICAL SLICES

M. Mantovani [1], D. Altenmüller[1], H. Fuellgraf [2], A. Moser[2], T.J. Feuerstein [1]

Electrical high frequency stimulation (HFS) of the globus pallidus internus or the subthalamic nucleus has beneficial motor effects in advanced Parkinson's disease (Benabid et al. 2001). The mechanisms underlying these clinical results, however, remain unclear. Since GABA plays an important role in CNS disorders of basal ganglia, in depression, anxiety and epilepsy, the modulation by HFS of its neocortical release may be (patho)physiologically relevant. Our aim was to characterize the actions and the possible mechanisms of HFS on GABA release in human neocortical tissue. Human neocortical slices were stimulated *in vitro* by electrical pulses with 130 Hz, 100 µs, for 10 min. Electrical HFS alone did not modulate basal GABA release (measured by HPLC) from neocortical slices. Increasing the open probability of voltage-gated sodium channels with veratridine (3 µM) in absence of HFS did also not change basal GABA release. Together with veratridine, however, application of electrical HFS induced a significant increase in GABA release. Maximum values of GABA release increased to approximately 300% over basal GABA release. The administration of the competitive $GABA_A$ receptor antagonist (+)-bicuculline (10 µM) or of the $GABA_A$ receptor channel blocker picrotoxin (32 µM) did not change basal GABA release. However, addition of (+)-bicuculline or picrotoxin during the HFS stimulation abolished the effect of HFS. Administration of the $GABA_B$ receptor agonist baclofen (100 µM) slightly reduced the HFS-evoked release of GABA. GABA release upon HFS was abolished by the chloride-transport blocker furosemide (100 µM), suggesting that HFS may require a plasmalemmal chloride gradient, which becomes operative through the $GABA_A$ receptor channel. Our results demonstrate for the first time in human neocortical slices that HFS significantly increased endogenous GABA release in the presence of veratridine. This effect seems to be dependent on activated voltage-gated sodium channels and excitatory $GABA_A$ autoreceptors due to an inverse chloride gradient.

[1] Section of Clinical Neuropharmacology, Neurozentrum, Albert-Ludwigs University, 79106 Freiburg, Germany
[2] Neurochemical Research Group, University of Lübeck, 23538 Lübeck, Germany

253

DIFFERENTIAL ALTERATION OF $GABA_A$-RECEPTOR SUBUNIT EXPRESSION IN THE HIPPOCAMPUS OF EPILEPTIC RATS THAT ARE SENSITIVE OR RESISTANT TO TREATMENT WITH PHENOBARBITAL

K.Bethmann[1], J.-M. Fritschy[2], C.Brandt[1] and W. Löscher[1]

Pharmacoresistance in epilepsy is most probably a multifactorial process. The regulation of the major inhibitory transmitter, GABA, and one of its receptors, $GABA_A$, plays an important role in epilepsy. Therefore we are interested whether $GABA_A$-receptor subunits are differentially expressed in hippocampal subregions between chronic epileptic rats that are either responding or not responding to treatment with the antiepileptic drug phenobarbital (PB). Non-epileptic controls were used for comparison. Female Sprague-Dawley rats were electrically stimulated via a bipolar electrode in the basolateral amygdala until they developed a self-sustained status epilepticus. During the following weeks, rats showed spontaneous recurrent seizures, which were treated with maximum tolerable doses of PB over two weeks. Before, during and after treatment, seizures were continuously monitored via EEG and video. Depending on the response to PB, rats were subdivided into PB-resistant and PB-responsive rats. After the pharmacological experiment, brains were immunhistochemically stained for the $GABA_A$-receptor subunits α1-5 and γ2. The optical density of the staining was then analyzed in the dentate gyrus (DG), the CA1, the CA3 and the hilus of the hippocampus in both hemispheres. There was a downregulation of all $GABA_A$-receptor subunits in most hippocampal regions in PB-resistant rats compared to controls. Downregulation was strongest for the α5 and weakest for the α4 subunit. PB-responsive animals also exhibited a mild decrease of $GABA_A$-receptor subunit immunoreactivity, mainly in the CA1 and CA3 regions. For α1, α3, α4 and α5 subunits, the reduction in PB-resistant rats was significantly larger in several hippocampal subregions than in PB-sensitive rats, indicating that altered subunit expression of $GABA_A$ receptors may be involved in the mechanisms underlying pharmacoresistance in these animals.

The study was supported by a grant (1 R21 NS049l592-01) from the NIH, Bethesda, MD, USA.

[1]Department of Pharmacology, Toxicology, and Pharmacy, University of Veterinary Medicine, Hannover, Germany
[2]Institute of Pharmacology and Toxicology, University of Zurich, Switzerland.

254

AUTORADIOGRAPHY OF CENTRAL $GABA_B$ RECEPTORS IN AN ANIMAL MODEL OF PAROXYSMAL DYSTONIA

S.E. Sander[1], J.N. Nobrega[2], R. Raymond[2], A. Richter[1]

$GABA_B$ receptors are widely expressed and distributed in the nervous system and have been implicated in a variety of disorders such as epilepsy and spasticity. In dystonia, a movement disorder in which muscle co-contractions cause repetitive twisting movements or abnormal postures, their pathophysiological role is still unknown. Studies in the *dt*^{sz} mutant hamster, an animal model of primary paroxysmal dystonia, demonstrated alterations of the GABAergic system, e.g., an ontogenetically decreased density of striatal GABAergic interneurons and changes in $GABA_A$ receptor binding in several regions of the CNS. In order to clarify if $GABA_B$ receptors are also involved in the pathophysiology of dystonia in the hamster mutant, we performed quantitative autoradiographic analysis of ligand binding to the selective $GABA_B$ receptor antagonist [^{3}H]-CGP54626. In the *dt*^{sz} hamster, dystonia is stress-inducible and occurs between the 16th and 70th day of life. Ten *dt*^{sz} hamsters were decapitated at the age of maximum expression of dystonic attacks (34-36 d) together with an age- and gender-matched control group without preceding induction of dystonia. The brains were removed, immediately frozen

and sliced at a temperature of -20°C. The slices were incubated with 2 nM [^{3}H]-CGP54626 and in order to define non-specific binding 10 µM CGP55845 was used. Densitometric analysis was performed with the MCID system. In none of the examined 32 brain regions was a significant difference (Bonferroni-corrected: p<0.0016) detectable between *dt*^{sz} hamsters and control animals. The results of our study propose that $GABA_B$ receptors are not altered under basal conditions in the *dt*^{sz} hamster. Further studies, including intrastriatal applications of $GABA_B$ receptor modulators, have to clarify the role of $GABA_B$ receptors in the manifestation of dystonic attacks.

This work was supported by DFG grant Ri 845/1-2.

[1]Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, 14195 Berlin, Germany
[2]Neuroimaging Research, Centre for Addiction and Mental Health, Toronto, Ontario M5T 1R8, Canada

255

EFFECTS OF THE KYNURENINE 3-HYDROXYLASE INHIBITOR RO 61-8048 AFTER STRIATAL MICROINJECTIONS IN AN ANIMAL MODEL OF PRIMARY PAROXYSMAL DYSTONIA

M. Hamann, F. Richter, A. Richter

Kynurenic acid is an endogenous antagonist of all ionotropic glutamate receptors with preferential affinity for the glycine site of the NMDA (N-methyl-D-aspartate) receptor and has been shown to reduce the glutamate release. Therefore, kynurenic acid is able to regulate overexcitation within the nervous system. In the *dt*^{sz} hamster, an animal model of primary paroxysmal dystonia, a common movement disorder with an unknown pathophysiology, there is evidence for a temporary overactivity of the glutamatergic system, coinciding with the occurrence of a stress-inducible dystonic episode. Previous investigations in the *dt*^{sz} mutant have shown that the systemic application of the kynurenine 3-hydroxylase inhibitor 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulfonamide (Ro 61-8048) reduced the severity of dystonia, corresponding to enhanced levels of kynurenic acid in different brain regions of the treated animals. To investigate the functional relevance of changes of kynurenic acid levels within the striatum, the effects of striatal microinjections of Ro 61-8048 on dystonia were investigated in the present study. The direct striatal administration of Ro 61-8048 leads to moderate antidystonic effects without leading to marked central side effects. Thus, the application of 30 µg/0.5 µl/hemisphere increased the latency to the onset of a dystonic episode. The administration of 60 µg/0.5 µl/hemisphere reduced the severity of dystonia in the first hour of the observation period. These preliminary results are in line with previous data, indicating that dysfunctions of the glutamatergic system contribute to dysfunctions within the basal ganglia network and thereby to the occurrence of a dystonic episode, although more pronounced effects were presumed in view of the marked antidystonic efficacy of Ro 61-8048 after systemic administration. As shown by previous experiments, dopaminergic overactivity within the striatum seems to be more relevant for the manifestation of severe dystonia in this animal model.

Institute of Pharmacology and Toxicology, Department of Veterinary Medicine, Freie Universität Berlin, Germany

256

BRAIN REGION-SPECIFIC REGULATION OF NEUROTROPHINS AND CORTICOTROPIN-RELEASING FACTOR (CRF) IN NORADRENALINE TRANSPORTER KNOCKOUT MICE (NAT$^{-/-}$)

B.Haenisch and H.Bönisch

Neurotrophins and neuropeptides are supposed to play a role in the pathophysiology of depression by being involved in anxiety-related behaviour and neuronal plasticity. Examining these targets might lead to further insights in the complexity of antidepressant actions like the delayed antidepressive effects. An important primary mechanism of action of many antidepressants, e.g. reboxetine, is the inhibition of the NAT. As NAT$^{-/-}$ mice behave like antidepressant-treated mice, we examined changes in the protein expression of some neurotrophins [nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophine-3 (NT-3)] and corticotrophin-releasing factor in the CNS of NAT $^{-/-}$ mice in comparison with NAT $^{+/+}$ mice. Protein levels were measured in different brain regions (olfactory bulb, cortex, cerebellum, brainstem, hippocampus, striatum, hypothalamus) by two-site enzyme-linked immunosorbent assay (ELISA). In NAT$^{-/-}$ mice NGF was significantly decreased in olfactory bulb, cerebellum and brainstem. Additionally, a tendency for downregulation could be observed in cortex, hippocampus and hypothalamus. The highest expression of NGF was detected in olfactory bulb, hippocampus and hypothalamus. Also BDNF and NT-3 showed the highest protein expression in hippocampus and hypothalamus. BDNF tended to be increased in striatum of NAT$^{-/-}$ mice while NT-3 showed an increased expression in the brainstem. Furthermore, knockout of the NAT significantly down-regulated NT-3 in the olfactory bulb and led to a slight decrease in NT-3 protein level in striatum and hypothalamus. For CRF the highest expression was detected in the hypothalamus. NAT$^{-/-}$ induced a significant decrease of CRF in the hippocampus. Our findings indicate that knockout of the NAT induces brain region-specific changes in the protein expression of some neurotrophins and CRF which supports a role of these targets in affective disorders like depression.

Institute of Pharmacology & Toxicology, University of Bonn, Reuterstr. 2b, 53113 Bonn, Germany

257

THE DOPAMINE D_3 RECEPTOR ANTAGONIST ST 198 AS A POTENTIAL COCAINE ABUSE THERAPEUTIC AGENT

O. Saur[1], M. Shih[2], C.S. Elsken[3], M.J. Forster[3], H. Stark[1]

The reinforcing effect of cocaine underlies among other factors an indirect stimulation of dopamine receptors especially in the nucleus accumbens. Dopamine transporter blockade yields in an increased concentration of synaptic dopamine levels. Unlike the other dopamine receptor subtypes dopamine D_3 receptors are located in high concentration in limbic areas of the brain whereas they are nearly abundant in most parts of the striatum. Due to this discrete localization dopamine D_3 receptors are a drug target for the treatment of drug abuse. Therefore the dopamine D_3 receptor selective full antagonist ST 198 (hD_2: K_i = 780 nM; hD_3: K_i = 12 nM) should attenuate dopamine D_3 receptor stimulation and thus reduce pleasurable feelings [1; 2]. Furthermore cocaine exposure leads to a selective over-expression of dopamine D_3 receptors without any changes in dopamine D_1 or D_2 receptor expression. This observation supports the involvement of dopamine D_3 receptors in the drug addiction processes. Blockade of these receptors could consequently antagonize behavioral sensitization processes [3].

In order to evaluate ST 198 as a potential medication for cocaine abuse mouse locomotor activity tests have been performed. The promising results of the dose response study of ST 198 alone and the cocaine interaction study paved the way for further tests.

[1] Mach UR, Hackling AE, Perachon S, Ferry S, Wermuth CG, Schwartz JC, Sokoloff P, Stark H; *Chembiochem* 2004; *5*, 508-18
[2] Le Foll B, Sokoloff P, Stark H, Goldberg SR; *Neuropsychopharmacology* 2004; 30, 720-30
[3] Le Foll B, Goldberg SR, Sokoloff P; *Neuropharmacology* 2005; *49*, 525-41

[1] Johann Wolfgang Goethe-Universität, Institut für Pharmazeutische Chemie, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany
[2] National Institutes of Health, National Institute on Drug Abuse, 6001 Executive Blvd., Rm. 4123, MSC 9551, Bethesda, MD 20892-9551, USA
[3] Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107-2699, USA

258

PLEXIN-B2, BUT NOT PLEXIN-B1, CRITICALLY MODULATES NEURONAL MIGRATION AND PATTERNING OF THE DEVELOPING NERVOUS SYSTEM IN VIVO

Deng S[1], Hirschberg A[1], Worzfeld T[1], Penachioni JY[2], Korostylev A[1], Swiercz JM[1], Vodrazka P[1], Mauti O[3], Stoeckli ET[3], Tamagnone L[2], Offermanns S[1], Kuner R[1]

Semaphorins and their receptors, plexins, have emerged as important cellular cues regulating key developmental processes. B-type plexins directly regulate the activity of several Rho GTPases as well as receptor tyrosine kinases and thereby modify the actin cytoskeleton in a variety of cell types. Recently, B-type plexins have been shown to be expressed in characteristic patterns in the nervous system over critical developmental windows. However, in contrast to the well-characterized plexin-A family, the functional role of plexin-B proteins in neural development and organogenesis in vertebrates *in vivo* is not known. Here, we have elucidated the functional contribution of the two neuronally-expressed plexin-B proteins, Plexin-B1 and Plexin-B2, towards the development of the peripheral- and the central- nervous system by generating and analysing constitutive knock-out mice. The development of the nervous system was found to be normal in mice lacking Plexin-B1, whereas mice lacking Plexin-B2 demonstrated defects in closure of the neural tube and a conspicuous disorganization of the embryonic brain. Upon analyzing mutant mice which bypassed neural tube defects, we observed a key requirement for Plexin-B2 in proliferation and migration of granule cell precursors in the developing dentate gyrus, olfactory bulb and cerebellum. Furthermore, we identified semaphorin 4C as a putative ligand for Plexin-B2 in binding and functional assays. Semaphorin 4C stimulated RhoA activation via plexin-B2 and enhanced proliferation and migration of granule cell precursors. These genetic and functional analyses reveal a key requirement for Plexin-B2, but not Plexin-B1, in patterning of the vertebrate nervous system in vivo.

[1]Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany
[2]Institute for Cancer Research and Treatment (IRCC), University of Torino School of Medicine, 10060 Candiolo, Torino, Italy
[3]Institute of Zoology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

259

CLASS I PHOSPHATIDYLINOSITOL 3-KINASES AND ASTROGLIAL PROLIFERATION

R. Müller[1], C. Fischer[1], J. Leemhuis, N. Klugbauer and D.K. Meyer
1: the authors contributed equally to this work

The family of phosphatidylinositol 3-kinases (PI3-kinases) phosphorylates membrane inositol lipids. The four members of the class I p110α, p110β, p110γ and p110δ are involved in cell attachment, proliferation and mobility. Little is known about the expression of the different isoforms in neural cells of the CNS and their contribution to the regulation of proliferation. In a first step, we examined the isoform-specific expression of class I PI3-kinases in cultured neocortical astroglial cells. By means of non-radioactive *in situ* hybridization, reverse transcription PCR and Western Blotting we could show that only p110α and p110β were expressed. To investigate the role of the both isoforms in proliferation, we expressed the respective dominant negative (dn) constructs coupled to EGFP via IRES in cultured neocortical astroglial cells. The adenoviral Tet-On system (BD Adeno-X Tet-On Expression from BD Biosciences) used allowed the graded expression of the gene of interest by application of doxycycline. The resulting infection rate was > 95%. In glial cells synchronized by transient serum removal for 24 h, the PI3-kinase inhibitor wortmannin and dnp110α as well as dnp110β significantly reduced the nuclear uptake of bromo-deoxyuridine (BrdU), indicating an

antiproliferative effect. Both dominant negative constructs reduced the activity of the small GTPases Ras and Rac without exerting an effect on Cdc42. In contrast, the 2 isoforms affected differently the activities of extracellular signal regulated kinases (ERKs), which are essential for proliferation. Whereas dnp110β reduced the phosphorylation of the ERKs dnp110α had no effect, although expression of both constructs diminished the phosphorylation of the PI3-kinase effector Akt. This finding suggested that both isoforms can influence proliferation via different signalling pathways. Further analysis by use of RNA interference is currently performed, to learn more about the interaction of p110α and p110β in the control of proliferation.
Institute of Pharmacology, Albert-Ludwigs-University, 79104 Freiburg, Germany.
The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

260

DETERMINATION OF NADH BY LASER-INDUCED FLUORESCENCE SPECTROSCOPY: APPLICATIONS IN PRECLINICAL NEUROSCIENCE

A. Rex, M. Hamann, A. Richter, F. Fink*, H. Fink

There is an increasing need for continuously monitoring changes in brain metabolism and neuronal activity, respectively. The majority of the energy, which is produced in neurons, is necessary for the maintenance of the physiological neuronal activity. The extent of the energy production is thus closely coupled to the neural activity. Measurement of NAD(P)H fluorescence by laser-induced fluorescence spectroscopy and with small glass fibre probes allows the determination of the mitochondrial activity, and therefore neuronal activity indirectly, in the brain with high temporal and spatial resolution (Rex & Fink, 2006, Las.Phys.Lett.,3:452-9). In vitro, dependence between the NADH concentration and NADH fluorescence was proven. Ex vivo investigations showed that under the selected spectroscopic conditions predominantly NADH fluorescence contributes to the fluorescence of the tissue and that the fluorescence intensity differs between brain regions. We could show in anaesthetized rats that the fluorescence intensity of NADH in the cortex is inversely proportional to the metabolic activity and that changes in the NADH fluorescence due to haemodynamical effects altering the optical properties of the tissue can be excluded. In further in vivo experiments administration of a serotonin 1A receptor agonist with known anxiolytic activity and inhibitory effects on neuronal activity in the hippocampus causes a reversible increase in the intensity of hippocampal NADH fluorescence in pharmacologically relevant doses. In awake and freely moving mutant dtsz hamsters, a model of paroxysmal dystonia, we could measure reversible changes of the NADH fluorescence during a dystonic episode. The magnitude of the change corresponds to the severity of the dystonic episode. The laser-induced fluorescence spectroscopy of the intrinsic and mainly mitochondrial bound NADH is a versatile applicable and reliable method which allows the spatial and temporal characterization of the metabolic state and thus the neuronal activity in the brain in vitro and in vivo.

Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, Koserstr. 20, 14195 Berlin
*Fachhochschule für Technik und Wirtschaft Berlin, University of Applied Sciences, Marktstraße 9, 10317 Berlin

261

BRAIN-SPECIFIC DELETION OF THE PACEMAKER CHANNEL HCN4

A. Blaich[1,2], F. Hofmann[2], A. Ludwig[1]

Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels are thought to play a role in several neuronal functions, e.g. determination of the resting membrane potential of neurons, generation of pacemaker potentials and learning and memory. The contribution of the four individual HCN isoforms (HCN1-4) to these diverse functions is only partially known. We analyzed the expression pattern of the HCN4 isoform in murine brain by immunohistochemistry and observed a strong and selective staining in the thalamus. This indicates a potential involvement of HCN4 in thalamus-dependent functions like control of sleep-wake cycle, motor activity and processing of sensory inputs. Ubiquitous deletion of HCN4 in mice results in embryonic lethality. To determine the physiological role of HCN4 in the adult brain we attempted to delete the HCN4 gene by using the Cre/loxP-system. Floxed HCN4 mice were crossed with transgenic animals expressing Cre recombinase selectively in the brain. The brain specific HCN4 knock out mice displayed a complete lack of the HCN4-transcript as shown by RT-PCR and northern blot analyses. This result was confirmed by western blotting. In addition, serial brain slices stained with the HCN4-antibody revealed a complete absence of HCN4 protein. Deletion of the closely related HCN2 isoform, which is also highly expressed in the thalamus, leads to absence seizures. Remarkably, telemetric EEG recordings from freely moving HCN4 knockout animals showed no spontaneous absence epilepsy. Behaviour analyses displayed a decrease in exploration (open field) and motor activity (rotarod and beamwalk) of the HCN4 knock out mice. In contrast, other thalamus-dependent functions like reaction to cutaneous stimuli and vision were apparently not impaired in the knock out mice. These results indicate that the HCN4 channel is involved in the control of motor activity and exploration.

[1]Institut für Experimentelle und Klinische Pharmakologie, Friedrich-Alexander-Universität Erlangen, [2]Institut für Pharmakologie und Toxikologie, Technische Universität München

262

HCN2 CHANNELS IN HIPPOCAMPAL INTERNEURONS MODULATE LONG-TERM POTENTIATION IN THE TEMPORO-AMMONIC PATHWAY

L. Matt[1], S. Michalakis[2], M. Weidinger[2], M. Biel[2], A. Ludwig[3], F. Hofmann[1], T. Kleppisch[1]

Deletion of the gene coding for the hyperpolarization-activated cyclic nucleotide-gated channel subtype HCN1 enhances long-term potentiation (LTP) in the hippocampal temporo-ammonic pathway and improves spatial learning performance. Hippocampal neurons also express substantial levels of the HCN2 channel isoform. To elucidate its function for synaptic plasticity, we studied LTP in hippocampal slices from wild-type mice and HCN2 null mutants ($HCN2^{-/-}$) using field EPSP recordings. As described for $HCN1^{-/-}$ mice, LTP was significantly enhanced in the temporo-ammonic input of $HCN2^{-/-}$ mice, while LTP in the more proximal Schaffer collateral/CA1 input of these mice was not altered. For $HCN1^{-/-}$ mice, this finding was accounted to a filter function of HCN1 channels in distal dendrites of pyramidal neurons dampening integration of excitatory synaptic inputs from the temporo-ammonic pathway. To further evaluate whether HCN2 channels serve a similar function, we examined LTP in animals with a conditional deletion of the HCN2 channel in principal hippocampal neurons. Incompatible with a critical function of HCN2 channels expressed in pyramidal cells, these mutants showed normal LTP in the temporo-ammonic and Schaffer collateral pathway. Remarkably, hippocampal slices from mice with a conditionally deleted HCN2 gene showed residual HCN2 immunoreactivity overlapping with the localisation of oriens-lacunosum moleculare (O-LM) interneurons. Patch-clamp recordings from such interneurons in wild-type mice revealed substantial hyperpolarization-activated (I_h) currents likely carried by HCN1 and HCN2 channels. Moreover, O-LM interneurons in $HCN1^{-/-}$ mice showed residual I_h currents matching the voltage dependence and activation kinetics of heterologeously expressed HCN2 channels. We suggest that a loss of function in GABAergic interneurons leads to dysinhibition and accounts for the enhanced LTP in $HCN2^{-/-}$ mice.

[1] Institut für Pharmakologie und Toxikologie, TU München, München, Germany
[2] Fakultät Chemie und Pharmazie, LMU München, München, Germany
[3] Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, FAU Erlangen, Erlangen, Germany

263

K_V7 (KCNQ) CHANNELS: TARGETS FOR THE THERAPY OF LEVODOPA-INDUCED DYSKINESIA?

A. Richter and S.E. Sander

Parkinson's disease is characterized by a progressive degeneration of dopaminergic nigral neurons, leading to decreased striatal dopamine levels. After long-term treatment with levodopa most patients develop dyskinesias. An increased activity of striatal projection neurons which express K_V7 channels seems to be critically involved in the pathophysiology. The $K_V7.2$-7.5 channel openers retigabine and flupirtine can decrease neuronal activity (so-called M currents) and have been shown to exert antidystonic effects in a model of inborn paroxysmal dystonia in which the striatal activity is increased. Analgetic effects of K_V7 channel openers might contribute to improve these disorders which are often accompanied by painful muscle spasms. Therefore, we examined these compounds in a model of levodopa-induced dyskinesia. Rats received injections of 8 µg 6-OHDA into the left medial forebrain bundle to induce lesions of dopaminergic neurons. Dyskinesia was provoked by treatment with 20 mg/kg levodopa and 15 mg/kg benserazide for 20 days. Three subtypes of dyskinesia (limb, axial and orolingual) were rated according to a score system from 0 to 4 every 30 min over 3 h. For drug testing, retigabine (2.5-5 mg/kg i.p.) and flupirtine (5-10 mg/kg i.p.) were administered prior or together with levodopa (or vehicle). In comparison to vehicle controls, 2.5 mg retigabine significantly reduced the severity of dyskinesia 110-140 min after injections. The antidyskinetic effects were longer lasting after treatment with 5 mg/kg (50-110 min). Flupirtine exerted significant antidyskinetic effects after administration of 10 mg/kg from 20-80 min. The antidyskinetic effects were not accompanied by marked central side effects. The results of our study suggest that the K_V7 channel openers retigabine and flupirtine, which are well tolerated in patients, are interesting targets for the treatment of levodopa-induced dyskinesia. Further studies have to clarify whether the effects of retigabine and flupirtine are selectively mediated by K_V7 channels and if these compounds are able to delay or prevent the development of levodopa-induced dyskinesias.

[1]Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, 14195 Berlin, Germany

264

THE P50 SUBUNIT OF THE TRANSCRIPTION FACTOR NF-κB IS INVOLVED IN ACUTE AND PERSISTENT INFLAMMATORY NOCICEPTION

H. Kühlein, A. Schmidtko, W. Gao, C. Ehnert, G. Geisslinger, E.Niederberger

The transcription factor nuclear factor-kappaB (NF-κB) is composed of homo- or heterodimers of different subunits, which are members of a family of structurally related proteins. NF-κB is an essential regulator of the expression of hundreds of genes, many of which play an important role in the regulation of inflammation. However, the *in vivo* role of the respective Rel/NF-κB proteins (p50, p52, p65, RelB, and c-Rel) in nociception is still unknown. To elucidate the role of the p50 subunit of NF-κB in acute and persistent nociception we investigated $NF\text{-}\kappa B\ p50^{-/-}$ mice. In animal behavioural tests of (i) acute mechanical and thermal stimulation (ii) acute chemical stimulation and (iii) persistent inflammatory hyperalgesia we were able to show that $NF\text{-}\kappa B\ p50^{-/-}$ mice display a decreased nociceptive behaviour compared to wild-type mice. Furthermore, we determined the mRNA level of the NF-κB dependent gene cyclooxygenase-2 (COX-2) in the spinal cord with RT-PCR, to clarify the underlying molecular mechanisms of the observed animal behaviour. We found that 8 hours after zymosan treatment the COX-2 mRNA levels were significantly increased in wild-type mice while no significant alteration was detectable in $NF\text{-}\kappa B\ p50^{-/-}$ mice, which was in accordance with data of the chronic nociception animal model. We suggest that the p50 subunit of NF-κB plays a constitutive role in acute pain, while the contribution to persistent pain might rely on activation of NF-κB by proinflammatory stimuli. In summary, this study illustrates that the p50 subunit of NF-κB is of importance in acute and persistent inflammatory pain and may therefore be exploited in the development of new analgesics.

pharmazentrum frankfurt/ZAFES, Institut für Klinische Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

265

TNF-ALPHA MODULATES MEMBRANE CONDUCTANCE IN DRG NEURONS OF RATS

J C Czeschik, T Hagenacker, M Schäfers, D Büsselberg

TNF-alpha is known to be an important factor in the genesis of neuropathic pain after a peripheral nerve lesion. It causes hyperalgesia by inducing spontaneous neuronal activity in the spinal ganglion, an effect that is especially mediated by the TNF receptor subtype TNFR1. The mechanisms how TNF-alpha leads to spontaneous activity are not fully understood yet. We have

focused on examining the influence of TNF-alpha on voltage-gated potassium, calcium and sodium channels and the membrane conductance of rat dorsal root ganglion neurones. All currents were recorded using the whole cell patch clamp technique. The internal and external solutions as well as adequate voltage protocols were chosen to isolate the currents: Changes of the membrane conducatance were recorded using IV-ramps. From a hyperpolarization of -90mV, cells were depolarized to a final depolarization of +20mV with 10mV/100ms. Voltage-activated channel currents were elicited by a depolarization from -80mV to 0mV. For the current voltage relation of calcium, depolarization steps started at -60mV and were increased by 10mV to a maximum depolarization of +30mV (equivalent for sodium and potassium channel currents). TNF-alpha was applied in a concentration of 100ng/ml and in some cases 1000ng/ml. Voltage-gated calcium and potassium channel currents and of nociceptive DRG neurones are not influenced by TNF-alpha in a concentration of 100ng/ml. Voltage-gated sodium channels currents were unchanged in 57% of the cells, while in 43% an increase of the current of 7% ± 4% occurred after application. The membrane conductance showed a decrease of 15% ± 12% after application of TNF-alpha at a depolarisation of +20mV. Therefore, we assume that TNF-alpha acutely modulates the membrane conductance by interfering with non-voltage-gated potassium channels. It is conceivable that the knowledge of these effects of TNF-alpha and TNFR1 will help to find ways to reduce the spontaneous activity of nociceptive neurons following nerve lesions, which could result in an improvement in the difficult therapeutical treatment of neuropathic pain.

266

RATS WITH AGE-RELATED DETERIORIATION IN REFERENCE-MEMORY SHOW DIFFERENCES IN ROS FORMATION

V. Siedschlag[1], M. Loeffler[1], J.C. Cassel[2], M. Majchrzak[2], H. Harati[2], M. Shan[3], R. Haag[3], R. Jackisch[4], T.J. Feuerstein[1]

Cluster analysis of water-maze reference-memory performance in rats distinguished subpopulations of young adult (3-5 months, Y), aged (25-27 months) moderately impaired (AMI) and aged severely impaired (ASI) rats. In this study possible relationships between alterations of memory and ROS formation in mitochondria obtained from whole rat brains were investigated. ROS formation was measured either in the absence (basal ROS) or presence of 100 µM 8-*tert*-butyl-gabapentin-lactam (8B-L), a substance which increases mitochondrial ROS formation. Basal ROS synthesis was significantly increased (about 60%) in AMI rats compared to Y and ASI rats, whereas ROS formation in ASI rats was similar to that in Y rats. ROS formation in the presence of 8B-L yielded the same differences at a higher level. Considering that moderate ROS synthesis reflects the efficiency of the respiratory chain, as inhibition of ROS generation corresponds to mild uncoupling and as small decreases in $\Delta\Psi_M$ prevent ROS formation, the following conclusion is possible: AMI rats might be able to compensate their age-related cognitive deficit by increasing the efficiency of mitochondrial ATP production in memory-related neurons – in parallel to the observed increase in mitochondrial ROS formation, thus reaching the performance levels of Y rats. ASI rats, however, may be characterized by a futile effort to increase the efficiency of mitochondrial ATP production which seems to lead to insufficient performance of the neurons involved in cognitive performance.

[1] Section of Clinical Neuropharmacology, Dept. Neurosurgery, Neurozentrum, Albert- Ludwigs University, 79106 Freiburg, Germany
[2] Laboratoire de Neurosciences Comportementales et Cognitives, FRE 2855, CNRS-Université Louis Pasteur, IFR 37 Neurosciences, GDR CNRS 2905, F-67000 Strasbourg, France
[3] Department of Chemistry, Free University of Berlin, 14195 Berlin, Germany
[4] Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, 79104 Freiburg, Germany

267

DOWN-REGULATION OF SURVIVIN BY PENTACYCLIC TRITERPENOIDS INDUCES APOPTOSIS IN HUMAN GLIOMA CELL LINES

K. Pitterle, T. Syrovets, B. Büchele, Th. Simmet

More than one third of the primary brain and central nervous system tumors belong to the family of gliomas. Among these, glioblastoma multiforme is the most common and aggressive glioma in adults. These tumors are largely chemoresistant. Despite aggressive treatment, which includes neurosurgery, radiotherapy, and chemotherapy, the majority of patients die within less than one year after diagnosis. Survivin, a protein belonging to the family of Inhibitors of Apoptosis (IAPs), is a dual regulator of cell proliferation and viability. It is overexpressed in most human tumors. Previous data indicate that survivin protects cells from chemotherapeutic damage and the levels of survivin correlate with an unfavourable prognosis of several malignant tumors. We found that survivin is constitutively expressed in the human glioma cell lines U373 MG and HS 683. In vitro treatment of the cell lines with the pentacyclic triterpenoids acetyl-β-boswellic acid (AβBA) and acetyl-11-keto-boswellic acid (AKβBA) isolated from *Boswellia carterii* led to a down-regulation of survivin at the protein level in a time-dependent manner. However, there were no changes in survivin mRNA, suggesting that AKβBA and AβBA exert their effect at the level of translation, rather than at the level of transcription. The pentacyclic triterpenoids decreased the proliferation and the viability of the glioma cell lines U373 MG and HS 683 as measured by XTT assays. Upon treatment with chemotherapeutic agents glioma cells can undergo autophagy. When we treated the glioma cell lines with AβBA or AKβBA, they showed no signs of autophagy as demonstrated by the lack of LC3-I to LC3-II conversion and the absence of acidic vacuoles. The cytotoxic effects of the triterpenoids are due to induction of apoptosis, as demonstrated by formation of DNA laddering and the cleavage of the procaspase 3. Both AβBA and AKβBA are lipophilic compounds, which can penetrate the blood-brain barrier and accumulate in brain tissue, as has been demonstrated in mice. Hence, the pentacyclic triterpenoids might represent a promising class of compounds for treatment of chemoresistant brain tumors.

Institute of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, D-89081 Ulm, Germany

268

BRAIN PHARMACOKINETICS OF ANTIEPILEPTIC DRUGS FOLLOWING INTRANASAL ADMINISTRATION

M. Czapp, H. Potschka

Despite the launch of several new antiepileptic drugs (AEDs) during the last two decades pharmacoresistance of epilepsy remains an unsolved problem. It is hypothesized that local overexpression of multidrug transporters (MDTs) in the epileptogenic tissue of pharmacoresistant individuals results in limited access of AEDs to the epileptic focus region. The MDT hypothesis renders a basis for the development of new strategies to overcome pharmacoresistance. Intranasal (IN) administration of AEDs implicates the circumvention of the blood-brain barrier via neural pathways. In the present study, we tested wether a delivery of AEDs to the brain parenchyma by IN administration can be achieved and if IN administration of AEDs has an anticonvulsant effect in the amygdala kindling model of temporal lobe epilepsy. For IN administration female Wistar rats were shortly anaesthetised with propofol and AEDs were placed 2 cm deep into both nares using a plastic tubing. To obtain information about the time course of the brain distribution microdialysis experiments were performed. We compared brain concentrations of AEDs after administration in an aqueous formulation with a mucoadhesive gel formulation. In addition, AED concentrations were analysed in homogenates of whole brains or separated brain regions. Pharmacokinetic data after IN administration were compared with those following intravenous (IV) administration. Furthermore, kindling experiments were performed to test anticonvulsant effects following IN administration. IN administration of phenobarbitone resulted in significantly higher levels in the dialysate as compared to IV administration. Following IN administration phenobarbitone was successfully delivered to all investigated brain regions. In kindling experiments an anticonvulsant effect was observed following IN administration. In future studies, we will investigate wether resistance can be overcome in a rat model of intractable epilepsy.

Department of Pharmacology, Toxicology, and Pharmacy, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany
Funding supported by: CURE (Citizens United for Research in Epilepsy)

269

Withdrawn

270

BEHAVIORAL ALTERATIONS IN A MODEL OF TEMPORAL LOBE EPILEPSY PRODUCED BY INTRAHIPPOCAMPAL KAINATE INJECTION IN MICE

I. Gröticke[1,2], K. Hoffmann[1], and W. Löscher[1,2]

Epilepsy patients frequently develop psychiatric and cognitive comorbidities, but suitable mouse models modelling this phenomenon still do not exist. Constituting a model of temporal lobe epilepsy, the unilateral injection of kainic acid (KA) intra-hippocampally into the CA1 region of mice causes a non-convulsive status epilepticus (SE) as well as subsequent long-term electroencephalographic and pathohistological changes, whereas little is known about the related behavioral phenotype. We stereotaxically injected KA into the right dorsal hippocampus of NMRI mice and implanted a bipolar electrode in the same place. Sham mice received intra-hippocampal saline instead. Furthermore, a naïve control group was included in the study. The first EEG and video recording was made immediately after surgery and lasted until the next morning. Following KA administration, mice slowly developed SE lasting for several hours, whereas sham-injected mice did not show any EEG alterations. In the following weeks, EEG recordings once a week revealed the occurrence of spike-wave discharges, and generalized motor seizures could be observed in 10 out of 12 KA-treated mice. KA- and sham-injected mice as well as control mice were subjected to a behavioral test battery evaluating general health, motor deficits, exploratory behavior, anxiety-related behavior and spatial learning abilities. None of these tests revealed any significant behavioral changes in KA-injected mice except for two subtests of the Morris water maze test. In these tests, KA-treated mice revealed deficits in remembering the previously well learned platform position during retention test of the water maze procedure, and the cued version seemed to prove an intact visus but difficulties to integrate new visual information. Compared to previous experiments with the pilocarpine model of temporal lobe epilepsy in mice, our study indicates that intra-hippocampal injection of KA produces a much more discrete epilepsy with almost no behavioral alterations in a battery of test systems.

[1]Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine, Bünteweg 17, 30559 Hannover, Germany and
[2]Center for Systems Neuroscience (ZSN), University of Veterinary Medicine, Bünteweg 17, 30559 Hannover, Germany

271

CHRONIC ALTERATIONS OF PHOSPHOLAMBAN FUNCTION CAUSE HEART FAILURE VIA TWO INDEPENDENT PATHWAYS

J. P. Schmitt and M. J. Lohse

Heritable mutations in phospholamban (PLN), an inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2), were identified in families with dilated cardiomyopathy (DCM) and progressive heart failure. The objective of our study was to better define the relations between specific alterations of PLN function and fatal cardiac disease. In transgenic mice bearing the DCM causing arginine 9 cysteine PLN mutation (PLN^{R9C}) and two, one or no endogenous PLN alleles, SR Ca^{2+} uptake rates ranged from subnormal to supranormal and directly correlated with the onset of DCM in a dose dependent manner. As a second variable of PLN function, PLN^{R9C} transgenic mice lacked the ability to regulate PLN activity via PKA dependent PLN phosphorylation. *In vivo*, myocyte SR Ca^{2+} uptake rates and hemodynamic function of these mouse hearts did not respond to beta-adrenergic stimulation. Unlike the superinhibitory effect of PLN^{R9C} in the presence of PLN^{wt} ($PLN^{+/+}$+$TgPLN^{R9C}$), SERCA2 inhibition by PLN^{R9C} alone was weak (22% of normal), and cardiomyocytes expressing PLN^{R9C} but no PLN^{wt}

($PLN^{-/-}$+$TgPLN^{R9C}$) displayed faster SR calcium uptake rates than wild-type myocytes. This acceleration of calcium kinetics due to PLN^{wt} ablation resulted in three-fold longer survival of $PLN^{-/-}$+$TgPLN^{R9C}$ mice compared to $PLN^{+/+}$+$TgPLN^{R9C}$ mice (66±19 weeks versus 21±6 weeks, p<0.001). However, at later age $PLN^{-/-}$+$TgPLN^{R9C}$ mice developed DCM, impaired cardiac contraction and premature death, although it is known that fast calcium kinetics do not promote heart failure and may even protect from cardiac dysfunction. Therefore, the heart failure phenotype in $PLN^{-/-}$+$TgPLN^{R9C}$ mice cannot be explained by the altered rate of SR calcium uptake as in $PLN^{+/+}$+$TgPLN^{R9C}$ mice, but strongly suggests a second independent pathway that leads to lethal DCM due to malfunction of PLN. We propose that impaired dynamics of PLN phosphorylation and dephosphorylation that adjust SR Ca^{2+} kinetics during low and high activity of the heart provide a second potent stimulus towards heart failure.

Institute of Pharmacology and Toxicology, University of Würzburg

272

RESTORED PHOSPORYLATION OF PHOSPHOLAMBAN DURING ISCHEMIA/REPERFUSION MEDIATES THE CARDIOPROTECTIVE ACTION OF TRAPIDIL

G.Kaber, O.Sichelschmidt, M.Martin, T.Hohlfeld, K.Schrör

Protein kinase A (PKA)-dependent phosphorylation of phospholamban (PLB) is largely reduced in ischemia/reperfusion (I/R) of myocardial tissue, resulting in cytosolic Ca^{2+}-overload and subsequent tissue injury. We have previously shown in rabbits that Trapidil (TRA) activates PKA-II and reduces I/R injury. This study investigates whether the PKA-II dependent phosphorylation of PLB is responsible for these beneficial effects.Langendorff-hearts of wild-type (WT) and PLB-knock-out (PLB-KO) mice were perfused at constant pressure (80 mmHg) and subjected to zero-flow ischemia for 60 min, followed by 45 min of reperfusion (n = 15-17). Hearts were treated with TRA at a concentration that is obtained in clinical use (10µM) or vehicle (VEH).I/R resulted in a significant increase in creatine kinase (CK) release in VEH-treated WT hearts. TRA reduced this effect by more than 70% (p<0.05). Left ventricular enddiastolic pressure (LVEDP) was also elevated in VEH-treated WT hearts, amounting to 39 ± 4 mmHg, and was significantly reduced to 24 ± 4 mmHg by TRA (p<0.05) The reduced PLB-phosphorylation after I/R was significantly improved by TRA and there was a faster Ca^{2+}-uptake into the sarcoplasmic reticulum (SR) in TRA-treated WT-hearts: 2.8 vs. 1,9 counts/µg*min (p<0.05).No such changes were seen in hearts of PLB-KO mice. Treatment with TRA did not alter CK release: 1591 ± 219 vs. 1563±155 mU/(g*min) nor did it reduce the elevated LVEDP: 36 ± 5 vs. 38 ± 5 mmHg (TRA vs. VEH). There was also no change in Ca^{2+}-uptake by TRA.These data indicate that restoration of PKA-II dependent phosphorylation of PLB by TRA improves Ca^{2+}-uptake into the SR and explains the cardioprotective actions of TRA in I/R. This is a novel and injury-specific mechanism of action for a cardioprotective compound.

Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf

273

INTERFERENCE WITH COMPARTMENTALIZED cAMP-DEPENDENT SIGNALLING NETWORKS IN CARDIAC MYOCYTES – IMPLICATIONS FOR THE TREATMENT OF HUMAN DISEASE

F. Christian[1*,+], M. Szaszak[1*,•], D. Lorenz*, J.-P. v. Kries*, W. Rosenthal*[,•] and E. Klussmann*[,•]

Compartmentalisation of protein kinase A (PKA) is achieved by direct interaction of its regulatory subunits (preferentially RII) with A kinase anchoring proteins (AKAPs). In cardiac myocytes, stimulation of β-adrenoceptors leads to activation of PKA, which phosphorylates several substrates and thereby increases heart contractility. The PKA-catalysed phosphorylations are dependent upon the tethering of PKA to the substrates by AKAPs. For example, AKAP18α interacts directly with L-type Ca^{2+} channels and thereby facilitates PKA phosphorylation of the channel. The phosphorylation increases channel open probability and causes an increased influx of Ca^{2+} into the cytosol. Although changes in cardiac myocyte contractility either cause or are associated with cardiovascular disease, surprisingly few drugs are available that modulate the cardiac myocyte cAMP system. Disruption of PKA-anchoring by AKAPs inside cells using anchoring disruptor peptides that bind competitively to the AKAP interaction sites on PKA-R subunits inhibit cAMP signalling processes in disease-relevant cells, suggesting that interference with PKA-anchoring is a suitable therapeutical concept. We identified a small molecule by screening of small molecule libraries (20,000 substances) that inhibited AKAP-RII interactions in cardiac myocytes. The compound inhibited β-adrenoceptor-stimulated increases of L-type Ca^{2+}-channel currents, an effect resembling that of β-blockers. The compound apparently affected AKAP-PKA interactions without interfering with other AKAP-dependent protein interactions such as that of AKAP18α and L-type Ca^{2+} channels. The data suggest that AKAP-PKA interactions are suitable targets for disruption by small molecules, and that AKAP-dependent protein interactions may constitute a novel class of drug targets. This approach may lead to alternative strategies for the treatment of diseases associated with altered cAMP signalling (e.g. cardiovascular disease) that are not addressed effectively by conventional pharmacotherapy relying predominantly on targeting receptors and enzyme activities.

[1] authors contributed equallly
* Leibniz-Institut für Molekulare Pharmakologie, Berlin, Germany
• Institut für Pharmakologie, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Freie Universität Berlin, Germany
+ Institut für Chemie und Biochemie, Freie Universität Berlin, Germany

274

ROLE OF INHIBITORY PROTEINS $G_{i\alpha2}$ AND $G_{i\alpha3}$ FOR CARDIAC L-TYPE CALCIUM CHANNEL GATING AND SURVIVAL OF TRANSGENIC MICE

C. Klein[1], J. Matthes[1], R. Piekorz[2], W.J. Koch[3], L. Birnbaumer[4], B. Nürnberg[2], S. Herzig[1]

Sympathetic overdrive via β_1-adrenoceptors (β_1-AR) is detrimental in heart failure. The prognostic consequences are less clear for β_2-adrenoceptors (β_2-AR). β_1-AR couple only to stimulatory G-proteins, while β_2-AR activate both inhibitory (G_i) and stimulatory G-proteins. We reported that lack of $G_{i\alpha2}$ is detrimental regarding survival of mice overexpressing the human β_2-AR (β_2TG): Homozygous or heterozygous gene deletion of $G_{i\alpha2}$ ($G_{i\alpha2}^{+/-}/\beta_2$TG) leads to marked and moderate reduction of survival, respectively (Förster et al., PNAS 2003;100:14475-80). Interestingly, activity of ventricular L-type calcium-channels (LTCC) was suppressed in $G_{i\alpha2}^{-/-}$ and $G_{i\alpha2}^{+/-}/\beta_2$TG mice in a PTX-sensitive manner, along with increased expression of $G_{i\alpha3}$-protein. We now further test the hypothesis that $G_{i\alpha2}$-protein expression is (cardio-)protective, while G_{i3}-protein is of functional relevance for LTCC activity. Single-channel activity is not altered in ventricular myocytes from mice lacking $G_{i\alpha3}$ (peak ensemble average current I_{peak}; wildtype: -38±7fA, n=15; $G_{i\alpha3}^{+/-}$: -40±3fA, n=15; $G_{i\alpha3}^{-/-}$: -47±7fA, n=16; p>0.05). On the other hand survival of β_2-transgenic mice lacking one or both alleles coding for $G_{i\alpha3}$ is similar to that of mice with β_2-AR overexpression alone. First experiments performed with β_2-transgenic mice lacking $G_{i\alpha3}$ ($G_{i\alpha3}^{-/-}/\beta_2$TG) suggest that LTCC activity is increased compared to wildtype, $G_{i\alpha3}^{+/-}$ and $G_{i\alpha3}^{-/-}$ mice, respectively (open probability P_{open}; $G_{i\alpha3}^{-/-}/\beta_2$TG: 11.5±0.2%, n=2; wildtype: 4.1±0.7%, n=15; $G_{i\alpha3}^{+/-}$: 3.6±0.4%, n=15; $G_{i\alpha3}^{-/-}$: 4.6±0.6%, n=16; p<0.01; I_{peak}; $G_{i\alpha3}^{-/-}/\beta_2$TG: -58±2fA, n=2). In marked contrast, the few survivors of the line of β_2TG mice totally lacking $G_{i\alpha2}$ ($G_{i\alpha2}^{-/-}/\beta_2$TG) displayed cardiac hypertrophy and reduction of single LTCC activity (e.g. I_{peak}; $G_{i2}^{-/-}/\beta_2$TG: -19±4fA, n=3; wildtype: -49±13fA, n=19; P_{open}: $G_{i\alpha2}^{-/-}/\beta_2$TG 1.1±0.2%, n=3; wildtype: 8.9±2.0%, n=19). $G_{i\alpha3}$ is of functional relevance for ventricular L-type calcium-channels when β_2-AR signalling is enhanced. Lack of $G_{i\alpha3}$ - in contrast to $G_{i\alpha2}$ - does not aggravate mortality in β_2-AR transgenic mice.

[1]Department of Pharmacology, University of Cologne, Germany; [2]Department of Biochemistry and Molecular Biology II, University of Düsseldorf, Germany; [3]Center for Translational Medicine, University Philadelphia, USA; [4]National Institutes of Health, Research Triangle Park, NC, USA

275

FUNCTIONAL STUDIES IN MICE OVEREXPRESSING A2A ADENOSINE RECEPTORS IN THE HEART

S. Grote-Wessels[1], I. Buchwalow[2], L. Fabritz[3], N. Zimmermann[4], W. Schmitz[1], F.U. Müller[1], P. Boknik[1]

Adenosine is an endogenous purine nucleoside playing an important role in myocardial protection during stress. A_{2A}-adenosine receptor (A_{2A}-AR) up-regulation has been found in failing vs. nonfailing human hearts, whereas A_1-AR and A_3-AR expression remained unchanged. Recent studies suggested protective effects of A_{2A}-AR stimulation against acute heart failure early after myocardial infarction. To investigate the physiological and possible pathophysiological role of the A_{2A}-AR, we generated mice with a heart-directed overexpression of the human A_{2A}-AR (TG). Expression of the transgene was verified by immunofluorescence staining and real-time PCR. In TG mice (14-20 weeks) expression of A_{2A}-AR in atria was associated with a positive inotropic and chronotropic effect of the A_{2A}-AR agonist CGS21680 (1µM) in the presence of the A_1-AR receptor antagonist DPCPX (1µM), whereas in WT the agonist showed no effects. The effect was completely blocked by the A_{2A}-AR antagonist ZM248315. After pretreatment with reserpine (5 mg/kg, 16 h before killing) the effect of CGS21680 remained unchanged. CGS21680 (in the presence of 1 µM DPCPX) increased Ca^{2+}-transient amplitudes as well as cell shortening of ventricular cardiomyocytes from TG, whereas in WT no effect was observed. Echocardiographic examination of anaesthesized mice (1.5%isofluran) showed a diminished contractile response to isoproterenol (2mg/kg) in TG as evidenced by decreased fractional shortening (TG, 44%±4* vs. WT, 56%±1, *p<0.05 vs. WT) and reduced ejection fraction (TG, 75%±5 vs. WT, 87%±2, *p<0.05 vs. WT), although frequency remained unchanged between TG and WT. The heart/body weight ratio remained unchanged in TG, indicating no compensatory heart hypertrophy at this point of examination. Thus we observed a responsiveness of TG hearts to A_{2A}-AR stimulation, which is not evident in WT hearts, indicating a functional overexpression of A_{2A}-AR. In conclusion our results provide evidence that A_{2A}-AR transgenic mice present an interesting tool to further elucidate the role of A_{2A}-AR in heart.(Supported by the BfArM)

[1]Institut für Pharmakologie und Toxikologie, Universität Münster, Universitätsklinikum, Domagkstr. 12, D-48149 Münster und [2]Institut für Pathologie, D-48149 Münster und [3]Medizinische Klinik und Poliklinik C Universität Münster, Universitätsklinikum D-48149 Münster und [4]Bundesinstitut für Arzneimittel und Medizinprodukte, Kurt-Georg-Kiesinger-Allee 3, D-53175 Bonn

276

CONDITIONAL GENE DELETION IN THE CARDIAC CONDUCTION SYSTEM OF MICE – GENERATION OF HCN4-CRE KNOCK-IN MICE

E. Hoesl[1,3], S. Feil[2], R. Feil[2], F. Hofmann[3], A. Ludwig[1]

The mechanisms underlying cardiac pacemaking are only partially understood. Several genes (e.g. HCN channels, L- and T-type calcium channels, ryanodine receptors) have been implicated to play an important role in cardiac pacemaking. However, the physiological function of these genes in cardiac rhythm generation is difficult to elucidate and complicated by the fact that knock-out of most of these genes is embryonic lethal. Conditional gene deletion by the Cre/loxP system is able to circumvent this embryonic lethality. To delete genes in the cardiac conduction system in a temporally-controlled manner, we attempted to generate mice expressing Cre recombinase specifically in cardiac pacemaking cells. The hyperpolarization-activated cyclic nucleotide-gated channel HCN4 is specifically expressed in the conduction system. Hence, we created a transgenic mouse line expressing a tamoxifen-inducible Cre recombinase under control of the HCN4 promoter by knock-in of Cre in the HCN4 locus. In the resulting mice, the correct insertion of the transgene was verified by Southern blot and PCR analysis. Western blot and immunohistochemistry demonstrated that expression of HCN4 from the non-targeted allele is unchanged. Efficiency and pattern of recombination was analyzed by lacZ staining. A highly selective Cre activity restricted to the sinoatrial and atrioventricular node was observed after injection of tamoxifen. Additional ECG recordings indicated that cardiac automaticity in these

transgenic mice is normal. These results indicate that HCN4-Cre knock-in mice should represent an excellent tool for the temporally regulated deletion of any loxP-flanked gene in the murine cardiac conduction system.
[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Friedrich-Alexander-Universität Erlangen, [2]Interfakultäres Institut für Biochemie, Eberhard Karls Universität Tübingen, [3]Institut für Pharmakologie und Toxikologie, Technische Universität München

277

BIOCHEMICAL CHANGES PRECEDING ATRIAL FIBRILLATION IN CREM-TRANSGENIC MODEL

M. Matus[1], S. Grote-Wessels[1], L. Fabritz[2], P. Kirchhof[2], W. Schmitz[1], FU Müller[1].

Atrial fibrillation (AF) is the most common cardiac arrhythmia affecting millions of people worldwide. Prolonged AF leads to electrical and structural remodelling and contractile dysfunction of atrial cardiomyocyte. New strategies for the management of AF will depend on the understanding of the mechanisms underlying atrial remodelling. We studied a transgenic mouse model with cardiomyocyte-specific expression of CREM-IbΔC-X, a human cardiac isoform of the transcription factor CREM (CREM-TG). CREM-TG mice develop AF and atrial hypertrophy at the age of 12-16 weeks. To characterize changes preceding AF we studied CREM-TG mice at the age of 6-7 weeks. At this early age enlargement of both left and right atrium was detected in CREM-TG animals, accompanied by prolongation of the P wave. Freely roaming CREM-TG mice did not display AF, but frequent atrial extrasystoles. In order to identify differentially expressed atrial proteins associated with AF, we performed a proteomics-based analysis of CREM-TG atria in comparison to wild-type littermate controls (WT). As assessed by 2-D differential gel electrophoresis, 16 protein spots were significantly downregulated and 29 spots significantly upregulated in CREM-TG compared to WT (n=3). Differentially expressed protein spots were subjected to peptide mass fingerprinting for identification by MALDI-TOF mass spectrometry in combination with MS/MS analysis. One of the upregulated proteins was GRP94 (1.9-fold upregulation in CREM-TG vs. WT), a calcium-binding, stress-induced glycoprotein localized in sarcoendoplasmic reticulum. We could confirm this upregulation of GPR94 in CREM-TG atria compared to WT using quantitative Western blotting. We conclude that changes in the expression of GPR94 precede the development of AF, suggesting a functional role of GPR94 in the pathophysiology of AF. (Supported by DFG.)
[1]Institut für Pharmakologie und Toxikologie und [2]Institut für Kardiologie und Angiologie, Universität Münster, Universitätsklinikum, Domagkstr. 12, Münster

278

MicroRNAs IN HEART FAILURE

C. Groß[1], T. Fischer[1], S. Just[2], W. Rottbauer[2], E. Schmitteckert[3], M. Castoldi[4], M. Muckenthaler[4] & S. Engelhardt[1]

MicroRNAs are endogenous, single-stranded RNA molecules, which post-transcriptionally regulate gene expression. By binding to specific messengerRNAs, microRNAs inhibit translation or lead to mRNA cleavage. For the human genome more than 500 genes encoding for microRNAs are known. The function of microRNAs in the adult myocardium is unclear. Using microRNA-arrays we identified several microRNAs that are differentially expressed in heart failure. The deregulation of these microRNAs was validated by Northern blot analysis in different stages of heart failure, in mouse models as well as in human myocardium. For one of the deregulated microRNAs, miR-21, we found early and strong up-regulation in cardiac disease. In modest heart failure the expression of miR-21 was three-fold (P<0.01), in manifest heart failure almost six-fold higher than in the healthy heart (P<0.01). Upregulation of miR-21 was induced in isolated cardiomyocytes by hypertrophic stimuli. By computational analysis of the miR-21 promoter region and luciferase based reporter assays we were able to identify two essential transcription factor binding sites that regulate the transcription of miR-21 in the heart. To study the cardiac function of miR-21, we generated recombinant adenoviral vectors and transgenic mice for the miR-21 precursor molecule. Under the control of a cardiac specific promoter these mice overexpress the mature form of miR-21. Furthermore, blocking miR-21 with morpholinos in zebrafish lead to a prominent cardiac phenotype.
miR-21 is deregulated in heart failure. Aberrant expression of miR-21 may play an important role during the development of heart failure.
[1]Rudolf Virchow Center, DFG-Research Center for Experimental Biomedicine, University of Wuerzburg
[2]Department of Internal Medicine, University of Heidelberg
[3]Institute of Pharmacology and Toxicology, University of Wuerzburg
[4]Department of Pediatric Oncology, University of Heidelberg

279

GEERATION OF ENGINEERED HEART TISSUE FROM MURINE EMBRYONIC STEM CELLS

C. Rogge, H. Behr, M. Didié, T. Eschenhagen, W.H. Zimmermann

Engineered Heart Tissues (EHT) from neonatal rat cardiomyocytes can be used as an in vitro heart model and for cardiac replacement therapy. The aim of this study was to develop a technology allowing the construction of EHT from murine embryonic stem cells (ESC). This would eventually facilitate the large scale application of the EHT-technology. Murine ESC-lines expressing the neomycin resistance under α-myosin heavy chain-promoter control (cardiomyocyte specific) were generated. Embryoid body (EB) cultures were established to study the cardiogenic potential of ESC. Circular EHTs (inner/outer diameter: 2/4 mm) were constructed with $1{,}5x10^6$ ES cells, collagen and matrigel. Cardiomyocyte selection was performed with G418 (200 µg/ml) for 20 days after an initial culture period of 8 days without G418. Contractile function was assessed under isometric conditions. Morphology was examined in paraffin sections, laser scanning and electron microscopy. EBs derived from 3 independent ESC-lines (A6, A8, A9) developed spontaneous contractions after 12 culture days (65±6, 18±4, and 26±5% of the respective EBs showed contractile areas; n=3 each). EHTs generated from the genetically modified ESC developed spontaneous contractions after 16-28 culture days, and could be maintained in culture for at least 6 months without loss of contractile function (twitch tension [TT] at 1 mM calcium at 28 days vs. 6 months: 0.23±0.03 vs. 0.19 mN; n=2-3). EHTs showed a positive inotropic effect under calcium (0.4-2.4 mM: TT +169±34%; n=15) and isoprenaline (1 µM: TT +57±11%; n=10) stimulation. Morphological analyses demonstrated the development of highly differentiated muscle bundles within EHTs. Taken together, EHT can be generated from genetically modified murine ESC. This novel technology allows large scaling of the EHT-technology for substance screening applications in vitro and experimental cardiac regeneration in vivo.
Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany

280

DYSTROPHIN-DEFICIENCY INCREASES THE SUSCEPTIBILITY TO DOXORUBICIN-INDUCED CARDIOTOXICITY

S. Deng [a], B. Kulle [b], M. Hosseini [c], G. Schlüter [c], G. Hasenfuss [d], A. Schmidt [d], L. Wojnowski [a]

The clinical use of doxorubicin (DOX) and other anthracyclines is limited by a dosage-dependent cardiotoxicity, which can lead to the development of cardiomyopathy and eventually to a congestive heart failure. The role of the individual genetic makeup in this disorder is poorly understood. We hypothesized that alterations in genes encoding cardiac cytoskeleton or sarcolemma proteins may increase the susceptibility to doxorubicin-related cardiotoxicity. Female dystrophin-deficient mice (MDX) and age-matched wild-type mice underwent chronic treatment with doxorubicin. Cardiac function and tissue damage were assessed by echocardiography and histopathology, respectively. Gene expression changes induced by DOX treatment were investigated using microarrays. DOX treatment resulted in mortality, cardiac insufficiency, and cardiac interstitial fibrosis. All these alterations were significantly more pronounced in DOX-treated MDX mice than in DOX-treated wild-type mice. The accompanying changes in gene expression were more numerous in MDX mice, including genes participating in the processes of oxidative stress, cell adhesion, cytoskeleton organization, inflammatory and immune response and cell death. In conclusion, dystrophin deficiency facilitates the development and progression of doxorubicin-induced cardiac injury and heart failure. The underlying mechanisms may involve changes in cell adhesion, in cytoskeleton, as well as inflammatory and immune responses. Genetic variants of cytoskeletal proteins in humans may affect the individual susceptibility to doxorubicin-induced cardiotoxicity. Conversely, cardiotoxic drugs may accelerate the manifestation of pre-clinical cardiomyopathies caused by deficiencies in cytoskeletal or sarcomeric proteins.
[a] Department of Pharmacology, Johannes Gutenberg University, 55101 Mainz, Germany
[b] Department of Biostatistics and Department of Mathematics, University of Oslo, Norway
[c] Department of Human Genetics, University of Göttingen, 37075 Göttingen, Germany
[d] Department of Cardiology and Pneumology, University of Göttingen, 37075 Goettingen, Germany

281

DECREASED INFLAMMATION AND ECM-REMODELLING BY LONG TIME TREATMENT WITH ROSUVASTATIN IN APOE DEFICIENT MICE

A. Marzoll, G. Dai, J.W. Fischer

Key factors that contribute to instability of atherosclerotic lesions are the extent of inflammation and remodelling of extracellular matrix (ECM). Cyclooxygenase-2 (COX-2) dependent prostaglandin synthesis is known to regulate both inflammation and ECM synthesis. This study aimed to analyze the effect of chronic rosuvastatin treatment on COX-2 expression and ECM-remodelling in ApoE-deficient mice with pre-existing atherosclerosis. 20 weeks old male ApoE-deficient mice received normal fat chow diet including rosuvastatin 20 mg/kg per day or normal chow as control. After 32 weeks animals were sacrificed. COX-2 RNA expression was determined by semi-quantitative RT-PCR. Plaque size, cell density, smooth muscle cell (SMC) content of fibrous cap, COX-2 expression, collagen, collagen neoepitopes and proteoglycan accumulation (decorin, biglycan, versican, perlecan) were determined in aortic root sections. Additionally, collagen, decorin and biglycan accumulation were determined in plaques of the innominate artery by immunohistochemistry and expression levels in the aorta were evaluated by western analysis. COX-2 mRNA and protein expression were decreased in lesions of rosuvastatin treated mice. Furthermore, cell density was reduced in aortic root lesions. Fibrous cap of treated animals showed a continuous cover of SMC in contrast to a discontinuous cover in controls. Rosuvastatin increased collagen, decorin and biglycan accumulation in lesions of the aortic root and the innominate artery. In contrast, versican and perlecan expression were not altered. Collagen neoepitopes which resulted from collagenase cleavage were decreased. Western analysis showed increased biglycan and decorin levels. These effects were independent of cholesterol lowering. No differences were observed with respect to intimal thickening. Chronic treatment with rosuvastatin increased total matrix accumulation as evidenced by decreased cell density. Specially, collagen and binding proteoglycans (biglycan, decorin) accumulated in statin induced ECM-remodelling. In addition, COX-2 expression was reduced suggesting decreased inflammatory activity. These changes are indicative of the development of a stable plaque phenotype.
Molekulare Pharmakologie, Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum Düsseldorf

282

MECHANISMS OF INCREASED OXYGEN RADICAL FORMATION IN ALCOHOLIC CARDIOMYOPATHY IN A MOUSE MODEL OF ACETALDEHYDE OVERLOAD

P. Wenzel, J. Müller, S. Schuhmacher, E. Schulz, M. Oelze, T. Isse, T. Kawamoto, T. Münzel and A. Daiber

Background: The major pathogenic factor of ethanol is acetaldehyde, which is formed upon oxidation of ethanol by the alcohol dehydrogenase and is further oxidized mainly by the mitochondrial aldehyde dehydrogenase (ALDH-2). Growing evidence suggests, that NADPH-oxidases are involved in the pathogenesis of alcoholic liver disease. We therefore sought to investigate the role of NADPH-oxidase derived superoxide in the development of alcoholic cardiomyopathy (ACM) using ALDH-$2^{-/-}$ mice as an animal model of acetaldehyde overload.
Methods: Male C57B6 and ALDH-$2^{-/-}$ mice were fed with a modified low-dose Liber-diCarli-diet (2%-ethanol) or liquid control diet (control). After 5 weeks, we determined malondialdehyde formation in internal organs by dot blot, NADPH oxidase activity by lucigenin (5microM) derived chemiluminescence, expression of NOS3 and NADPH-oxidase subunits $p67^{phox}$ and rac1 in heart membrane fractions by Western blotting, cardiac fibrosis by Sirius Red staining and vascular function of isolated aortic rings in isometric tension studies.
Results: Compared to control (e.g. control-fed C57B6 mice), 2%-ethanol-fed ALDH-$2^{-/-}$ mice showed a significant increase of malondialdehyde yield in internal organs, the highest of which

appearing in heart tissue, increased heart/body and lung/body weight ratio, significantly increased cardiac NADPH activity (2fold) and expression of NOS3 (1.7 fold), $p67^{phox}$ (1.9 fold) and rac1 (4.5 fold) and increased cardiac fibrosis. Interestingly, neither control-fed-ALDH-$2^{-/-}$ nor 2%-ethanol-fed C57B6 mice yielded results significantly different from control. Vascular responses to nitroglycerin were impaired in control-fed-ADLH-$2^{-/-}$ and in 2%-ethanol-fed C57B6 and to an even greater extent in 2%-ethanol-fed ADLH-$2^{-/-}$.
Conclusions: Our results lead us to speculate, that NADPH-oxidases contribute to the development of ACM. The crucial role of acetaldehyde in this picture is underpinned by the fact, that neither control-fed ALDH-$2^{-/-}$ nor 2%-ethanol-fed C57B6 mice developped signs of cardiomyopathy or organ damage, except for the impaired vascular nitroglycerin response, indicating the important role of ALDH-2 for bioactivating nitroglycerin.
II. Medizinische Klinik der Johannes-Gutenberg-Universität Mainz, Germany (P.W., J.M., M.O., S.S., E.S., T.M., A.D.)
Department of Environmental Health, University of Occupational and Environmental Health, Yahatanishi, Kitakyushu, Japan (I.T., T.K.)

283

L-VOCC- AND SOC-DEPENDENT Ca^{2+}-INFLUX DURING M_3-RECEPTOR-STIMULATION TRIGGERS DIFFERENT SIGNAL TRANSDUCTION PATHWAYS MEDIATING TONIC CONTRACTION OF PORCINE CORONARY SMOOTH MUSCLE.

J. Weirich, L. Dumont, G. Fleckenstein-Grün

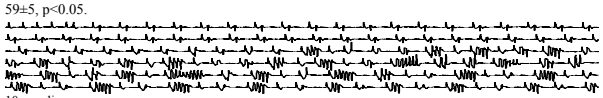
Previously, we could demonstrate M_3-receptor-mediated tonic contraction of porcine coronary smooth muscle (CSM) in the absence of L-VOCC-dependent Ca^{2+}-influx, i.e. in the presence of verapamil. Under these conditions, Ca^{2+}entry through store-operated Ca^{2+}channels (SOC) was the main source of activator $Ca^{2+}$and contraction was strongly dependent on the production of arachidonic acid (AA) by phospholipase A_2 (PLA_2) and on the AA metabolites emerging from the COX-pathway as well as on the activity of PKC and Rho-kinase (RhoK). Here, we studied by means of specific inhibitors the signal transduction pathways mediating the M_3-receptor-induced (270 nM Ach) tonic contractile response (increase in isometric tension) of CSM strips in the presence of L-VOCC-dependent Ca^{2+}-influx, i.e. in the absence of verapamil. Again, GF109203X (10 µM), an inhibitor of PKC, as well as Y-27635 (5 µM), an inhibitor of RhoK, minimized the Ach-meditated response to 10±4% and to 15±4% of control ($p<0.001$), respectively. However, 100 µM 4-BPB, an inhibitor of (PLA_2), did not affect isometric tension significantly. Likewise, 10 µM indomethacin, the inhibitor of the cyclooxygenase (COX) pathway of AA metabolism, reduced the response only to 49±9% of control ($p<0.01$), whereas 50 µM nordihydroguiaretic acid (NDGA) an inhibitor of the lipooxygenase (LOX) pathway, markedly reduced the response to 8±2% of control ($p<0.001$).
Conclusion: 1) only the Ca^{2+}-influx through SOCs is able to activate PLA_2. Thus, Ca^{2+} entering particular Ca^{2+}-entry pathways (e. g. via SOCs or via L-VOCCs) and Ca^{2+}-dependent enzymes (e. g. PLA_2) may be associated to specific subsarcolemmal compartments in CSM. 2) COX inhibitors, at least of the indomethacin-like type, preferentially suppress SOC-dependent contractile responses. 3) irrespective of the source of activator Ca^{2+}, both, PKC as well as of RhoK are key enzymes for M_3-receptor-mediated tonic contraction in CSM, probably converging to a common signalling step, thus, sensitizing the contractile apparatus to activator Ca^{2+}.
Study Group for Ca^{2+}Antagonism, Department of Physiology, University of Freiburg, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany

284

EFFECT OF CARBACHOL ON ATRIAL AND VENTRICULAR RATE AND RHYTHM IN DELTAKPQ-SCN5A MICE WITH LONG QT SYNDROME 3

L. Fabritz, D. Damke, S. Laakmann, A. Blana,, L. Fortmüller, M. Tekook, D. Volkery, G. Breithardt, E. Carmeliet, P. Carmeliet, P. Kirchhof

Arrhythmias in long QT 3 syndrome (LQT3) occur predominantly at rest. Increased vagal tone during sleep has been suggested as an arrhythmogenic trigger in LQT3 but studies in LQT3 patients are scarce.
Methods: Telemetry Holter ECGs were recorded in freely roaming adult mice with the knock-in deletion **Δ**KPQ-SCN5A and LQT3 and in WT littermates at baseline, during stress protocols, and during administration of carbachol 0.5mg/kg with or without chronic oral propranolol. Action potential duration (APD) was measured in monophasic action potentials recorded both at fix RV pacing frequency and during spontaneous rhythm at baseline and during infusion with Carbachol 10^{-7} to 10^{-5}.
Results: During administration of carbachol, both **Δ**KPQ-SCN5A and WT developed sinus bradycardia. **Δ**KPQ-SCN5A(8/8) but not WT(0/8) developed prolonged ventricular bigemini or runs of ventricular tachycardia during in vivo telemetry, $p<0.06$, see example of bradycardia, bigemini and start of runs, these continued for 30 min. In addition, runs of atrial tachycardia were observed in **Δ**KPQ-SCN5A during administration of carbachol. In contrast, no arrhythmias were documented during stress tests by isoproterenol, swimming and warm air jets. Chronic oral propranolol did not protect from carbachol induced arrhythmias in **Δ**KPQ-SCN5A, $p<0.05$.
In the isolated heart, carbachol did not prolong APD at fix pacing frequency, but induced bradycardia in both WT and **Δ**KPQ-SCN5A and pauses>5s in **Δ**KPQ ($p<0.05$ vs. WT). Carbachol-induced bradycardia prolonged spontaneous APD in **Δ**KPQ-SCN5A LQT3 compared to WT APD and compared to **Δ**KPQ-SCN5A LQT3 APD at faster heart rate, **Δ**KPQ-SCN5A LQT3, n=16, carbachol 87±5ms versus baseline 59±5, $p<0.05$.



10s per line
Conclusions: Bradycardia induced by parasympathetic stimulation is proarrhythmic and ß-adrenoreceptor block does not protect from arrhythmias induced by parasympathetic stimulation in the **Δ**KPQ-SCN5A LQT3 mouse model.
Department of Cardiology and Angiology, University Hospital Münster, Münster, Germany, Center for Transgene Technology, KU Leuven, Leuven, Belgium

285

AN IMPROVED PROTEOMICS-BASED APPROACH FOR THE ANALYSIS OF MODIFICATIONS INVOLVED IN THE CARDIOPROTECTION BY PHARMACOLOGICAL PRECONDITIONING

N. Dyballa, S. Metzger, O. Toma, W. Schlack, N.C. Hauck-Weber

Background: The volatile anaesthetic desflurane exerts cardioprotection by preconditioning *in vivo*[1]. Using a proteomic approach, we aim to identify new targets playing a trigger or mediator role in desflurane-induced preconditioning (DES-PC). Last year we presented first results obtained from a subtractive analysis of wide-range two-dimensional gel electrophoresis (2D-PAGE) of rat heart crude extracts. We got a general idea of protein distribution in control and preconditioned myocardium. But to obtain maximum information from the gel patterns it was necessary to improve the standard 2D-PAGE protocol. Materials and Methods: Optimised proteome profiling was carried out with different strategies concerning sample solubilisation and isoelectric focussing (IEF). Because the choice of buffer components is an important factor in 2D-PAGE, we improved protein solubility by the addition of the denaturing agent thiourea as well as the detergent ASB-14. To overcome streaking resulting from unprotected thiol groups in alkaline regions we blocked cysteine containing proteins with another reducing agent instead of DTT. Results: By the addition of thiourea and ASB-14 to the standard buffer we could enhance protein spot abundance by 18 %. The organic disulfide dithiodiethanol reduced horizontal streaking between spots in the alkaline pH range as well as the number of spots caused by non-specific protein oxidation so that the gels exhibit well-rounded spots. Furthermore, IEF as 1st dimension of 2D-PAGE was developed. Instead of the classical in-gel rehydration procedure we applied sample just prior to IEF by repeated cup-loading during a low voltage step. This enables an improved recovery of high M_r and alkaline proteins and allows for a more detailed analysis of ribosomal and nuclear proteins. Conclusion: Altogether, the refined 2D-PAGE procedure provides a solid basis for the identification of protein alterations induced by DES-PC. Further investigations will focus on the identification of proteins with functional role in pharmacologic-relevant signalling pathways. Especially post translational modifications will be evaluated by software-based image acquisition.
Supported by Deutsche Forschungsgemeinschaft, GRK 1089, TP 5

286

A ROLE FOR CASPASE-1 IN HEART FAILURE

S. Merkle[1], S. Frantz[2], M. P. Schön[1,3], J. Bauersachs[2], M. Buitrago[1], R. J. A. Frost[1], E. Schmitteckert[4], M. J. Lohse[4], S. Engelhardt[1]

Apoptosis of cardiomyocytes is increased in heart failure and has been implicated in disease progression. The activation of pro-apoptotic caspases represents a key step in cardiomyocyte apoptosis. In contrast, the role of pro-inflammatory caspases (caspases 1, 4, 5, 11, 12) is unclear. Here, we study the cardiac function of caspase-1. Gene array analysis in a murine heart failure model showed upregulation of myocardial caspase-1. In addition, we found increased expression of caspase-1 protein in murine and human heart failure. Mice with cardiomyocyte-specific overexpression of caspase-1 developed heart failure in the absence of detectable formation of IL-1β or IL-18 and inflammation. Transgenic caspase-1 induced primary cardiomyocyte apoptosis before structural and molecular signs of myocardial remodeling occurred (TUNEL, +410±120%, $P<0.01$). In contrast, deletion of endogenous caspase-1 was beneficial in the setting of myocardial infarction-induced heart failure. Furthermore, caspase-1-deficient mice were protected from ischemia-reperfusion-induced cardiomyocyte apoptosis (TUNEL, -70%±4%, $P<0.05$). Studies in primary rat cardiomyocytes indicated that caspase-1 induces cardiomyocyte apoptosis primarily through activation of caspases-3 and -9. In contrast to previous findings which imply a pro-inflammatory role of caspase-1, these data suggest a primary proapototic role for caspase-1 in cardiomyocytes. Our findings indicate a functional role for caspase-1-mediated myocardial apoptosis contributing to the progression of heart failure.
[1]Rudolf Virchow Center, DFG-Research Center for Experimental Biomedicine, University of Wuerzburg
[2]Department of Internal Medicine, University of Wuerzburg
[3]Department of Dermatology, Venereology and Allergology, University of Wuerzburg
[4]Institute of Pharmacology and Toxicology, University of Wuerzburg

287

IDENTIFICATION OF PI16 AS A NEW CARDIAC ANTIHYPERTROPHIC GENE BY A GENETIC YEAST SCREEN FOR SECRETED PROTEINS

R.J.A. Frost[1, 2], M.J. Lohse[2] and S. Engelhardt[1]

Cardiomyocyte hypertrophy and fibrosis are of central importance for the development of congestive heart failure. Experiments with conditioned media demonstrated communication between cardiomyocytes and fibroblasts via secreted factors. The aim of our work is the identification and characterization of proteins secreted from the heart. Using a genetic secretion trap screen with a murine cardiac cDNA-library we identified 54 non-redundant proteins putatively secreted from the heart. We then studied regulation of the corresponding mRNAs during the development of heart failure. Specifically, the mRNA expression of protease inhibitor 16 (PI16) was strongly upregulated both in murine cardiac hypertrophy (aortic banding: +242±82%, $p<0.01$) and heart failure (β_1-adrenergic receptor transgenic mice: +470±55%, $p<0.01$). We also found significant upregulation of PI16 mRNA in human heart failure (+323±160%). PI16 is a putative 489 amino acid protein with so far unknown function. We generated a specific polyclonal antibody and found also at the protein level a strong and early upregulation of PI16 expression in heart failure. *In vitro* stimulation of neonatal rat cardiomyocytes with fetal calf serum strongly induced PI16 protein expression. In cryosections of mouse hearts PI16 is found to accumulate in the extracellular space. After transfection of neonatal rat cardiomyocytes with a PI16-expressing adenovirus PI16 is secreted into the culture medium and inhibits isoproterenol/phenylephrine induced cardiomyocyte hypertrophy (isoleucine incorporation -25% at MOI 1.6, $p<0.01$). Transgenic mice overexpressing PI16 in a cardiomyocyte specific manner (PI16 TG) have smaller hearts with normal heart function as determined by cardiac catheterization. After aortic banding PI16 TG mice revealed a similar reduction of the ventricle weight compared to wild-type mice. In line with these findings, individual cardiomyocytes from PI16 TG mice were smaller (-16%, $p<0.01$) and less fibrosis occurred. Taken together, using a genetic yeast screen of a cardiac cDNA-library we identified 54 proteins putatively secreted from the heart. PI16, a newly identified secreted protein from cardiomyocytes shows strong upregulation at early stages of cardiac failure and inhibits growth of cardiomyocytes *in vitro* and *in vivo*.
University of Würzburg, [1]Rudolf-Virchow-Center, DFG-Research Center for Experimental Biomedicine and [2]Institute of Pharmacology

288

CONDITIONAL HEART-SPECIFIC EXPRESSION OF WILD-TYPE AND PHOSPHO-SITE MUTATED PROTEIN PHOSPHATASE INHIBITOR-1

Katrin Wittköpper, Ali El-Armouche, Peter Boknik, Thomas Eschenhagen

Inhibitor-1 (I-1) acts as an amplifier of β-adrenergic signaling downstream of PKA by inhibiting type-1 phosphatases (PP1) only in its PKA-phosphorylated form at Thr35. I-1 is also phosphorylated by the Ca^{2+}-dependent PKCα at Ser67 probably reducing its activity. To define the *in vivo* phospho-regulation of I-1 in the heart, we aimed at generating transgenic mouse lines with temporally regulated heart-specific expression (Tet-Off system) of myc-tagged (i) wild-type I-1 (WT), (ii) a constitutively active and truncated form of I-1 generated by phosphomimetic mutagenesis at Thr35 (I-1-T35D) and (iii) a non-phosphorylatable mutant at Ser67 generated by replacing Ser by Ala (I-1-S67A). First we tested the functionality of the myc-tagged I-1 mutants *in vitro* by generating recombinant proteins and performing PP1 activity assays before and after PKA *in vitro* phosphorylation. As expected, I-1 WT and I-1-S67A inhibited PP1 activity only in their PKA phosphorylated form with IC_{50} values of 36±2 and 16±2 nM, respectively, whereas the inhibitory capacity of I-1-T35D was almost independent of PKA with an IC_{50} of 260±2 nM. "Single" transgenesis was achieved by pronucleus injection of each construct containing a TetO-attenuated promoter driving expression of the corresponding I-1 cDNA (responder). The founders were crossed with I-1 KO until they were on a I-1 null background. The Tet-off system requires a second transgenic line with a cardiac-specific promoter (α–MHC) driving the tetracycline controlled transactivator gene (tTA). This line was also bred into an I-1 null background. Crossing the 3 responder lines with the tTA line revealed double transgenic/KO (dTG/KO) mice, which were expected to express the corresponding I-1 form only in the absence of doxycycline. Indeed, Western blotting revealed that doxycycline-free water feeding was sufficient to elicit high transgene expression in the adult mouse heart, whereas transgene expression in dTG/KO fed with doxycycline *in utero* and after birth was not detectable. This system appears to be highly suitable to temporally control cardiac I-1 transgene expression and thus to evaluate the impact of I-1 phospho-regulation *in vivo*.

Institutes of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf and University of Münster, Germany

Induzierbare und herzspezifische Expression von verschiedenen Formen des Phosphatase-Inhibitor-1 auf einem Phosphatase-Inhibitor-1 Knockout Hintergrund

289

PROTEIN PHOSPHATASE 5 SUBSTRATES IN THE MAMMALIAN HEART

F Werner[1], N Gründker[2], P Boknik[2], U Gergs[1], S Rothemund[3], W Schmitz[2], J Neumann[1]

PP5, a serine/threonine protein phosphatase expressed in all mammalian tissues including the heart, is thought to play a role in cell cycle, stress response and atrial natriuretic peptide (ANP) signaling. PP5 activity is regulated by an auto inhibitory domain and can be highly stimulated by 200µM arachidonic acid. Little is known about the physiological substrates and functions of PP5. Therefore, recombinant PP5 was expressed in a bacterial expression system and *in vitro* assays were carried out to identify putative substrates. Arrays of peptides taken from putative serine phosphorylation sites were synthesized on a cellulose membrane support via SPOT technique and were phosphorylated *in vitro* by PKA in presence of radioactive ATP. Dephosphorylation of these membrane-bound peptides in the presence of PP5 alone or after addition of arachidonic acid was studied. Putative substrates found included the IP_3 receptor (10% dephosphorylation relative to control), the L-type Ca^{2+} channel α1 (12%) and β2 subunits (31%), phospholamban (41%), ryanodine receptor (23%), β_2 adrenoceptor (13%), myosin binding protein C (51%) and the glycogen binding subunit of PP1 (26%). Dephosphorylation of threonine phosphorylated peptides was analyzed with an anti-phosphothreonine antibody. Here PP5 dephosphorylated DARPP-32 (17%) and PP1 inhibitor-1 (18%). In addition, we studied the role of PP5 using mice with heart specific overexpression of PP5. In these mice cardiac contractility as well as β–adrenergic signaling is reduced *in vivo*. By Western Blotting utilizing phospho-specific antibodies we noted a reduced phosphorylation of phospholamban at serine-16 and of the ryanodine receptor at serine-2808 (80% and 30% dephosphorylation, respectively). Moreover, measurement of ANP stimulated cGMP synthesis revealed a desensitization of the ANP receptor. In summary, we were able to identify amino acid sequences containing putative phosphorylation sites derived from cardiac proteins, which were dephosphorylated by PP5 *in vitro*. The findings in transgenic mice indicated possible *in vivo* targets of PP5.

[1]Institut für Pharmakologie und Toxikologie, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), [2]Institut für Pharmakologie und Toxikologie, Universitätsklinikum Münster, [3]IZKF Leipzig, Universität Leipzig, Germany

290

HEART SPECIFIC OVEREXPRESSION OF PP2A LEADS TO IMPAIRED CONTRACTILITY BUT PROTECTS AGAINST ISCHEMIA

N. Schulz[1], U. Gergs[1], J. Neumann[1]

Transgenic mice with heart-specific overexpression of the catalytic subunit of protein phosphatase 2A (PP2A) under control of the α-myosin heavy chain promotor developed cardiac hypertrophy and impaired contractility. The hearts of these mice also suffered from ventricular dilatation and a diminished response to β-adrenergic stimulation. The time to 50% decay of the Ca^{2+}-transient and time to 90% relaxation of the cardiomyocytes was prolonged compared to wild type mouse hearts. To further investigate the influence of increased phosphatase activity, transgenic (n=6) and wild type (n=6) hearts were exposed to ischemic stress. Therefore, isolated hearts were perfused using a work-performing modus. After equilibration hearts were subjected to a period of 120 minutes no-flow ischemia followed by reperfusion. There was a significant decrease in systolic pressure in wild type hearts after ischemia (86,8% of pre-ischemic value) wheras transgenic hearts showed a complete recovery to pre-ischemic values. Additionally, contractile parameters were reduced in wild type hearts after ischemia (+dp/dt_{max}: 34,5% of pre-ischaemic value; -dp/dt_{max}: 36,9% of pre-ischaemic value) but not in transgenic hearts. Furthermore time-parameters for relaxation were shorter in transgenic hearts ($t_{90\%}$: 77,2% of pre-ischaemic value, $t_{50\%}$: 78,3% of pre-ischaemic value), while wild type hearts returned to pre-ischaemic values. These data indicate that the increased phosphatase activity led to impaired contractility but improved post-ischaemic recovery. It is tempting to speculate that PP2A is involved in reperfusion damage.

[1] Institut für Pharmakologie und Toxikologie, Martin-Luther-Universität Halle-Wittenberg, Magdeburger Str. 4, D-06184 Halle (Saale)

291

SEROTONIN INCREASES PHOSPHORYLATION OF PHOSPHOLAMBAN IN THE HUMAN ATRIUM

J. Neumann [1], A. Simm [2], RE. Silber [2], U. Gergs [1]

In the human heart serotonin (5-HT) increases the contractile force through 5-HT_4 receptors. Functional 5-HT_4 receptors were detected in atria and ventricle and they may play a proarrhythmic role. Moreover, in human heart failure 5-HT_4 receptor mRNA was increased compared to nonfailing hearts. Due to the cardiostimulant effects of 5-HT it is suggested that 5-HT may contribute to arrhythmias in patients treated with β–adrenoceptor antagonists. It is known that 5-HT_4 receptors can increase cAMP and hence activate protein kinase A (PKA) through G_S-protein mediated activation of the adenylyl cylase. Therefore, phosphorylation of regulatory proteins like phospholamban should be increased after 5-HT administration as it is known for β–adrenergic stimulation. Here, we studied the effect of 5-HT on force of contraction and phosphorylation of phospholamban in isolated electrically driven atrial preparations from human hearts. The preparations were obtained from patients undergoing cardiac bypass surgery due to coronary heart disease. Preparations were studied under isometrical conditions driven at 0.5 Hz. Application of 2 µM 5-HT exerted a positive inotropic effect: force of contraction was increased by 514% (n = 9). A further set of preparations was incubated either with 5-HT (2 µM) or buffer as control and after 5 minutes atria were frozen in liquid nitrogen. The phosphorylation of phospholamban at serine-16 and threonine-17 was analysed by Western-Blotting using phospho-specific antibodies. We noted an increased phosphorylation at serine-16 as well as at threonine-17 in 5-HT treated atrial preparations compared to control (n = 3-4). In summary, we demonstrated a correlation between the positive inotropic effect of 5-HT and the phosphorylation of phospholamban, indicative of activation of the adenylyl cyclase pathway by 5-HT in human atria.

[1]Institut für Pharmakologie und Toxikologie, [2]Klinik für Herz – und Thoraxchirurgie, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany

292

DIFFERENTIAL PHOSPHORYLATION-DEPENDENT REGULATION OF CONSTITUTIVELY ACTIVE AND MUSCARINIC-RECEPTOR ACTIVATED $I_{K,ACH}$ CHANNELS IN PATIENTS WITH CHRONIC ATRIAL FIBRILLATION

Voigt N, Christ T, Wettwer E, Bock M, Knaut M, Strasser RH, Ravens U, Dobrev D

In chronic atrial fibrillation (cAF) the potassium current $I_{K,ACh}$ develops agonist-independent constitutive activity. We hypothesized that abnormal phosphorylation-dependent regulation underlies the constitutive $I_{K,ACh}$ activity. We used voltage-clamp technique and biochemical assays to study $I_{K,ACh}$ regulation in atrial appendages from 55 sinus rhythm (SR) and 26 cAF patients. In voltage-clamped atrial myocytes, basal current was higher (at -100 mV: cAF, -20.7±1.2 pA/pF, n=48/20 [myocytes/patients] vs. SR, -12.8±0.7 pA/pF, n=108/48, P<0.05), whereas the M-receptor (2 µM carbachol) activated $I_{K,ACh}$ was lower (-6.3±1.0 pA/pF vs. -11.0±1.0 pA/pF; P<0.05) in cAF than in SR. Blockade of type-2A phosphatase and the subsequent shift to increased M-receptor phosphorylation (and inactivation) reduced M-receptor activated $I_{K,ACh}$ in SR but not in cAF pointing to an impaired function of G-protein-coupled receptor kinase. Using subtype selective kinase inhibitors we found that in SR the M-receptor activated $I_{K,ACh}$ requires phosphorylation by protein kinase C (PKC) and calmodulin-dependent protein kinase II (CaMKII), but not by PKA. In cAF, constitutive $I_{K,ACh}$ activity results from abnormal channel phosphorylation by PKC but not by CaMKII, whereas the additional M-receptor mediated $I_{K,ACh}$ activation occurs apparently without involvement of these kinases. In cAF, the higher protein level of PKCε but not PKCα, PKCβ_1 or PKCβ_2 is likely to contribute to the constitutive $I_{K,ACh}$ activity. These results indicate that the occurrence of constitutively active $I_{K,ACh}$ in cAF results from abnormal PKC function, whereas the additional M-receptor mediated $I_{K,ACh}$ activation does not require contribution of PKC or CaMKII. Selective drug targeting of constitutively active $I_{K,ACh}$ channels may be suitable to reduce the ability of AF to sustain.

Departments of Pharmacology and Toxicology (N.V.,T.C.,E.W.,U.R.,D.D.), Cardiology (M.B., R.H.S.) and Cardiosurgery (M.K.), Dresden University of Technology, 01307 Dresden, Germany

293

REGULATION OF ATRIAL CALCIUM HANDLING IN ATRIAL TACHYCARDIA AND CONGESTIVE HEART FAILURE DOG MODELS OF ATRIAL FIBRILLATION

R Wakili*,#, Y-H Yeh§, S Kääb*, U Ravens#, S Nattel§, D Dobrev#.

Background: Atrial tachycardia remodeling (ATR) and congestive heart failure (CHF) produce different substrates for atrial fibrillation (AF). We hypothesized that abnormal phosporylation-dependent regulation of key Ca^{2+}-handling and myofilament proteins contributes to the arrhythmogenic substrates in ATR and/or CHF.

Methods: ATR was induced by 7-day atrial tachypacing at 400/min; CHF by 2-wk ventricular tachypacing at 240/min. Induced AF duration was increased in both ATR and CHF groups. Total and phosphorylated levels of proteins were analyzed by Western blot in right atrial samples from 14 control (Ctl), 10 ATR and 10 CHF dogs. Results: Protein levels of SERCA were lower in CHF than in Ctl and ATR dogs, with no significant change in its endogenous inhibitor phospholamban (PLB; see table). Phosphorylation (and inactivation) of PLB at Thr-17 (CaMKII-site) was higher in CHF than in Ctl with no change in phosphorylation of PLB at Ser-16 (PKA-site) suggesting increased SERCA function. PKA phosphorylated myosin binding protein C (MyBP-C) was decreased in CHF and ATR, whereas PKA phosphorylation of troponin-I (Tn-I) was higher in CHF only indicating altered Ca^{2+} sensitivity of myofilaments. Conclusions: In CHF, but not in ATR, abnormal Ca^{2+}-handling contributes to the arrhythmogenic substrate favoring AF maintenance and accounting for impaired contractility.

PROTEIN	CONTROL	ATR	CHF
SERCA	1.20 ± 0.10	1.06 ± 0.08	0.75 ± 0.08*
Phospholamban (PLB)	1.34 ± 0.07	1.17 ± 0.09	1.13 ± 0.15
PLB-Thr17 / PLB	0.36 ± 0.08	0.49 ± 0.14	0.81 ± 0.12#
PLB-Ser16 / PLB	0.46 ± 0.14	0.47 ± 0.16	0.53 ± 0.11
MyBP-C-Ser282 / MyBP-C	0.45 ± 0.09	0.26 ± 0.05#	0.14 ± 0.03#
Tn-I-Ser23/24 / Tn-I	2.87 ± 0.12	3.15 ± 0.21	3.67 ± 0.19*

*p<0.05 vs. Ctl and ATR; #p<0.05 vs. Ctl; values are arbitrary units normalized to GAPDH (mean±SEM)

*Medical Hospital I, Klinikum Großhadern, University of Munich, 81377 Munich, Germany
§Montreal Heart Institute, Université de Montréal, Montreal PQ, Canada
#Department of Pharmacology and Toxicology, Technical University of Dresden, 01307 Dresden, Germany

294

A NEW CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA-ASSOCIATED *CASQ2* MUTATION CAUSES HYPERGLYCOSYLATION, LOWER CALCIUM BINDING, AND DEFECTIVE SR CALCIUM RELEASE

D. Wehrmeister[1], F.U. Müller[1], J. Neumann[2], A.V. Postma[3], W. Schmitz[1], E. Schulze-Bahr[4], A.A. Wilde[3], U. Kirchhefer[1]

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a highly malignant, inherited disorder characterized by stress-induced ventricular arrhythmias in the absence of structural alterations of the heart. Mutations of the *CASQ2* gene are associated with an autosomal recessive or dominant form of CPVT. The importance of calsequestrin (CSQ) is underscored by its high Ca^{2+} storage capacity and its structural and functional contribution to the Ca^{2+} release of the sarcoplasmic reticulum (SR). Here, we characterized a new *CASQ2* mutation, K206N, which transmitted autosomally and is associated with CPVT and sudden cardiac death in a young patient. To study the functional effects of this mutation, we expressed CSQ^{K206N}, in comparison to the native form (NT) of CSQ, in Sf9 insect cells by use of a baculovirus and in HEK293 cells or isolated neonatal mouse cardiomyocytes with the help of an adenoviral expression system. The mutation resulted in the insertion of an additional glycosylation site. As expected, the hyperglycosylated form of CSQ ran at a higher molecular mass on SDS-PAGE. Incubation with endoglycosidase H reversed completely the hyperglycosylation of the high mannose form CSQ^{K206N}. Hyperglycosylation of CSQ^{K206N} may favor the degradation of the protein which was corroborated by a lower protein expression at higher MOIs (>10) of CSQ^{K206N} compared to CSQ^{NT} in all cell lines tested. Moreover, Ca^{2+} binding properties were tested by an overlay technique using $^{45}Ca^{2+}$. Ca^{2+} binding was reduced at 1 mM added Ca^{2+} by 54% in homogenates of Sf9 insect cells infected with CSQ^{K206N} compared to CSQ^{NT} (n=4, P<0.05). Measurement of Ca^{2+} transients was performed in isolated neonatal mouse cardiomyocytes, which were loaded with indo-1 and stimulated at 0.5 Hz. Application of 10^{-6} mol/L isoproterenol resulted in a higher rate of spontaneous Ca^{2+} release and Ca^{2+} oscillations in CSQ^{K206N} compared to CSQ^{NT} cardiomyocytes. However, protein expression of SERCA2a was unchanged between CSQ^{K206N} and CSQ^{NT} cardiomyocytes. In conclusion, CSQ^{K206N} leads to hyperglycosylation, a reduced Ca^{2+} binding, and a defective SR Ca^{2+} release. We suggest that an impaired folding of the mutated CSQ may trigger the development of CPVT in patients.

This study was supported by the Deutsche Forschungsgemeinschaft (Ki 653/13-1).

[1]Institut für Pharmakologie und Toxikologie, Universität Münster, Universitätsklinikum, 48149 Münster; [2]Institut für Pharmakologie und Toxikologie, Martin-Luther-Universität Halle-Wittenberg, 06112 Halle; [3]Experimental and Molecular Cardiology Group, Academic Medical Center, Amsterdam, The Netherlands; [4]Medizinische Klinik und Poliklinik C, Universität Münster, Universitätsklinikum, 48149 Münster

295

CARDIAC DYSFUNCTION AND DECREASED SURVIVAL OF BIGLYCAN-DEFICIENT MICE AFTER MYOCARDIAL INFARCTION

[1]Westermann D, [2]Mersmann, [3]Melchior A, [3]Freudenberger T, [4]Petrik C, [5]Lüllmann-Rauch R, [6]Young MF, [7]Levkau B, [8]Baba H, [3]Unger Th, [2]Zacharowski K, [1]Tschöpe C, [3]Fischer JW.

The small Leucin-rich repeat proteoglycan biglycan (BGN) is involved in regulation and organization of collagen fibril formation. After myocardial infarction (MI) extensive remodelling of extracellular matrix (ECM) and inflammation occurs. The role of BGN in cardiac ECM remodelling and possible contribution to regulation of cardiac hemodynamic function was investigated in BGN-deficient (BGN-KO) mice either (i) *in vivo* after experimental myocardial infarction (MI) and (ii) *in vitro* by characterization of myofibroblasts. MI was induced by ligation of left anterior descending coronary artery. Morphology and differences in left ventricular (LV) hemodynamic function, assessed by pressure-volume measurements *in vivo,* were analysed 5 and 21 days after MI. (ii) Differences in migration and proliferation as well as collagen gel contraction were analysed in isolated myofibroblasts from BGN-KO mice. BGN expression was increased at 3, 7 and 14 days after MI. Notably, infarct scar was significantly reduced in BGN-KO mice at 5 days. Within 21 days post MI mortality among BGN-KO mice increased due to frequent ventricular ruptures. Surviving BGN-KO animals displayed impaired LV-function at 21 days shown by enlarged dilatation after MI. BGN-KO fibroblasts were characterized by increased proliferation and collagen gel contraction. In conclusion these data show that BGN is required for adaptive remodelling and collagen organization which are acquired for infarct scar stabilization and preservation of cardiac function. Furthermore BGN might have a function in controlling the proliferative response during hypertrophic remodelling.

[1]Abteilung für Kardiologie und Pneumologie, Charite-Universitätsklinikum Berlin, [2]Molekulare Kardioprotektion & Entzündung, Abteilung für Anästhesie, Heinrich-Heine-Universität Düsseldorf; [3]Molekulare Pharmakologie, Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität Düsseldorf, [4]Center for Cardiovascular Research, Institut für Pharmakologie und Toxikologie, Campus Charite-Mitte, Charite-Universitatsmedizin Berlin, [5]Institut für Anatomie, Christian-Albrechts-Universität Kiel, [6]Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892, [7]Institut für Pathophysiologie und [8]Institut für Pathologie, Universitätsklinikum Essen.

296

MYOCARDIUM SPECIFIC IDENTIFICATION OF CREB TARGET GENES VIA CHROMATIN IMMUNOPRECIPITATION (ChIP)

W.K.Liem[1], M.D. Seidl[1], K. Tenbrock[2], W. Schmitz[1], F.U. Müller[1]

The transcription factor CREB mediates transcriptional activation in response to various second messengers including cAMP. Transcriptional activation by CREB requires its binding to the cAMP-response element (CRE; TGACGTCA and variants thereof) in the promoter region of the respective target genes. CREs were identified in the promoters of multiple genes, which are therefore potentially regulated by CREB. However, little is known about the binding of CREB to potential CRE binding sites in specific tissues. We attempt to determine CREB target genes in H9c2 myoblasts and in rat and mouse myocardium to identify CREB-occupied CRE sites, using the ChIP assay to study the interaction of DNA and proteins in an instantaneous and physiological representation of nuclear events. Samples were incubated with formaldehyde to crosslink consensus-element bound transcription factors to the DNA. The DNA was sheared and the fragments crosslinked to CREB were isolated via an immunoprecipitation with a CREB-specific antibody. The enrichment of isolated CRE binding sides of different genes was quantified by Real-Time PCR. The binding of CREB to the c-fos promoter was increased 7 fold in myoblasts and 2 fold in the rat and 40 fold in the mouse heart against a ChIP with an IgG antibody as a control. The binding of CREB to the transcription factor ICER showed a more than 30-fold significant increase in the mouse heart. No binding of CREB was detected in the negative control gene GAPDH (not regulated by CREB) and in the antibody free control reactions. In conclusion, the ChIP technique is an appropriate method to study DNA-protein interaction in heart tissues. Moreover, it appears to be possible to analyse thousands of promoters in the heart simultaneously, combining the ChIP assay with microarray techniques (ChIP on chip). This might be a valuable tool to elucidate the gene regulation in the heart for example in pathophysiological conditions. (Supported by the DFG.)

[1]Institut für Pharmakologie und Toxikologie, Universität Münster, Universitätsklinikum, Domagkstr.12, D-48149 Münster.
[2]Institut für experimentelle Dermatologie, Universität Münster, Universitätsklinikum, Röntgenstr. 21, D-48149 Münster.

297

ATP MODIFIED CONTRACTION IN THE HUMAN HEART

U Gergs[1], P Boknik[2], W Schmitz[2], A Simm[3], RE Silber[3], P Illes[4], J Neumann[1]

Extracellular ATP has many properties in the cardiovascular system. These include negative and positive inotropic effects (species dependent), negative chronotropic and dromotropic as well as anti-hypertrophic effects. High extracellular ATP can occur locally during ischemia or by a local release from platelets. ATP can act via P2-purinoceptors which are further divided in $P2X_{1-7}$ -and $P2Y_{1-14}$-receptors. The inotropic effects of ATP in the human heart have hitherto not been reported. We studied contractile effects in isolated electrically driven (1Hz) atrial preparations from patients undergoing cardiac bypass surgery. ATP (100µM) rapidly decreased force of contraction (NIE) and thereafter, more slowly increased force of contraction. The maximum positive inotropic effect (PIE) amounted to 152% of pre drug value (n=9, range: 114 to 206%), was stable after 5 min and could be washed out rapidly. The PIE did not affect the time parameters of contraction. This argues against a cAMP-mediated PIE of ATP, as β-adrenoceptor-stimulation under the same conditions shortens these time parameters. Moreover, a similar PIE was noted with 100µM ATPγS, which is resistant to degradation. This argues against an adenosine-mediated effect of ATP. In a second set of experiments preparations were preincubated for 30 min with P2-antagonists, and in their continuous presence, ATP was applied. However, the PIE as well as the NIE of ATP could not be blocked with either suramin (100µM and 500µM), PPADS (50µM), reactive blue 2 (500µM) or TNP-ATP (100µM), which are used to block subtypes of P2X- and/or P2Y-receptors. From these data we suggest that ATP may decrease and increase force of contraction via $P2X_{4-6}$ and/or $P2Y_4$-receptors. In summary, we describe a P2-purinoceptor mediated biphasic effect of ATP on force contraction in the human heart. The exact receptor subtype needs to be elucidated as well as the involved signal transduction pathway.

[1]Institut für Pharmakologie und Toxikologie, [3]Klinik für Herz– und Thoraxchirurgie, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), [2]Institut für Pharmakologie und Toxikologie, Universitätsklinikum Münster, [4]Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Universität Leipzig, Germany

298

CHRONIC ADMINISTRATION OF ROFECOXIB DOES NOT INDUCE HISTOLOGICAL LESIONS IN THE RAT MYOCARDIUM

D. Kouvelas [1], C. Pourzitaki [1], E. Delvizi[2], A. Alvanou[3], T. Papamitsou[3], E. Tsoukali[2]

Selective cyclooxygenase-2 (COX-2) inhibitors are suspected of increasing risk for cardiovascular complications due to their vasoconstrictive and thrombogenic actions. The purpose of the present study was to investigate whether chronic administration of rofecoxib, a COX-2 inhibitor, affects the myocardium. Therefore, levels of creatine phosphokinase (CPK) and troponin were measured during administration of rofecoxib. The rat myocardium was also histologically examined. Furthermore, the pharmacokinetic reaction of this COX-2 inhibitor was monitored. Male Wistar rats were divided into four groups, each group receiving rofecoxib (25 mg/kg, s.c.) once daily, either for 10, 20, 30, or 45 days. After that, animals were sacrificed, and blood samples were taken for determination of CPK, troponin, and levels of rofecoxib. Levels of

CPK and troponin were colorimetrically detected; levels of rofecoxib were determined using a high pressure liquid chromatography (HPLC) with UV detection. For histological investigation of the rat myocardium, hematoxylin-eosine stained slices were examined by photonic microscopy. Chronic administration of rofecoxib caused steadily increasing levels of both, CPK and troponin. However, the myocardium did not show signs of histological lesions in any group of rats. Observing the concentration of rofecoxib by time indicated a steady state after 10 days of administration that lasted for 30 days. After 45 days the concentration continued increasing. This further rise of drug concentration possibly points to a loss of metabolic control due to cumulative hepato- and/or nephrotoxic effects of the drug. Taken together, even if rofecoxib caused elevated levels of CPK and troponin, a histological damage to the myocardium could not be observed.

[1]Laboratory of Pharmacology, [2]Laboratory of Toxicology and Forensic Medicine, [3]Laboratory of Histology and Embryology, Medical School, Aristotle University of Thessaloniki

299

ACUTE PHASE OF DAUNORUBICIN CARDIOMYOPATHY IS ASSOCIATED WITH AN ADAPTIVE AUGMENTED FUNCTION OF ISOLATED CARDIOMYOCYTES

J. Klimas[1], D. Kucerova[1], A. Gazova[2], J. Kmecova[1], P. Krenek[1], P. Boknik[3], J. Kyselovic[1]

Usage of anthracyclines impairs cardiac function. We tested whether the reduced left ventricular performance is due to a decreased function of isolated cardiomyocytes in the acute phase of daunorubicine-induced cardiomyopathy. Wistar rats were treated for two weeks with daunorubicin (DAU, 3mg/kg, i.p., dosage in 48 h interval), control rats (CON) received vehicle. The hearts of DAU rats showed a loss of heart weight (see table, mean±SEM) and this was associated with a loss of cardiomyocytes on histological level. We observed an impaired contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) of left ventricle using left ventricular catheterization. However, isolated cardiomyocytes of DAU (paced at 0.5 Hz) exhibited augmented cell function. Expression of RyR was increased in left ventricular homogenates from DAU rats (100±26% vs 212±40%, P<0.05, n=8-10, per group).

	CON	DAU
LV catheterization (n)	7	7
dP/dt_{max} (mmHg/s)	4976±503	2893±435*
dP/dt_{min} (mmHg/s)	-4144±304	-2868±297*
Isolated cells (animals/cells)	5/40	5/45
Cell shortening (%)	5.1±0.4	6.7±0.5*
Time to 50% relaxation (ms)	138.8±6.2	117.2±7.0*

We found a contradiction between the function of the whole heart and isolated cardiomyocytes during the acute phase of daunorubicin-induced cardiomyopathy. We suppose that the elevated function of single cells as adaptive response to loss of cardiomyocytes cannot rescue the decreased left ventricular function at this stage.

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University, 832 32 Bratislava, Slovak Republic; [2]Institute for Pharmacology, Medical Faculty, Comenius University, 811 08 Bratislava, Slovak Republic, [3]Institut für Pharmakologie und Toxikologie, Universitätsklinikum Münster, 48149 Münster, Germany

300

Regulation of hyaluronic acid (HA) in vascular smooth muscle cells (VSMC) by cytokines

Rabausch B., de Groot J., Fischer J.W.

Accumulation of hyaluronic acid is characteristic for atherosclerotic and restenotic lesions. HA is synthesized by three HA-synthase (HAS) isoforms (HAS1-3). HA regulates fundamental processes such as migration and cell proliferation. However, the functional significance of the individual HAS-isoenzymes is unknown. The aim of the present study was to identify factors that regulate HAS3 and to investigate the function of HAS3 in human arterial smooth muscle cells (SMC). SMC from coronary arteries were used at 60-80% confluency. Interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), classical proinflammatory cytokines, mediate proatherogenic processes. Therefore we used IL-1ß and TNFα to investigate the regulation of HA. HA synthesis and the pericellular HA-coats were strongly stimulated in quiescent SMC after incubation with IL-1ß (10 ng/ml). However only HAS3-mRNA is specifically induced by IL-1β and TNFα. HA synthesis and HAS3-mRNA induction by IL-1β could be prevented by dexamethasone (100 nM) and the NFκB inhibitor Bay11-7082 (10 µM), suggesting IL-1β induces HA and HAS3 via NFκB activation in VSMC. Other factors such as PDGF-BB, vasodilatory prostaglandins and thrombin had no effect on HAS3 expression. Subsequently SMC were transfected with HAS3-siRNA or non-silencing control-siRNA to specifically study the function of HAS3. HAS3-siRNA significantly reduced HAS3-mRNA levels to 50±22% of control cells. Suppression of HAS3 expression caused (i) decrease in cell spreading to 59.4±9.2% (n=4, p<0.05), (ii) reduction of FCS induced migration as determined by a modified boyden chamber assay (57±8%, n=3, p<0.05) and (iii) inhibition of FCS induced DNA synthesis to 63± 8% of controls (n=3, p<0,05) as determined by [^{3}H]-thymidine incorporation.

These findings demonstrate for the first time that HA synthesis by HAS3 (i) occurs specifically in response to the proinflammatory cytokines such as IL-1ß, (ii) participates in the regulation of cell shape and spreading, (iii) supports migration and proliferation. Therefore HAS3 mediated HA-synthesis might support the proliferative and migratory phenotype of SMC in inflamed regions of the atherosclerotic plaques.

Institut für Pharmakologie und Klinische Pharmakologie, Moleculare Pharmakologie, Universitätsklinikum Düsseldorf, Universitätsstraße1, 40225 Düsseldorf

301

HEME OXYGENASE-1: A NOVEL KEY PLAYER IN THE DEVELOPMENT OF TOLERANCE IN RESPONSE TO ORGANIC NITRATES

A. Daiber[a], P. Wenzel[a], M. Oelze[a], M. Coldewey[a], M. Hortmann[b], A. Seeling[c], D. Stalleicken[d], J. Lehmann[c], H. Li[b], U. Förstermann[b], T. Münzel[a]

Background: Previously we and others have proposed that nitrate tolerance as well as cross-tolerance is likely due to increased production of reactive oxygen species (ROS) leading to an inhibition of the nitroglycerin (GTN)-metabolizing enzyme mitochondrial aldehyde dehydrogenase (ALDH-2) as well as to decreased nitric oxide (NO) bioavailability and/or impaired NO signaling. Importantly, pentaerithrityl tetranitrate (PETN) treatment has been shown to induce no tolerance and to possess antioxidant properties *in vitro* and *in vivo*.

Methods: Wistar rats were chronically infused with PETN (10.5µg/kg/min for 3d) or GTN (6.6µg/kg/min for 3d). Blood pressure (BP) was assessed by telemetry-assisted catheter method, endothelial function was assessed by isometric tension recording, protein expression by Western blot and mRNA levels by RT-PCR analysis. ROS were measured by chemiluminescence, ALDH-2 activity was determined by HPLC and heme oxygenase-1 activity by plasma bilirubin levels.

Results: Chronic PETN treatment, in contrast to GTN, did not induce nitrate tolerance or cross-tolerance. The protein and mRNA expression of the antioxidant enzymes heme oxygenase-1 (HO-1) and ferritin was increased (1,6 and 1,5-fold, respectively) in response to PETN but not GTN infusion (p<0,05). In contrast to GTN therapy, NO signalling, ROS formation and the activity of the GTN/PETN metabolizing enzyme ALDH-2 were not significantly modified upon chronic PETN treatment. PETN and GTN caused a small decrease in BP on the first day of infusion which was still present on the 6th day of PETN infusion but resulted in an increased BP for GTN on day 6. The inhibitor of HO-1 expression apigenin induced a tolerance-like impaired PETN potency in PETN *in vivo* treated rats whereas the HO-1 inducer hemin dramatically improved tolerance in GTN *in vivo* infused rats.

Conclusions: *In vivo* PETN-treatment in Wistar rats induces neither nitrate tolerance nor cross-tolerance. These beneficial effects may be explained, at least in part, by induction of the antioxidant enzyme HO-1 and ferritin, and by the lack of stimulation of ROS production by PETN and are not shared by GTN.

[a] Klinikum der Johannes Gutenberg-Universität Mainz, II. Medizinische Klinik, Kardiologie, 55101 Mainz, Germany
[b] Institut für Pharmakologie, Johannes Gutenberg-Universität Mainz, 55131 Mainz
[c] Institut für Pharmazie, Friedrich-Schiller-Universität Jena, Philosophenweg 14, D-07743 Jena, Germany
[d] Alpharma-Isis GmbH & Co. KG, 40764 Langenfeld, Germany

302

MECHANISMS UNDERLYING RECOUPLING OF ENOS BY HMG-COA REDUCTASE INHIBITION IN AN ANIMAL MODEL OF DIABETES MELLITUS

M. Brandt[1], P. Wenzel[1], A. Daiber[1], M. Oelze[1], E. Closs[3], J. Xu[3], T. Thum[4], J. Bauersachs[4], G. Ertl[4], M.-H. Zou[3], U. Förstermann[2] and T. Münzel[1]

Objective: NADPH-oxidases and endothelial nitric oxide synthase (eNOS) uncoupling are important sources of superoxide in diabetes mellitus. HMG-CoA-reductase-inhibition has been shown to upregulate GTP-cyclohydrolase-I, the key enzyme for tetrahydrobiopterin *de-novo*-synthesis and to normalize tetrahydrobiopterin levels in hyperglycaemic endothelial cells. We therefore sought to determine how HMG-CoA-reductase-inhibition contributes to eNOS-recoupling *in vivo* in a rat model of diabetes mellitus.

Research design and methods: In male Wistar rats, diabetes mellitus was induced by a single *i.v.* injection of streptozotocin (STZ, 60 mg/kg). After 7 weeks of atorvastatin feeding (20 mg/kg/d), we analyzed vascular function by isometric tension studies, levels of circulating endothelial progenitor cells (EPCs) by FACS, superoxide formation by lucigenin enhanced chemiluminescence and dihydroethidium-staining, vascular levels of the phosphorylated VAsodilator Stimulated Phosphoprotein (P-VASP), tyrosine nitrate of the prostacyclin synthase (PIGS-3NT), expression of GTP-cyclohydrolase-I, dihydrofolate reductase, eNOS and the NADPH-oxidase subunits rac1, $p47^{phox}$, $p67^{phox}$, nox1 and nox2 by Western blot and RT-PCR and vascular tetrahydrobiopterin levels by HPLC.

Results: In hyperglycemic rats, atorvastatin normalized impaired responses to endothelium-dependent vasodilators, levels of circulating EPCs, vascular superoxide formation, PGIS-3NT, P-VASP, expression of NADPH-oxidase subunits, eNOS and GTP-cyclohydrolase-I and tetrahydrobiopterin levels significantly. In addition, dihydroethidium-staining revealed, that the decrease of vascular superoxide production by atorvastatin was at least in part due to eNOS recoupling.

Conclusion: Apart from normalizing NADPH-oxidase mediated superoxide formation, atorvastatin reduced vascular oxidative stress by recoupling eNOS due to regeneration of the key enzyme for tetrahydrobiopterin-synthesis, GTP-cyclohydrolase-I, leading to an amelioration of endothelial dysfunction in the setting of diabetes mellitus.

[1] Second Medical Clinic, Department of Cardiology and Angiology, Johannes Gutenberg University Mainz, Germany
[2] Department of Medicine and Endocrinology, University of Oklahoma Health Science Center, Oklahoma City, OK, USA
[3] Department of Pharmacology, Johannes Gutenberg University Mainz, Germany
[4] Medical Clinic I, Department of Cardiology, Bavarian Julius-Maximilians-University Würzburg, Germany

303

ROLE OF RHOGEF PROTEINS IN DOCA-SALT INDUCED HYPERTENSION

*M. Lukasova, *A. Wirth, *N. Wettschureck, #S. Gutkind, *S. Offermanns

There is increasing evidence that the Rho/Rho-kinase pathway is involved in the pathogenesis of several cardiovascular disorders, including hypertension. Various receptor classes among which are also G-protein-coupled receptors (GPCRs) are able to activate Rho through guanine nucleotide exchange factor (GEF) proteins which catalyze the exchange of GDP for GTP on Rho GTPases resulting in their activation. GPCRs mediate RhoA activation primarily via the $G\alpha_{12}/G\alpha_{13}$-mediated activation of a subfamily of RhoGEF proteins including LARG, PDZ-RhoGEF and p115 RhoGEF. Based on our findings that smooth muscle specific $G\alpha_{12}/G\alpha_{13}$ double deficient mice show normal basal blood pressure but severely impaired hypertensive response to DOCA-salt treatment, we have investigated the role of these three RhoGEF proteins in DOCA-salt induced hypertension.

The expression of RhoGEF proteins was analyzed using quantitative RT-PCR with cDNAs prepared from aortic smooth muscle layers of control and DOCA-salt treated animals. LARG was found to be the most abundantly expressed RhoGEF protein in the aortic media. DOCA-salt treatment led to a significant upregulation of LARG in aortic media on the mRNA level. Uni-nephrectomized LARG $^{-/-}$, PDZ-RhoGEF $^{-/-}$ and wildtype mice received a subcutaneous pellet releasing 50mg DOCA for 21 days and 1% NaCl in drinking water. Telemetric blood pressure measurements were performed in awake

animals. The basal blood pressure of LARG $^{-/-}$ and PDZ-RhoGEF $^{-/-}$ mice was not different from that of wildtype animals. DOCA-salt treatment led to a development of hypertension in wildtype and PDZ-RhoGEF $^{-/-}$ mice whereas DOCA-salt induced hypertension was severely reduced in LARG $^{-/-}$ mice. Therefore we conclude that LARG is an important mediator of DOCA-salt induced increase in vascular tone.

*Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg. #National Institute of Health, NIDCR, USA.

304

ACUTE EFFECTS OF PACLITAXEL AND SIROLIMUS ON THE VASOMOTOR RESPONSE OF RAT MESENTERIC ARTERIES

K. Leineweber, M. Vogelsang, G. Heusch

Background: Paclitaxel (PAC)- and sirolimus (SIR)-eluting stents reduce the occurrence of angiographic restenosis and the need for repeated revascularisation after percutaneous coronary interventions. However, recent clinical studies suggest that they might also induce adverse effects: impaired vasodilation and possibly vasospasm.

Aim: To investigate whether or not the vascular responses to serotonin (5HT, 10 nM-10 µM) and carbachol (CARB, 10 nM-10 µM) might be altered in rat mesenteric arteries (RMA) with intact and mechanically denuded endothelium (+/- E), preincubated (30 min) with 10 µM PAC or 10 µM SIR.

Results: In RMA+E, PAC and SIR reduced the 5HT-induced maximum of contraction (normalized to 120 mM KCl-induced vasoconstriction, constriction = 100%) and shifted the 5HT concentration response curve (CRC) to the right, whereas the CARB-induced vasodilaton (of 80% 5HT-preconstricted vessels, relaxation = 0%) was not affected (see Table). In RMA-E, PAC and SIR had no effect on 5HT-induced maximum of contraction. Only SIR shifted the 5HT-CRC to the right. Whereas PAC did not affect vascular responsiveness to CARB, SIR increased the CARB-induced maximum of relaxation and shifted the CARB-CRC to the left (see Table).

PAC (n=6)	RMA-E	RMA+E	RMA-E/PAC	RMA+E/PAC
5HT-LogEC$_{50}$ [M]	-6.95±0.02	-6.29±0.01*	-6.99±0.04	-6.12±0.01#
Max. of constriction (%)	159±3	143±1*	152±5	127±1#
CARB-LogEC$_{50}$ [M]	-7.97±0.80	-7.79±0.03	-7.62±0.31	-7.80±0.06
Max. of relaxation (%)	26±6	97±1*	31±3	98±2
SIR (n=6)	RMA-E	RMA+E	RMA-E/SIR	RMA+E/SIR
5HT-LogEC$_{50}$ [M]	-7.08±0.02	-6.27±0.01*	-6.75±0.02*	-6.01±0.02#
Max. of constriction (%)	154±2	141±1*	158±2	121±3#
CARB-LogEC$_{50}$ [M]	-8.10±0.23	-7.67±0.07	-7.17±0.16*	-8.04±0.12
Max. of relaxation (%)	14±1	99±2*	40±2*	94±3

*)p<0.05 vs. RMA-E, #)p<0.05 vs. RMA+E

Conclusion: PAC and SIR have no detrimental effects on vascular responsiveness after short-term administration to normal or endothelium-denudated RMA: in RME+E the 5HT-induced vasoconstriction is reduced (PAC and SIR), while in RMA-E the CARB-induced vasorelaxation is enhanced (SIR).

Institut für Pathophysiologie, Universität Duisburg-Essen, Universitätsklinikum Essen

305

ANP PRESERVES ENDOTHELIAL BARRIER FUNCTION IN VIVO – FIRST INSIGHTS INTO THE UNDERLYING MECHANISMS

M.F. Bubik, F. Hoffmann, B.A. Mayer, S. Zahler, A.M. Vollmar, and R. Fürst

We have recently shown that the cardiovascular hormone atrial natriuretic peptide (ANP) can preserve *in vitro* barrier function of inflammatory activated endothelial cells. Here, we aimed (i) to test the *in vivo* relevance of our *in vitro* findings and (ii) to provide first insights into the underlying mechanisms utilized by ANP. To mimic an inflammatory activated endothelium, rats were treated with histamine (1.3 µg/animal, 30 min). The hematocrit level was used to judge endothelial permeability *in vivo*. We found that pre-treatment with ANP (4.3 µg/animal, 15 min) minimizes the histamine-induced impairment of endothelial barrier function. To gain insight into the underlying mechanisms, we used human umbilical vein endothelial cells (HUVEC) and focused on two subcellular systems crucially involved in the regulation of endothelial permeability: the cell-cell adhesion (VE-cadherin) and the cell-contraction (myosin light chain, MLC) system. Alterations of VE-cadherin localization (immunocytochemistry) and tyrosine phosphorylation (immunoprecipitation, Western blot) were analyzed: both the typical histamine-induced (1 µM, 15 min) VE-cadherin retraction from the cell membrane and the increase of tyrosine phosphorylation were inhibited by ANP (1 µM, 30 min). Furthermore, alterations of MLC phosphorylation (immunocytochemistry, Western blot) were investigated: the histamine-evoked increase of MLC phosphorylation was strongly diminished by ANP. In summary, we could for the first time demonstrate that ANP preserves the histamine-evoked impairment of endothelial barrier function *in vivo*. Moreover, we provide a first insight into the underlying mechanisms: we could show that ANP influences both the endothelial cell-cell adhesion (VE-cadherin) and cell-contraction (MLC) system. These results warrant further investigations into the ANP-evoked signaling pathways which regulate these systems.

Department of Pharmacy, Pharmaceutical Biology, University of Munich, Germany

306

GENE EXPRESSION PROFILING OF HUMAN CORONARY SMOOTH MUSCLE CELLS AFTER DIFFERENTIATION IN VITRO

P. Mayer, H.K. Heim, A. Harst

Human coronary smooth muscle cells (HCASMCs) can be kept in primary culture. Usually the cells will be arrested by serum starvation before an experiment because it is assumed that this makes the cultured cells more similar to the quiescent smooth muscle cells in vivo. Vice versa, the proliferating state in culture (in the presence of full medium) may correspond to the proliferating and migratory state of injury and inflammation including arteriosclerosis. To compare the two states (arrested and proliferating) we performed genome-wide gene profiling experiments on DNA chips (Agilent) and confirmed the results by Real-Time PCR. It turned out that serum starvation caused induction (up-regulation) of many genes; down-regulation was observed to a much lesser extent. This argues for an active differentiation process and against an unspecific damage due to serum starvation. Disregarding genes with a very low (and therefore questionable) absolute expression level, 80 genes were markedly changed (by a factor of nine or more), 19 of these were down-regulated and 61 were up-regulated. Of these 80 genes, 35 were tested by Real-Time PCR and were (with only two exceptions) all confirmed to be regulated as indicated on the chip. This demonstrates that the gene chip data were highly reliable, at least for the strongly regulated genes. As expected, cell cycle genes were down-regulated by serum starvation whereas signalling proteins and contractile elements were up-regulated, further underscoring that a differentiation process had taken place. Unexpectedly, however, the largest number of strongly induced genes was related to extracellular matrix proteins. This may indicate that smooth muscle cell could not only contribute to vascular stenosis by dedifferentiation and proliferation but also by excessive production of extracellular material in the quiescent state. Furthermore it became obvious from our study that proliferating HCASMCs produce several cytokines (e.g. IL8, LIF) and may thereby take a more active role in (arteriosclerotic) inflammation than previously thought.

Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM), Kurt-Georg-Kiesinger-Allee 3, 53175 Bonn, Germany

307

ROLE OF HB-EGF EXPRESSION IN THE THROMBIN-MEDIATED DIFFERENTATION OF SMOOTH MUSCLE CELLS

A. Pérez Sastre[1], H.P. Reusch[2] and M. Schaefer[1]

The phenotypic modulation of vascular smooth muscle (VSM) cells during the transition from a proliferative to a contractile phenotype is characterized by an increased expression of contractile proteins. The signalling cascades and transcriptional regulators of this transition are poorly defined. Previous studies have demonstrated that the thrombin-induced expression of smooth muscle-specific contractile proteins require a biphasic activation of ERK1/2 with a delayed phase occurring about 45-240 min after thrombin application. Here we demonstrate the necessity of *de novo* RNA and protein synthesis for the second phase of ERK1/2 activation. Actinomycin D or cycloheximide treatment abolished the delayed phase of thrombin-induced ERK1/2 phosphorylation indicating a requirement of RNA synthesis and protein translation for the second phase of ERK1/2 phosphorylation. Pretreatment of the cells with cytochalasin D (a potent inhibitor of actin polymerization) and brefeldin A (disrupts the structure and function of the Golgi apparatus) abolished the second phase of ERK1/2 phosphorylation. Therefore, we conclude that the newly synthesized protein that critically controls the second phase of ERK1/2 phosphorylation may be a transmembrane protein. Semi-quantitative RT-PCR of protease-activated receptor 1 (PAR1), heparin-binding EGF (HB-EGF) and the EGF receptor were performed after thrombin stimulation and revealed that proHB-EGF mRNA levels were significantly increased. The time-course of HB-EGF expression closely coincided with the second phase of thrombin-induced ERK1/2 phosphorylation. Inhibition of the second phase of ERK1/2 activation by heparin (a scavenger of HB-EGF) or by siRNA-mediated knockdown of HB-EGF expression support the concept that *de novo* expression of HB-EGF is a critical intermediate for triggering the long-lived second phase of ERK1/2 phosphorylation. In addition, the thrombin-induced expression of smooth muscle-specific α-actin was prevented in the presence of heparin. We conclude that *de novo* HB-EGF expression and EGF receptor activation by HB-EGF promote the thrombin-induced delayed and long-lasting signalling into the Ras/Raf/MEK/ERK-cascade and a subsequent expression of contractile proteins.

[1] Institut für Pharmakologie, Charité - Universitätsmedizin Berlin, Thielallee 67-73, 14195 Berlin
[2] Abteilung für Klinische Pharmakologie, Ruhr-Universität Bochum, Universitätsstr. 150, 44801 Bochum

308

TEMPORALLY-CONTROLLED CELL FATE MAPPING OF VASCULAR SMOOTH MUSCLE CELLS DURING ATHEROGENESIS IN THE MOUSE

R. Feil[1], R. Lukowski[2], F. Hofmann[2], S.M. Schwartz[3], M.E. Rosenfeld[3], S. Feil[1]

Atherosclerosis is one of the major causes of death in the western world, but its pathogenesis is poorly understood. One question is how particular cell types, such as vascular smooth muscle cells (VSMCs), contribute to atherogenesis. In this study, temporally-controlled Cre/lox-mediated cell fate mapping was used to investigate the role of VSMCs in atherosclerotic plaque formation in apolipoprotein E-deficient mice. Prior to development of atherosclerosis, VSMCs were genetically labelled and their fate was followed during disease progression. These analyses indicated that VSMCs derived from the media can contribute to plaque growth and express marker proteins of macrophages and/or chondrocytes suggesting an in vivo trans-differentiation potential of medial VSMCs. These results also demonstrate that the inducible site-specific recombination technology is a useful tool to follow the fate of selected cell types in health and disease.

[1] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
[2] Institut für Pharmakologie und Toxikologie, TU München, Germany
[3] Department of Pathology, University of Washington, Seattle, U.S.A.

309

VASODILATOR EFFECTS OF FIBRINOGEN IN HUMAN ARTERIES

J.Hochfeld[2], M.Bas[1], N.Kirchhartz[2], C.Tüllmann[2], S.Kumpf[2], T.Suvorava[2], M.Oppermann[2], T.K.Hoffmann[1], V.Balz[1], G.Kojda[2]

Hyperfibrinogenamia has been found in angiotensin-converting-enzyme-inhibitor (ACEi)-induced angioedema, the pathophysiology of which is linked to bradykinin. Little is known about arterial vasomotor effects of fibrinogen and interactions with bradykinin. So far, there is just one published study showing transient vasomotor effects of fibrinogen concentrations up to 4 µM in human saphenous vein segments.

Vascular activity of fibrinogen and its effect on bradykinin-induced vasodilation and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) were investigated in small (0.8 - 1.4 mm diameter) porcine coronary (SPCA) and human internal mammary artery (IMA) segments. Fibrinogen (1-15 µM) induced a concentration-dependent vasodilation in preconstricted SPCA (n=13) reaching a vasodilatator effect of 70 ± 4.7 %. Likewise, fibrinogen induced a 52.1 ± 9.1 % (n=7) vasodilation in IMA rings. Fibrinogen vasorelaxations of SPCA and IMA were completely inhibited by the glycoprotein (GP) IIb/IIIa binding antibody abciximab (4 µg/ml). Abciximab also concentration-dependently inhibited the β_3-integrin-antibody mediated westernblot detection of the human smooth muscle cell vitronectin receptor ($\alpha_v\beta_3$-integrin). These results suggest that fibrinogen induces vasodilation by binding to this receptor. Furthermore, fibrinogen increased the vasodilator

potency of bradykinin by 10-fold (P<0.0001) and increased bradykinin-induced VASP-phosphorylation (P<0.01). In SPCA, endothelial denudation or treatment with the NO-synthase inhibitor L-NAME halved fibrinogen relaxations at fibrinogen concentrations ≥10 µM (P<0.01). Vasodilation to 10 µM fibrinogen of 32.7 ±4.8 % was strongly reduced by the potassium channels blocker glibenclamide to 11.3 ±4.6 % (P<0.0001). Our data indicate that fibrinogen dilates human arteries by stimulation of the smooth muscle vitronectin receptor. Furthermore, fibrinogen strongly potentiates bradykinin-induced vasodilation suggesting that this acute-phase protein may contribute to endothelial actions of bradykinin such as those resulting in ACE-inhibitor-induced angioedema.

[1]Hals-,.Nasen- und Ohrenklinik, [2]Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany

310

DETERMINATION OF VASCULAR CONTRACTILITY IN CREM KNOCKOUT MICE

M.D. Seidl[1], G. Schütz[2], W. Schmitz[1], F.U. Müller[1]

The transcriptional regulation by transcription factors of the CREB/CREM-family (cAMP responsive-element binding protein and modulator) represents a pivotal mechanism of gene control in response to various second messengers including cAMP and cGMP. CREB was implied in the regulation of gene expression in vascular smooth muscle cells, however, the functional role of CREB and CREM in the vasculature is not understood in detail. In order to study the vascular function of CREM, we analysed the contractility of aortic rings of male CREM-deficient mice (KO) and of wild type (WT) controls after stimulation with the α-adrenoceptor agonist phenylephrine and with prostaglandin $F_2\alpha$. Vasorelaxation was studied after precontraction with phenylephrine and stimulation with the muscarinic receptor agonist carbachol, the β-adrenoceptor agonist isoproterenol or with the nitrogen oxide donor sodium nitroprusside. In detail, the mean EC_{50}±SEM was 65±20 vs. 48±11 nmol for phenylephrine, 587±27 vs. 642±49 nmol for prostaglandin $F_2\alpha$, 128±21 vs. 78±6.8 nmol for carbachol, 2.2±1.4 vs. 0.8±0.7 µmol for isoprenaline and 12±1.6 vs.13±1.2 nmol for sodium nitroprusside in KO (n=3-7) vs. WT (n=3-9) mice, respectively. Overall statistical analysis (TWO-WAY ANOVA) of the specific response curves revealed no significant differences in aortic contractility between the experimental groups. In conclusion, inactivation of CREM did not alter the effects of phenylephrine, prostaglandin $F_2\alpha$, carbachol, isoproterenol, or sodium nitroprusside on the contractility of aortic rings in mice suggesting that CREM does not play a major role in the regulation of vasotonus in mice. Further investigations are necessary to prove this suggestion and to clarify the role of CREM in the vascular system. (Supported by the IZKF Münster).

[1]Institut für Pharmakologie und Toxikologie, Universität Münster, Universitätsklinikum, Domagkstr.12, D-48149 Münster.
[2]Abteilung Molekularbiologie der Zelle I, Deutsches Krebsforschungszentrum Im Neuenheimer Feld 280 D-69120 Heidelberg.

311

ENRICHMENT OF HUMAN VASCULAR SMOOTH MUSCLE CELLS WITH CHOLESTEROL *IN VITRO* INDUCES EXPRESSION OF CYCLOOXYGENASE-2

Baumgärtel-Allekotte, D; Rauch, BH; Schrör, K.

Accumulation of cholesterol in the vessel wall is an early event in atherosclerosis and triggers inflammatory processes which play a central role in the development of the disease. Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme for prostaglandin production. Proinflammatory prostaglandins contribute to the pathogenesis of arteriosclerosis. The present study we establish an *in vitro* model of cholesterol-enriched cultured human vascular smooth muscle cells (SMC) and demonstrate that accumulated intracellular cholesterol induces expression of COX-2 in these cells. Cultured human aortic SMC were enriched with cholesterol by using cholesterol:methyl-β-cyclodextrin complexes (Chol-MbCD). Cholesterol content was determined by Oil Red O staining and quantified by a flourometric assay. COX-2 mRNA and protein were determined by RT-PCR and Western blotting. 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, was determined by enzyme immunoassay. Incubation of human SMC with Chol-MbCD (10 µg/ml cholesterol) for 24 hours resulted in the formation of intracellular lipid droplets. Total cellular cholesterol concentration increased about 2-fold, whereas the percentage of cholesteryl esters did not significantly change. Incubation with Chol-MbCD concentration-dependently (1 - 30 µg/ml cholesterol) increased COX-2 mRNA and protein expression. A 2-fold increase of 6-keto-$PGF_{1\alpha}$ in the medium of cholesterol-enriched SMC indicated functionality of the cholesterol-induced COX-2. MbCD alone did not affect COX-2 expression. Incubation with Chol-MbCD is a useful method to mimic increases in cholesterol levels of human vascular SMC and is associated with expression of functionally active COX-2. This model may be a useful tool to study interactions between inflammatory mediators, such as prostaglandins, and cholesterol enrichment under well-defined *in vitro* conditions.

Universitätsklinikum Düsseldorf, Institut für Pharmakologie und Klinische Pharmakologie Universitätsstraße 1, 40225 Düsseldorf, Germany

312

MORPHOLOGICAL CHANGES OF PAROTID GLAND IN EXPERIMENTAL HYPERLIPIDAEMIA

J.Daskala, E.Chatzigianni, A.Goudeli, S Anagnostopoulou, E.Tigka and C.Tesseromatis

Patients with hypothyroidism and hyperlipidemia(elevated plasma levels of triglycerides(TG), total cholesterol(TC) and low-density lipoprotein cholesterol), are considered in high risk to undergo atherosclerosis and may manifest sicca or Sjoegren's like Syndrome with xerostomia, xerophthalmia and fatty degeneration of parotid parenchyma. The aim of the study was to investigate the role of hyperlipidaemia in the microstructure of parotid gland and the possible amelioration through statin treatment in Wistar rats. Forty Wistar rats(111.06±3.36g) were divided into 4 groups (A1, A2 controls,B1 B2 experimental). Control animals consumed normal cereal rodents diet while the experimental hyperlipidaemic mixture(50% cereals, 20% butter, 10,5% sucrose, 10% casein, 2,5% cholesterol, 1% vitamins, 0,15% propylthiouracil, 0,1% choline -hyperlipidaemia + hypothyroidism). The A2 and B2 were treated with simvastatin 40mg/kg/daily p.o.for 3 months. Statistical analysis: x^2 and t-test.

Results

Groups(n=10)	A1	A2	B1	B2
TC mg/dl	54.71±7.81	65.25±8.16	391.14±55.88**	346.25±43.28**
HDL mg/dl	17.10±2.43	20,12±2.51	40.86±5.84	36,37±4.55
LDL mg/dl	25.29±3.61	27,25±3.40	324,86±46.41	285,25±36.65
TG mg/dl	62,29±8.90	89.12±11.14	128±18.29	123±15.37
Parotid weight mg	39.57±17.47	43.16±12.50	35.89±10.22	32.02±11.43
Parotid width mm^2	44.14± 8.76	48.25±11.01	53.43±17.47	43.75±34.24

**p< 0.05, **p< 0.01, ***p< 0.001*
*(TC): A1/B1***, A2/B2***, B1/B2*, A1/B2****
*(TG): A1/B1**, A2/B2*, B1/B2*, A1/B**
*(HDL): A1/B***, A2/B2*, B1/B2*, A1/B2****
*(LDL):A1/B1***, A2/B2*, B1/B2*, A1/B2****
(PW): A1/B1 , A2/B2*, B1/B2*, A1/B2**
(PWD): A1/B1, A2/B2*, B1/B2*, A1/B2**

The histological findings (sections under HE) demonstrated changes in the parotid gland morphology of the hyperlipidaemic groups, such as the presence of chronic inflammation, fibrosis, lipocytes, foci of lymphocytic infiltration, nuclei abnormalities, diversity of vessel wall thickness, signs giving the appearance of sicca syndrome. The influence of statin tended to potentate the chronic inflammatory infiltrate while the lipocytes were decreased.

In conclusion hyperlipidaemia affects the parotid structure and may be responsible for a degeneration process similar to the sicca syndrome.Institut fuer Pharmakologie Uni.Med., Athen, M. Assias 75 11527 Griechenland

313

SUPERIORITY OF RADIOTELEMETRY OVER THE TAIL-CUFF METHOD IN EVALUATING CONTROL AND DRUG-INDUCED VALUES IN CARDIOVASCULAR FUNCTIONS IN RATS

I. Abu-Taha, B. Lemmer

Background - Different methods are used to determine blood pressure in rats, i.e. intrarterial catheter (AC), tail-cuff (TC) and radiotelemetry (RT). Whereas in AC and TC animals have to be restricted in their activity, RT permits a free movement of the animal during measurement. The aim of our study was to compare RT with TC. Methods – Male (n=4) normotensive WKY and spontaneously hypertensive SHR rats were kept under a light: dark cycle of 12:12 hours (lights on at 07:00). Radiotransmitters (TA11PA-C40; DSI, St. Paul, Minnesota, USA) were implanted into abdominal aorta to monitor systolic and diastolic blood pressure (SBP, DBP), heart rate (HR) and motor activity (MA). To measure SBP with TC the Harvard Blood Pressure Monitoring System (Edenbridge, England) was used. Experiments in each rat were performed at 09:00h (rest phase ,L) and at 21:00h (activity phase, D) for 60 min. During TC animals are kept in a the supplied restrainer. Measurements were done: (1) by RT, (2) by TC (4 readings every 5 min), (3) and by RT with simultaneously monitoring in the TC restrainer , (4) during metoprolol treatment (bid, 2 days, once prior study; 8 and 16mg/kg for WKY and SHR, resp).Results - TC greatly increased HR and BP as monitored by RT in both strains and in both L and D with a more pronounced effect in WKY in L and SHR in D. TC measurements of SBP were also significantly increased in both WKY and SHR. Metoprolol reduced TC-induced increase in HR in both L and D, but left the BP increase unaffected.

Conclusion - Our data demonstrate that TC leads to a stress-induced increase in HR and BP in both normotensive and hypertensive rats. Thus, TC is not suitable to evaluate control and drug-induced values in cardiovascular functions.

Institute of Pharmacology and Toxicology, University of Heidelberg, Maybachstr. 14, D-68169 Mannheim, Germany

314

RADIOTELEMETRIC STUDIES ON CARDIOVASCULAR FUNCTION WITH L-NAME AND TELMISARTAN IN WT AND eNOS-/- KNOCK OUT MICE

M. Arraj and B. Lemmer

Background: The effects of L-NAME and of AT1 receptor antagonist telmisartan were studied on blood pressure in endothelial NOS knock out mice (eNOS-/-) in comparison to wild-type C57BL/6 mice (WT) by radiotelemetry.

Experimental design: Blood pressure transmitters [PA-C20 device (DSI, St. Paul, Minnesota, USA)] were implanted subcutaneously. (1) eNOS-/- and WT mice kept under a light:dark schedule of 12:12 h (light on 07:00 am) were treated with L-NAME in tap water in different doses: 0.5 mg/ml, 1.5 mg/ml and 5 mg/ml for three days. Thereafter, treatment was discontinued for two weeks. (2) eNOS-/- and WT mice were injected intraperitoneally with 3 mg/kg telmisartan at 07:00 pm in 48 hours intervals for 6 days.

Results: Under control conditions the eNOS-/- mice are mild hypertensive in comparison to WT. L-NAME increased systolic (SBP) and diastolic (DBP) blood pressure in WT mice to the level of eNOS-/- mice from the first day of dosing and in dose-independent manner, whereas L-NAME had no effects on SBP and DBP in eNOS-/- mice. In contrast to WT mice, telmisartan decreased SBP and DBP in eNOS-/- mice slightly already after two days from the beginning of the treatment. However, eNOS-/- mice did not reach the normotensive blood pressure values of WT mice.

Conclusion: L-NAME increases blood pressure in WT mice, whereas it has no effects on blood pressure in eNOS-/- mice. AT1 antagonists play a modest role in blood pressure regulation in mild hypertensive eNOS-/- but not in normotensive mice.

Institute of Pharmacology and Toxicology, Ruprecht-Karls-University of Heidelberg, Maybachstr. 14, D-68169 Mannheim, Germany

315

Withdrawn

316

EFFECT OF SILYMARIN, MELATONIN AND N-ACETYLCYSTEINE ON BLEOMYCIN-INDUCED LUNG INJURY IN RATS.

M. Th Ghoneim*, M. G. El-Sakkar*, T. Abdel-Azim*, & A. A. Sheeta**

Pulmonary fibrosis is the end stage of some pulmonary diseases such as COPD. The aim of the present study was to investigate the protective effect of Silymarin, Melatonin, and N-acetylcysteine (NAC) on lung fibrosis induced in rat model. Lung fibrosis was induced by endotracheal instillation of a single subleathal dose of bleomycin HCl (5 mg /kg body weight) (BLM). Drugs given as follows: Silymarin was given in a dose of 50 mg, Melatonin in a dose of 10 mg and NAC in a dose of 486.6 mg/kg/day respectively. Treatments started orally daily for 14 days after the day of BLM instillation. Bronchoalveolar lavage fluid (BALF) was used to measure total protein concentration, lactate dehydrogenase activity (LDH), and total glutathione level. Lung tissue homogenates were used to measure myeloperoxidase (MPO) activity, lipid peroxide (LPO) content, and total lung collagen. BILF did not change the body weight but caused an increase in lung weight. Treatment of BILF with either silymarin, melatonin or NAC slightly decreased the lung weight. The lung hydroxyproline increased by BILF but decreased by treatment with silymarin, melatonin or NAC. In the BALF, the total cell count and neutrophil cell count were significantly increased; BALF due to lung fibrosis was not reduced by any of the drugs used. Silymarin, melatonin or NAC significantly attenuated the increased LDH activity in BALF induced by BILF. Glutathione was decreased in BALF in BILF. Treatment with silymarin, melatonin or NAC increased glutathione. Only Silymarin significantly attenuated the increased lung MPO activity. The lipid peroxide content in the lung significantly increased by BILF, treatment with silymarin, melatonin or NAC attenuated this elevation. Conclusion: Treatment with silymarin can inhibit lung fibrotic progression induced by bleomycin. Melatonin and NAC are partially effective against lung fibrosis.

Departments of Pharmacology* & Forensic Medicine and Clinical Toxicology**, Faculty of Medicine, 51521 El-Messalla, Alexandria, , Egypt.

317

ARGINASE ISOENZYMES SHOW DIFFERENTIAL EXPRESSION AND FUNCTION IN HUMAN AND RAT PULMONARY FIBROBLASTS

Warnken M, Haag SD, Matthiesen S, Racké K

Arginase was shown to be up-regulated in asthmatic diseases. Since arginase provides ornithine, a precursor for polyamine synthesis and proline, an essential substrate for collagen synthesis, it has been suggested that arginase might be a key enzyme in airway remodeling processes. The present study aimed to characterize the expression of arginase isoenzymes in rat and human pulmonary fibroblasts and to explore whether arginase inhibition affects collagen synthesis. Primary human lung fibroblasts, rat tracheal and lung fibroblasts were obtained by an outgrowth technique from resected tissue. Arginase I and II mRNA were detected by RT-PCR. Collagen in culture supernatant was determined by densitometric analysis of Western blots using a specific antibody against collagen I. In rat tracheal and lung fibroblasts mRNA for both arginase isoenzymes could be detected, with arginase I giving a stronger signal at 30 PCR cycles than arginase II at 35 PCR cycles. In primary human lung fibroblasts no mRNA encoding arginase I (40 PCR cycles), but clear expression of mRNA encoding arginase II (30 PCR cycles) was detected. Collagen I was determined after fibroblasts had been cultured in proline free medium for 5 days of which the last 2 days were additionally serum free. The specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (norNOHA, 100µM) was present for the last 2 or 4 days. Collagen content in media of primary human lung fibroblasts was not affected by norNOHA, whereas in supernatant of rat tracheal fibroblasts collagen I was time-dependently reduced, by 19±5 % after 48 h, 29±7 % after 96 h (n=5-6, $p< 0.05$, each). In conclusion, arginase isoenzymes show species specific expression patterns. Arginase appears to contribute significantly to the supply of proline for collagen synthesis in rat tracheal fibroblasts, in which arginase I is the predominant isoenzyme, but not in primary human lung fibroblasts, in which arginase II is the only expressed isoenzyme.

Department of Pharmacology & Toxicology, University of Bonn

318

ARGINASE II EXPRESSION IS UPREGULATED BY PDGF IN HUMAN LUNG FIBROBLASTS WITHOUT CONTRIBUTING TO PROLIFERATION

Haag S, Racké K, Matthiesen S, Subepithelial fibrosis is a crucial alteration in chronic inflammatory and obstructive airway diseases like asthma and COPD. Arginase I and II, shown to be upregulated in asthmatic diseases, have been suggested to contribute to airway remodeling processes by providing substrates for the synthesis of polyamines and collagen. Platelet-derived growth factor (PDGF) plays a key role in chronic tissue remodeling, therefore we studied whether PDGF alters expression of arginase in primary human lung fibroblasts and whether arginase activity is relevant for their proliferation. Primary human lung fibroblasts were isolated by an outgrowth technique from resected human lung material. Serum-deprived cells were cultured with or without test substances for 30 h in presence of [^{3}H]-thymidine for the last 24 h. PDGF increased [^{3}H]-thymidine incorporation in a concentration dependent manner by 83 ± 17% at 1 ng/ml and by 398 ± 9% at 100 ng/ml (each n=9). RT-PCR revealed that primary human lung fibroblasts express mRNA encoding arginase II, but not arginase I. PDGF concentration-dependently up-regulated expression of arginase II by 55 ± 6% at 1 ng/ml and by 252 ± 40% at 100 ng/ml (n=3-6). The PDGF receptor specific tyrosine kinase inhibitor AG1296 (5 µM) prevented the proliferative effect of PDGF and largely blocked the PDGF-induced up-regulation of arginase II indicating that both effects are transferred via the PDGF-receptor signaling pathway. Interestingly, specific inhibition of arginase by N^{ω}-hydroxy-nor-L-arginine (1 µM to 100 µM) did not significantly affect proliferation of primary human lung fibroblasts, neither in absence nor presence of PDGF. These results were shown to be cell specific, since in human alveolar epithelial cell line A549 PDGF did neither stimulate arginase II expression nor proliferation. In conclusion, though PDGF enhances proliferation and arginase II expression in primary human lung fibroblasts, arginase as provider of substrate for polyamine synthesis is not crucially involved in proliferation processes of human airway fibroblasts.

Funded by DFG Ra 400/12-2

Department of Pharmacology & Toxicology, University of Bonn, Germany.

319

ESTABLISHMENT OF IN VIVO MODELS OF COPD

R. Puljic, A. Pahl

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world. It is defined as a disease state characterised by airflow limitation that is not fully reversible. It is both progressive and associated with an abnormal inflammatory response of the lungs. Cigarette smoking is by far the most common cause of COPD. Recent reports have underlined the role of T-cells as a potentially important factor in the chronic inflammatory process leading to COPD. There is an increase in the total number of T-lymphocytes in lung parenchyma, peripheral and central airways of patients with COPD, with the greater increase in CD8+ than CD4+ cells. The mechanism by which T-lymphocytes accumulate in the airways is not yet understood. In the present study we developed an acute and a chronic mouse model of COPD. In the acute model mice were exposed to single intranasal instillation of LPS which mimicked acute exacerbations of COPD patients. Similar to human patients a rapid influx of neutrophils into the lung of LPS-challenged mice was observed. This influx was accompanied by increased levels of chemokines in BAL fluid. In a sub-acute model four days after single intranasal LPS-administration no neutrophils could be detected in the BAL. In contrast, T-cells started to infiltrate the lung. Therefore we established a chronic mouse model by administering LPS three times weekly for five weeks. After a recovery period of ten days changes in cell composition of various tissues were analysed. Neutrophils were detected neither in the BAL nor in lung tissue. In contrast, a huge increase of T-cells in the BAL was observed. Furthermore, the number of CD8+ cells increased in relation to CD4+ cells. CD8+ cells in this model were further characterised with regard to their cytotoxic effector activity. Additionally, the role of IL-17 a pro-inflammatory cytokine produced by T-lymphocytes was analysed. In summary, we established an acute and a chronic mouse model of COPD mimicking specifically different aspects of human pathology to study the role of T-cells in COPD.

Department of Experimental and Clinical Pharmacology, University of Erlangen-Nuremberg.

320

EFFECTS OF THE HYALURONANSYNTHASE INHIBITOR 4-METHYLUMBELLIFERONE ON ESOPHAGEAL SQUAMEOUS CANCER CELLS

Twarock S; Nagy N; Fischer JW

Abundant production of hyaluronic acid (HA) in the vicinity of gastrointestinal cancer cells is a hallmark in tumor development. Esophageal cancer is a rare but severe kind of gastrointestinal cancer which is differenciated in adenocarcinoma and squameous cell carcicoma of the esophagus. 4-methyl-umbelliferone (4-MU) is a selective inhibitor of the hyaluronan synthases (HAS1-3) and is thought to have promising properties as an anticancer agent. Aim of the present study was to (i) evaluate the effect of 4-MU on the proliferation and migration of human oesophageal cancer cells and (ii) to examine the morphological changes and underlying mechanisms. For this purpose cell lines of squameous cell carcinoma derived from human oesophagus (OSC1 and OSC2) were used. In OSC1, HAS2- and HAS3-mRNA are highly expressed, while HAS1-mRNA was not detectable. OSC2-cells express only HAS3 but no HAS1 and HAS2. In both cell types pericellular HA was detected by affinity histochemistry. 4-MU (300µM) reduced proliferation of OSC1 to 58,6%+-8,5% and OSC2 to 74,1% +- 5,1%. Migratory activity of the cells was reduced to 45%+-4% for OSC1 and to 52%+-8% for OSC2. OSC1 cells undergo a typical shape shift which starts one hour after incubation and is fully observable after 24 hours. The 4-MU treated cells show a flat, shrinked and clustered phenotype and appear to have tighter cell contacts. Moreover, 4-MU leads to a decrease in integrine downstream signalling as can be seen by immunhistochemical staining of phoshorylated focal adhesion kinase (pFAK). In conclusion the present data show that OSC1 and OSC2 predominantly express HAS2 and HAS3. The hyaluronan synthase inhibitor 4-MU reduces migration and proliferation of OSC1 and OSC2 cells. OSC1 cells show a distinct morphological change which is accompanied by dephosphorylation of pFAK. 4-MU shows a strong influence on the interaction of tumor cells with hyaluronanmatrix suggesting a possible use of 4-MU in the control of invasive cancer.

Molekulare Pharmakologie, Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum Düsseldorf, 40225 Düsseldorf

321

EFFECT OF HERBAL EXTRACTS ON THE PERISTALTIC REFLEX OF RAT SMALL INTESTINE

B. Yuece[a], J. Wallbach[a], A. Sibaev[a], S.N. Okpanyi[b], O. Kelber[b], B. Goke[a], M. Storr[a,c]

Background: Dyspepsia and motility related disorders of the gastrointestinal tract are often treated with herbal extracts. The clinical efficacy of STW 5 (Iberogast®), a fixed combination of standardized herbal extracts, in this indication is clinically proven, but its possible mechanisms of action are still not fully resolved.

Aim: Aim of the present study was to investigate whether STW 5 and the individual herbal extracts influence myenteric neuronal pathways underlying the peristaltic reflex of rat small intestine.

Methods: Myenteric pathways of the peristaltic reflex were studied in 10 cm ileum segments. Peristaltic activity was initiated by electrical stimulation in the middle of the segments. Ascending and descending reflex responses were recorded using force transducers. Contractile forces and latency of the response were evaluated. Drugs were added in a cumulative manner. Concentrations are given as dilution of the standardized fluid herbal extract in organ bath medium.

Results: All reflex responses were blocked by tetrodotoxin and atropine indicating that they are mediated by neuronal, cholinergic mechanisms. STW 5 increased ascending reflex responses at lower concentrations and reduced ascending and descending reflex responses in higher concentrations. Reflex latency was prolonged in higher concentrations. The herbal extracts had individual, reproducible effects on the elicited reflex responses, showing concentration dependency (e.g. peppermint leaf extract significantly reduced ascending contraction: 1:1000: -10.1 ± 4.0 %; 1:500: -8.8 ± 6.6 %; 1:250: –17.3 ± 4.7 %; 1:167: –39.5 ± 13.5 %; 1:83: -55.4 ± 15.5; 1:50: -58.9 ± 15.6). All observed effects were fully reversible after washout.

Conclusion: Herbal extracts and their fixed combination STW 5 (Iberogast®) modulate peristaltic reflex responses in a reproducible and reversible manner. This study gives new insights on possible mechanisms by which STW 5 might cause the beneficial effects reported in randomized controlled double blind studies.

[a]Medizinische Klinik 2, University of Munich, Munich, Germany, [b]Scientific Department, Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany, [c]Department of Physiology and Biophysics, University Calgary, Calgary, Canada

322

INFLUENCE OF APPLE JUICES ON INFLAMMATORY CHANGES IN THE RAT COLON

J. Minn[1], H. Dietrich[2], F. Will[2], D. Schrenk[1], H.J. Schmitz[1]

The aim of this study was to investigate the influence of apple juice on a DSS induced colitis in male rats. To investigate the time-course of the inflammation the animals were kept on drinking water containing 3% dextrane-sulfate-sodium (DSS; m.w. 36.000-50.000) for ten days followed by seven days drinking water. After decapitation tissue samples were taken from day 1-17 of the treatment. To investigate the influence of different apple juices on the inflammation apple pulp, cloudy apple juice, clear apple juice or a polyphenol-free control juice were offered to the animals for seven days before and after the ten day treatment with 3% DSS-solution. Tissue samples were taken at the end of the second juice application. The analyzed parameters were bodyweight gain, ratio of colon weight/colon length, myeloperoxidase (MPO) activity and transforming growth factor β1 (TGF-β1) levels. A ten days application of 3% DSS-solution led to a uniform, moderate inflammation in the colon. At the eighth day of DSS-treatment the animals showed diarrhea and rectal bleeding. Both declined two or three days after the end of DSS-treatment. Inflammation was evident from the eighth day of DSS-treatment and was still visible seven days after the end of DSS-treatment. The healing process, as indicated by an increase in TGF-β1, started two or three days after the end of DSS-treatment. Within the DSS treated groups apple pulp, cloudy apple juice and clear apple juice enhanced the increase in TGF-β1. By comparison of the DSS treated groups with their corresponding control groups the following parameters showed that apple pulp and cloudy apple juice attenuated the DSS induced colitis while clear apple juice had no effect on the inflammation: bodyweight gain, ratio of colon weight/colon length and MPO-activity. Our findings indicate that apple pulp and cloudy apple juice can attenuate DSS induced inflammation in the rat colon, and may thus play a role in the prevention of colo-rectal cancer.

[1]Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany, [2]Research Institute Geisenheim, Geisenheim, Germany

323

A ROLE OF CA^{2+}-ACTIVATED K^+ CHANNEL (BK) A SUBUNIT IN K^+ HOMEOSTASIS AND FLOW-DEPENDENT RENAL K^+ EXCRETION

T. Rieg[1,3], V. Vallon[3,4], M. Sausbier[2], U. Sausbier[2], B. Kaissling[5], P. Ruth[2], and H. Osswald[1]

In addition to ROMK channels, large conductance Ca^{2+}-activated K^+ (BK) channels are expressed in the apical membrane of the aldosterone sensitive distal nephron (ASDN), where they could contribute to renal K^+ secretion. To gain further insights, BK channel α-subunit knockout mice ($BK^{-/-}$) and wild-type mice (WT) were studied. First, flow-induced K^+ secretion was assessed by acute pharmacological blockade of vasopressin V_2 receptors, which inhibits water reabsorption in the ASDN (connecting tubule (CNT) and downstream). While this maneuver induced similar diuresis in both genotypes, a concomitant increase in urinary K^+ excretion and a significant correlation between urinary flow rate and K^+ excretion was only observed in WT. Second, $BK^{-/-}$ and WT presented similar urinary K^+ excretions. This was associated, however, with higher plasma aldosterone concentrations and stronger expression of ROMK in the apical membrane of the ASDN (particularly CNT) in $BK^{-/-}$ than in WT under control-K^+ diet and particularly in response to a high-K^+ diet. Moreover, despite of greater plasma concentrations of K^+ and aldosterone, which are key activators of renal K^+ excretion, $BK^{-/-}$ restricted urinary K^+ excretion in response to a low-K^+ diet to the same extent as WT. Finally, high-K^+ intake significantly increased the renal expression of the BK channel in WT. These studies indicate a role of the BK channel α-subunit in flow-induced K^+ secretion as well as K^+ homeostasis. ROMK may compensate in the absence of functional BK channels.

[1] Institute of Pharmacology and Toxicology, Medical Faculty of the Eberhard Karls University, 72074 Tübingen, Germany
[2] Institute of Pharmacy, Department of Pharmacology and Toxicology, Eberhard Karls University, 72076 Tübingen, Germany
Departments of [3] Medicine and [4] Pharmacology, University of California San Diego & VA San Diego Healthcare System, San Diego, CA 92161
[5] Institute of Anatomy, University of Zürich, 8057 Zürich, Switzerland

324

PKCα -DEPENDENT PHOSPHORYLATION OF THE mRNA STABILIZING FACTOR HUR CONTROLS THE STABILITY OF CYCLOOXYGENASE-2

A. Doller[*], A. Huwiler[* †], R. Müller[*], H. H. Radeke[*], J. Pfeilschifter[*] and W. Eberhardt[* ‡]

In this study, we investigated the molecular mechanisms underlying the ATP analog adenosine 5´O-(thiotriphosphate) (ATP γS)-induced nucleo-cytoplasmic shuttling of the mRNA stabilizing factor HuR in human mesangial cells (hMC). Using synthetic protein kinase C (PKC inhibitors and siRNA approaches we demonstrate that knock-down of PKC α efficiently blocked the ATP-dependent nuclear HuR export to the cytoplasm. The involvement of PKC α in HuR shuttling is furthermore highlighted by the high cytosolic HuR content detected in fibroblasts stably overexpressing PKCα when compared to mock-transfected cells. The ATP-induced recruitment of HuR to the cytoplasm is preceded by a direct interaction of PKC α with nuclear HuR as demonstrated by immunoprecipitation experiments. *In vitro* phosphorylation assays revealed that HuR is a direct substrate of PKC α. Mapping of putative PKC target sites identified serines 158 and 221 being indispensable for HuR-phosphorylation by PKC α. RNA pull-down assay and RNA electrophoretic mobility shift assay furthermore demonstrated that the HuR shuttling by ATP is accompanied by an increased HuR-binding to COX-2 mRNA. Physiologically, the ATP-dependent increase in RNA binding is linked with an augmentation in COX-2 mRNA stability and subsequent increase in PGE_2 synthesis. Regulation of HuR via PKC□-dependent phosphorylation emphasizes the importance of posttranslational modification for stimulus-dependent HuR shuttling.

[*] pharmazentrum frankfurt/ZAFES, *Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany*
[†] Present address: Institut für Pharmakologie, Universität Bern, CH-3010 Bern, Switzerland

325

ADAM10 IS INVOLVED IN IFN-γ INDUCED CXCL16 CLEAVAGE IN HUMAN MESANGIAL CELLS

A. Schramme[1], S. Schmehl [1], I. Hauser[2], L. Schäfer[1], H.-J. Gröne[3], J. Pfeilschifter[1] and P. Gutwein[1]

Inflammatory cell infiltrates are a hallmark of chronic kidney diseases (CKDs). Understanding the molecular mechanism that regulate renal leukocyte recruitment suggest chemokines and chemokine receptors as new targets for specific pharmacological intervention. We have analyzed the expression and cleavage of CXCL16 in the human kidney cells. Here we analyzed the CXCL16 expression in mesangial cells in vitro. We can show that INF-γ induces the cleavage of CXCL16 and increased amounts of soluble CXCL16 were found in the supernatant of the cells. Blocking ADAM10 activity with a specific inhibitor, we were able to identify ADAM10 as the responsible metalloproteinase, cleaving CXCL16. In vivo we found CXCL16 expression in the healthy kidney mainly in podocytes, mesangial cells and distal tubular cells. Interestingly increased CXCL16 expression was found in the glomerulus of kidney transplated patients. Importantly CXCR6 expressing immune cells were found in high numbers in the inflammed kidney of transplanted patients. Analyzing urine samples of patients with inflammatory kidney diseases, we found compared to healthy volunteers increased amounts of soluble CXCL16 in the urine of the patients with kidney diseases. Taken together this data suggest an important participitation of the chemokine CXCL16 and its receptor CXCR6 in the recruitment of leukocytes into the kidney during inflammatory processes.

[1] Pharmazentrum Frankfurt, [2] Nephrologie Frankfurt, [3] DKFZ, Heidelberg

326

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 3-OXO-TIRUCALLIC ACID IN CHICKEN EMBRYONIC PLASMA USING A FLUORINATED STATIONARY PHASE: APPLICATION IN PHARMACOKINETIC STUDIES

B. Büchele, W. Zugmaier, F. Genze, Th. Simmet

The oleogum resins from various *Boswellia* species commonly named frankincense are primarily known for their contents of pentacyclic triterpenoids such as boswellic acids. Yet these oleogum resins contain additional pharmacologically active compounds belonging to the family of tetracyclic triterpenoids such as tirucallane derivatives. It has been suggested that tetracyclic triterpenpoids might significantly contribute to the overall biological effects of extracts from *Boswellia serrata* oleogum resin. On the other hand, phytopharmaceutical preparations such as extracts from *Boswellia serrata* are believed to be safe and are commonly used in complementary and Ayurvedic medicine. On this background, it seems important to control frankincense-based phytopharmaceuticals for their contents of 3-oxo-tirucallic acid to avoid harming effects for the patients. In any case, it will be essential to investigate the pharmacodynamic and pharmacokinetic properties of 3-oxo-tirucallic acid in greater detail. To our knowledge, so far no HPLC determination of 3-oxo-tirucallic acid in plasma and consequently no pharmacokinetics have been reported yet. Here we describe a rapid, sensitive and selective HPLC separation method combined with photodiode array detection that allows analysis of the tetracyclic triterpenoid 3-oxo-tirucallic acid delivered in a special water-soluble polyvinylpyrrolidone microsuspension. Using a newly developed fluorinated stationary phase, we achieved separation of 3-oxo-tirucallic acid from nearby eluting plasma compounds, collected from chick chorioallantoic membrane vessels. Specifically for studies on human cancer xenografts, this testing system is advantageous because it reduces the number of mammalian animal experimentation due to pre-selection and it requires only small amounts of limited and precious compounds; last but not least, it is economically efficient. The standard curve was linear from 0.2 - 26 µmol/l 3-oxo-tirucallic acid. By a novel combined serial extraction method on diatomaceous earth and graphitized carbon black the compound was isolated from chick embryonic plasma. Recovery, sensitivity and robustness of the 3-oxo-tirucallic acid determination with the developed procedure yielded favourable results. In the range from 0.65 to 2.2 µmol/l plasma, the mean recoveries for 3-oxo-triucallic acid were 86%. The lower limit of quantitation was 0.65 µmol/l plasma. The intra-assay coefficient of variation was 8.1% and 4.0% at 0.65 µmol/l and 2.2 µmol/l, respectively; the inter-assay coefficient of variation was 10.5 % and 7.1 % at the afore-mentioned concentrations. We conclude that the proposed HPLC procedure allows reliable and convenient measurement of 3-oxo-tirucallic acid in plasma.

Institute of Pharmacology of Natural Products & Clinical Pharmacology, University of Ulm, D-89081 Ulm, Germany

327

CISPLATIN ELICITS ITS ANTI-INVASIVE ACTION ON HUMAN CANCER CELLS VIA UPREGULATION OF TISSUE INHIBITOR OF MATRIX METALLOPROTEINASES-1

R. Ramer, K. Eichele and B. Hinz

Cancer cell invasion is one of the crucial events in local spreading, growth and metastasis of tumors. A distinctive feature in this process represents the degradation of basement membranes which is determined by the ratio of matrix metalloproteinases (MMPs) to tissue inhibitors of MMPs (TIMPs). However, pharmacological modulation of this system is poorly understood. The present study therefore investigates the mechanism underlying the anti-invasive action of the chemotherapeutic cisplatin. In human cervical carcinoma cells (HeLa), cisplatin caused a time- and concentration-dependent suppression of cell invasion through Matrigel without a concomitant effect on cellular motility. Inhibition of invasion by cisplatin was accompanied by upregulation of TIMP-1, whereas levels of MMP-2, MMP-9 and TIMP-2 remained unchanged. Cisplatin´s effects on TIMP-1 expression and invasion were associated with phosphorylations of p38 and p42/44 mitogen-activated protein kinases and were abrogated by specific inhibitors of both pathways. A reversal of cisplatin-elicited actions was also achieved by inhibition of protein kinase C, but not by suppression of the phosphatidylinositol 3-kinase pathway. The impact of TIMP-1 in the anti-invasive action of cisplatin was proven by transfecting cells with small interfering RNA targeting TIMP-1 which completely reversed suppression of invasion by cisplatin. A functional relevance of TIMP-1 upregulation was substantiated by findings showing

a concentration-dependent inhibition of Matrigel invasion by recombinant TIMP-1. The essential role of TIMP-1 in the anti-invasive action of cisplatin was confirmed using another human cervical carcinoma cell line (C33A) and human lung carcinoma cells (A549). The TIMP-1-dependent anti-invasive action was mimicked by cannabinoids (R(+)-methanandamide, Δ^9-tetrahydrocannabinol), but not by other chemotherapeutics (5-fluorouracil, paclitaxel) or cytotoxic substances (cycloheximide, C_2-ceramide). Altogether, our data demonstrate a hitherto unknown mechanism by which cisplatin exerts its anti-metastatic properties on highly invasive cancer cells.

Supported by DFG (SFB 539, TP BI.6) and Deutsche Krebshilfe e.V.

Department of Experimental and Clinical Pharmacology and Toxicology, Friedrich Alexander University Erlangen-Nürnberg, D-91054 Erlangen, Germany

328

STRUCTURAL GENOMICS AND DRUG DISCOVERY: CASE STUDIES ON HUMAN METABOLIC ENZYMES

U. Oppermann, K.L Kavanagh, K. Guo, S. Ng, N. Shafqat, J. Dunford, A.Rojkova, E. Pilka, V. Hozjan, G. Kochan, X. Wu, P. Lukacik and all other staff at SGC

The Structural Genomics Consortium (SGC) is a public-private partnership with the mandate to substantially increase structural information on human proteins of medical interest. The SGC is organized as a charity company and the results are immediately made available to the public domain. The goals for the first 3 years of operations were to deposit around 380 novel structures, which will be reached ahead of schedule. The laboratories are located at University of Toronto, University of Oxford and the Karolinska Institute. The SGC has adopted a protein family based approach with biological themes (as implemented in Oxford) reaching from protein phosphorylation, transmembrane signalling to metabolic enzymes. This approach has resulted in structure determination and molecular characterization of a large set of targets of medical relevance and interest in drug discovery. As examples of this high-throughput structure determination and target characterization approaches the results from the Metabolic Enzymes group will be presented and discussed. We have determined among others the structures and mechanism of Nitrogen-containing bisphosphonates (Target: farnesyldiphosphate synthase) used in osteoporosis, the high resolution structure and ligand binding of 11beta-hydroxysteroid dehydrogenase type 1, as target in metabolic disease, and a novel class of epigenetic regulators of the 2-oxoglutarate dependent oxygenases with implications in cancer biology.

Structural Genomics Consortium, Biology group 1: Oxidoreductases and Metabolic Enzymes, University of Oxford, Oxford OX3 7LD, UK
Email: udo.oppermann@sgc.ox.ac.uk

329

DEXAMETHASONE INDUCES ENDOTHELIAL MAPK PHOSPHATASE-1 VIA ACTIVATION OF AP-1 AND CREB AND THE FORMATION OF REACTIVE OXYGEN SPECIES

R. Fürst, S. Zahler, and A.M. Vollmar

Glucocorticoids, such as dexamethasone (Dex), are highly valuable drugs for treating diseases with an inflammatory component. The anti-inflammatory properties of glucocorticoids are thought to be mediated via a glucocorticoid receptor-mediated inhibition of pro-inflammatory transcription factors. However, we could recently introduce the MAPK phosphatase (MKP)-1 as a novel mediator of the anti-inflammatory action of Dex in the human endothelium (Fürst et al., *FASEB J.* 2006; in press). Nothing is as yet known about the signaling pathways responsible for the up-regulation of MKP-1 by Dex. Thus, aim of the present study was to elucidate the underlying molecular mechanisms by which Dex increases MKP-1 expression in human endothelial cells (HUVEC). This increase could be a result of a MAPK-activated feedback loop. Therefore, we hypothesized that MAPK-activated transcription factors could be involved. Indeed, by using Western blot analysis, we showed that all three MAPKs are activated by Dex and, by applying pharmacological inhibitors of these kinases, that they are crucial mediators of MKP-1 induction. As judged by electrophoretic mobility shift assay, Dex activates the transcription factors AP-1 and CREB. We used a decoy approach to causally link Dex-induced AP-1 and CREB activation to the increased MKP-1 expression. Furthermore, we investigated which MAPK could account for the increased AP-1 and CREB activity. JNK and ERK were found to be responsible for an activation of AP-1, whereas ERK and p38 MAPK are involved in CREB activation. Moreover, we could show that reactive oxygen species (ROS formation was measured by loading cells with dihydrofluorescein diacetate) seem to participate in these signaling processes. In summary, we for the first time provide insights into the underlying signaling mechanisms by which Dex induces its novel anti-inflammatory target MKP-1 in the human endothelium: Dex upregulates MKP-1 both via ERK- and JNK-mediated AP-1 activation and via ERK and p38 MAPK-mediated CREB activation. Moreover, ROS are involved in these Dex-induced signaling pathways.

Department of Pharmacy, Pharmaceutical Biology, University of Munich, Germany

330

THE MULTI PDZ DOMAIN PROTEIN MUPP1: A SCAFFOLDING PROTEIN CONTROLLING THE ACROSOME REACTION IN MAMMALIAN SPERMATOZOA

F. Ackermann[1], N. Zitranski[1], D. Meyer[1], D. Heydecke[1], B. Wilhelm[2], T. Gudermann[1] and I. Boekhoff[1]

Upon adhesion to the zona pellucida, mammalian sperm undergo regulated exocytosis of the acrosome. Despite the difference in size, some parallels can be drawn concerning the signal transduction processes controlling the sperm acrosome reaction and synaptic vesicle exocytosis. Since components of intracellular signal transduction pathways are often organized in multiprotein signalling complexes, attempts were made to identify scaffolding proteins expressed in the acrosomal region of mammalian spermatozoa. Using RT-PCR approaches and immunohistochemical as well as Western blot analyses, the Multi-PDZ domain Protein MUPP1, which comprises 13 potential protein interaction modules, was identified in mouse testis tissue. Immunocytochemical experiments combined with immunogold electron microscopy revealed that MUPP1 is exclusively detectable within the acrosomal region of different mammalian spermatozoa and is most prominent at the outer acrosomal membrane. To assess the possible function of MUPP1 during the acrosome reaction, a functional assay with the photosensitive calcium chelator NP-EGTA-AM and an inhibitory anti-MUPP1 antibody was used. The results of these approaches revealed that antibody treatment significantly reduces acrosome reaction compared to sperm incubated with IgG alone. These results together with the observation that MUPP1 co-migrates in detergent-insoluble lipid rafts along with proteins involved in acrosomal exocytosis, like syntaxin-2, indicates that MUPP1 in different mammalian species may assemble similar, if not identical, signaling molecules controlling acrosomal exocytosis.

[1] Institut für Pharmakologie und Toxikologie, und [2] Institut für Anatomie und Zellbiologie, Philipps-Universität Marburg

331

CHRONIC ADMINISTRATION OF RESVERATROL INDUCES A REDOX-DEPENDENT SENESCENCE-LIKE GROWTH ARREST BY TRANSIENT ACCUMULATION OF CELLS IN S-PHASE

E. H. Heiss, Y. D. C. Schilder and V. M. Dirsch

Induction of senescence, an irreversible growth arrest, in cancer cells is regarded as a mean to halt cancer progression. The phytoalexin resveratrol (Rv) is known to possess a variety of cancer preventive, cancer therapeutic and chemosensitizing properties. We report here that chronic treatment with Rv in a subapoptotic concentration of 30 µM induces senescence- like growth arrest in HCT 116 colon carcinoma cells. In contrast to the widely accepted antioxidant property of Rv we demonstrate that one causative stimulus for senescence induction by Rv is an increased level of ROS including hydrogen peroxide and superoxide. Increased ROS levels partly originate from mitochondria and temporally coincide with the retardation of cells in S-phase of the cell cycle accompanied by activation of the ATR/ATM/ Chk1/ cdc2 checkpoint. To elucidate a potential cause effect relationship between ROS and S-phase accumulation we employed the antioxidants NAC (general antioxidant) and mito Q (mitochondrially directed antioxidant) as well as the ATR/ATM inhibitor caffeine and measured in parallel cell cycle distribution and intracellular ROS levels upon exposure to Rv. Whereas caffeine was able to almost completely overcome both S-phase accumulation and increased ROS levels after RV treatment, the antioxidants had –despite successful ROS reduction- only a minor but still significant rescuing effect on Rv-mediated S-phase arrest or cdc2 phosphorylation. We therefore hypothesize that S-phase arrest precedes elevation of ROS after Rv treatment and that there is a positive and self-sustaining feedback loop between both events. Repetitive administration of Rv might therefore mimic chronic oxidative stress and induce a senescence response in cancer cells.

Department of Pharmacognosy, University of Vienna, Austria

332

SOMATIC GENE TRANSFER OF COX-2 AND PGI_2 –SYNTHASE IN A MOUSE MODEL OF HYPOXIA INDUCED PULMONARY HYPERTENSION

E.I. Monjón Tortosa, M. Roth[1], J. Nguyen[2], T. Gessler[1], T.Schmehl[1], B. Fuchs, R.T. Schermuly[1], W. Seeger[1], J. Hänze[1], F. Grimminger[1], L. Fink, N. Weissmann[1]

Aims: Pulmonary arterial hypertension is a progressive pulmonary disorder associated with increased pulmonary vascular resistance, vascular remodelling and right heart failure. In a hypoxia-driven murine model we aimed to reduce pulmonary hypertension and vascular remodelling by transfer of pDNA encoding Cyclooxygenase 2 (COX-2) and Prostacyclin-synthase (PGI_2-synthase).

Methods: Mice were exposed to normobaric hypoxia (10% O_2) for 21 days. In two groups with n=6 mice (pMG plasmid encoding COX-2 and PGI_2–synthase versus empty pMG plasmid) pDNA was orotracheally applied on day 1, 7 and 14 of the hypoxic period. Right-ventricular pressure and right heart weight were measured on day 21. Lungs were fixed, paraffin-embedded, and stained with IHC double-staining for smooth muscle actin and Factor VIIIa. Artery muscularization was assessed by histomorphometrical analysis (using a computerized system, QWin, Leica).

Results: After 21 days, right-ventricular pressure was significantly lower in the treated mice. Moreover, right heart weight was reduced in tendency. Histomorphometrical analysis showed a lower degree of muscularisation of the small vessels (20-70µm) in the transfer group.

Conclusions: Orotracheally applied somatic gene transfer of pDNA encoding COX-2 and PGI_2-synthase was able to reduce right ventricular pressure and vascular remodelling in a murine model of pulmonary hypertension.

Institut für Pathologie, Giessen
[1]Medizinische Klinik II, Uniklinikum Giessen
[2]Institut für Pharmazeutische Technologie, Universität Marburg

333

ANALYSIS OF CYTOTOXIC AND PROINFLAMMATORY EFFECTS OF TWO siRNA-STABILIZING POLYETHYLENIMINES (PEI) IN MURINE LUNG CELLS

A. Beyerle[a,c], A.-G. Lenz[a], H. Schulz[a,c], S. Höbel[b], B. Urban-Klein[b], A. Aigner[b,c], T. Stöger[a,c]

Gene therapy is a promising approach in the treatment of genetic disorders in man. It aims at either supplementing a defective mutant allele with a functional one or repressing the expression of a pathologically induced transcript. In vivo a key step is the delivery of nucleic acids, i.e. DNA or siRNA molecules. Polyethylenimines (PEIs) are polymers known to form noncovalent interpoly-electrolyte complexes with nucleic acids and have been successfully used for non-viral gene delivery. Due to its property to efficiently stabilize nucleic acids by complexation, PEI allows the delivery of intact siRNAs into different target cells upon topical or systemic administration. In particular, the delivery by inhalation holds promise for the treatment of a wide range of pulmonary and non-pulmonary disorders and offers numerous advantages over more invasive modes of administration. For systemic siRNA delivery to induce RNAi in vivo, commercially available jetPEI has already been established, however, other PEIs with lower cytotoxicity and higher transfection efficacy may be desirable. In this study we

compare the cytotoxic and proinflammatory effects of two different PEIs and PEI/siRNA polyplexes in murine target cells of the lung: the macrophage-like cell line RAW264.7 and the alveolar epithelial-like cell line LA4. Particle-related cytotoxcity was determined using assays for cell viability (WST-1) and for the release of the cytosolic enzyme lactate dehydrogenase (LDH). Furthermore, as an indicator of an inflammatory response, the formation of four different proinflammatory cytokines (TNF-α, IL-1β, IL-6, and KC) by the cells was quantified by ELISA. In both cell lines, cytotoxicity was concentration dependent. The influence on the metabolic and mitochondrial activity and the LDH release of the polyplexes (PEI/siRNA) was found to be lower than that of the polycations themselves. When evaluating the proinflammatory effects of both polycations and their polyplexes, we observed no increase in IL-1β and only slightly elevated levels of TNF-α, IL-6 and mouse KC, with these effects being not concentration dependent. We conclude that both PEIs and PEI complexes are non-toxic at low concentrations relevant for RNAi induction. Further approval of these results in *in vivo* models would allow their use as non-viral nanocarriers for gene therapy applications.

[a] GSF- National Research Center for Environment and Health, Institute for Inhalation Biology, Munich-Neuherberg, Germany
[b] Department of Pharmacology and Toxicology, Philipps-University, School of Medicine, Marburg, Germany
[c] DFG Forschergruppe 627 "Nanohale"

334

PEG-PEI/siRNA AND PEI/siRNA COMPLEXES FOR siRNA DELIVERY TO INDUCE RNA INTERFERENCE (RNAi)

A. Malek[1,3], O. Merkel[2,3], F. Czubayko[1,3], T. Kissel[2,3] and A. Aigner[1,3]

RNA interference (RNAi) has emerged as a powerful strategy for the inhibition of gene expression in vitro and in vivo. It is mediated through small interfering RNAs (siRNAs) which trigger specific mRNA degradation. The instability and poor delivery of unmodified siRNA molecules into target cells and target tissues, however, so far severely limits the use of siRNAs in vivo. Polyethylenimines (PEIs) are synthetic polymers with a protonable amino group in every third position and a high cationic charge density. Thus, PEIs are able to form non-covalent interpolyelectrolyte complexes with DNA or siRNAs allowing their protection from nucleolytic degradation, efficient cellular uptake through endocytosis and intracellular release due to the 'proton sponge effect'. To increase biocompatibility and cellular delivery, PEG-PEIs, i.e. PEIs covalently coupled to polyethylenglycol (PEG), with vastly different structures were synthesized. More specifically, PEI 5 kDa and PEI 25 kDa were PEGylated with similar composition (~ 50 % PEG content), but with PEG chain length and graft density varying within a wide range. Here, we explore the applicability of these PEIs and PEG/PEIs for siRNA delivery in vitro and in vivo. While all (PEG-)PEIs mediate siRNA protection against nucleases, although to a different extent, the comparison of DNA transfection and siRNA targeting efficiencies in cell culture reveals differences between (PEG-)PEI/DNA and (PEG-)PEI/siRNA complexes regarding their bioactivity. Furthermore, between different (PEG-)PEIs, differences in complex toxicities are observed. In vivo tissue distribution experiments upon i.v. injection of ^{32}P-end labeled, complexed siRNAs reveals the rapid, exponential siRNA clearance from the blood and the delivery of intact siRNAs into various tissues including liver, spleen, kidney and lung. The efficient PEI-mediated delivery of siRNAs to the lung will allow to study the potential of targeting tumor relevant genes in lung metastases, which represents one project within the DFG Forschergruppe 627 "Nanohale".

[1]Dept. Pharmacology and Toxicology, Philipps-University, School of Medicine, Marburg, Germany
[2]Dept. Pharmaceutics and Biopharmacy, Philipps-University Marburg, Germany
[3]DFG Forschergruppe 627 "Nanohale"

335

IN VITRO STUDIES ON THE EFFECTS OF DEXAMETHASONE IN OCULAR INFLAMMATION USING A CANINE CORNEAL CELL PRIMARY CULTURE

A. Werner, M. Braun and M. Kietzmann

Ocular inflammations in ophthalmology are routinely treated with glucocorticoids such as dexamethasone. Most ophthalmic drugs have been approved for the use in humans but not in animals. The penetration of dexamethasone into the eye has been studied in different species (rabbit, dog, cat, horse) but not in humans, therefore the animal studies are used as a model for men. Interspecies differences in their pharmacokinetics can be found, nevertheless, high drug concentrations are generally measured in the anterior structures of the eye, especially in the cornea. This work was conducted to study the cellular effects dexamethasone exhibits on the three major cell types of the cornea (e.g. endothelium, fibroblasts, epithelium) in an *in vitro* system. Primary canine corneal endothelial, epithelial cells and fibroblasts were taken into cell culture. They were stimulated either with lipopolysaccharide (LPS) or sodium dodecyl sulfate (SDS) to provoke an inflammatory-like reaction which was measured as an increased prostaglandine E_2 (PGE_2) production. The cells were treated with different dexamethasone concentrations (1.0, 0.1 and 0.01 µg/ml). An enhanced PGE_2 concentration was measured after the stimulation with LPS and SDS. Dexamethasone reduced the LPS-induced PGE_2 production in a dose-dependent manner. The SDS-induced PGE_2 concentration was less clearly reduced by dexamethasone, caused by a higher variability of the results. Nevertheless, a trend towards a dose-dependent PGE_2 reduction due to the dexamethasone treatment was detectable. The primary cell culture of the three canine corneal cell types is an interesting system to evaluate the effects of dexamethasone. Still, further studies are needed to study the molecular effects of dexamethasone on these cells. Also a three-dimensional reconstruction of the cornea using these primary cells is being performed to compare theses results with a situation that is still closer to that *in vivo*.

Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

336

COMPARISON OF PERCUTANEOUS PERMEATION WITH EPIDERMAL LIPID COMPOSITION IN FOUR DIFFERENT SPECIES

J. Stahl, F. Niedorf, M. Kietzmann

Interspecies and interindividual variations are a major problem in the estimation of percutaneous permeation in toxicological risk-assessment as well as in dermatological and pharmacological research. As some lipid classes have been described to significantly influence the epidermal permeability barrier, these variations may be attributed to differences in the skin lipid profiles in different mammalians. Therefore we quantified multiple lipid classes in canine and porcine abdominal, in rat back and bovine udder skin, which are accepted skin models in pharmacology and pharmacy or which are important in veterinary medicine. The epidermal lipids were extracted from heat separated epidermis using a chloroform-methanol mixture. The content of extractable lipids of each individual was determined and the different lipids were isolated and quantified by high-performance thin-layer chromatography using a sequence of three solvent systems. Significant differences in the total lipid content between the species were found, with the highest amount in bovine udder skin, followed by dog, pig and rat skin. Some lipid fractions showed significant variations (e.g. cholesteryl ester with the highest quantity in dogs and the lowest in bovine udder skin), while others were detectable in only some species (ceramide 4 could only be found in the epidermis of pigs) or showed no significant differences (free fatty acids). Additionally we examined the *stratum corneum* barrier strength of the above mentioned species in a standardised *in vitro* set-up using four substances with different molecular weight and lipophilicity (flufenamic acid, ibuprofen, indomethacine, and salicylic acid). We conclude that a correlation between the lipid profile and the permeability of drugs through the skin may exist.

Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

337

EFFECTS OF THE ANTIDEPRESSANT CITALOPRAM ON CRE/CREB-DIRECTED GENE TRANSCRIPTION IN TRANSGENIC REPORTER GENE MICE

C. Noll, U. Böer, Cierny I., W. Knepel

The selective serotonin reuptake inhibitor citalopram has been used as an effective antidepressant in major depressive disorder. In contrast to its immediate action which positively influences energy and intrinsic drive, the antidepressant action occurs with a time lag of 2-3 weeks. This delay of the therapeutic response can be explained on the molecular level by a neuronal adaptation based on a changed pattern of expressed genes. Gene expression is regulated by transcription factors like the cAMP-responsive element (CRE) binding protein (CREB). A prerequisite for the activation of CREB is its DNA-binding and phosphorylation on serine-133. CREB has been reported to be important for adaptive brain functions like learning and memory and also might mediate the action of antidepressants. However, there are no studies available which investigated the effect of citalopram on CRE/CREB-driven gene transcription in vivo by means of transgenic mice. In our laboratory a transgenic mouse line was generated expressing a luciferase reporter gene controlled by four copies of the CRE from the rat somatostatin gene promoter. In the present study the effect of citalopram on the CRE/CREB-directed gene transcription in vivo was investigated. For this purpose transgenic mice were treated acutely (24h) and chronically (21d) with citalopram (2x25mg/kg/d). Luciferase activity was measured in homogenates of different brain regions. Acute treatment significantly increased CRE/CREB-directed transcription in the olfactoric bulb by 167%, in the cerebellum by 188%, in the pons by 162%, in the colliculi by 152%, in the prefrontal cortex by 194%, and in the cortex by 213%. CREB phosphorylation on Ser-133 measured by western blot analysis in whole brain homogenates was also slightly increased. By contrast, chronic treatment did not have an effect on CRE/CREB-directed transcription. Thus, the antidepressive action of citalopram occurring with a delay seems not to be mediated by CREB, however, as CRE/CREB-directed transcription is activated by acute treatment it could be involved in the immediate action on energy and intrinsic drive.

Department of Molecular Pharmacology, Georg-August-University Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

338

A SMALL *CLOSTRIDIUM BOTULINUM* C3 FRAGMENT COMPRISING AMINO ACIDS 154-182 IS SUFFICIENT TO PROMOTE AXONOTROPHIC EFFECTS IN MURINE HIPPOCAMPAL NEURONS

M. Höltje, G. Ahnert-Hilger, I. Just, F. Hofmann

We were able to demonstrate that *Clostridium botulinum* C3 exoenzyme ($C3^{bot}$) may not exclusively base on enzymatic inactivation of Rho by ADP-ribosylation. Using primary cultures of murine hippocampal neurons we report that $C3^{bot}$ possesses an additional axonotrophic function independent from its enzymatic activity (Ahnert-Hilger et al., 2004). Based on these observations we further investigated this remarkable and till then unobserved effect. Therefore, we truncated down the full length $C3^{bot}$ protein and constructed overlapping peptide fragments that harboured no enzymatic activity. By doing so we wanted to identify the region of $C3^{bot}$ responsible for the axonotrophic effect. Application of $C3^{bot}$ derived fragments should help to answer the question whether the enzyme independent effects relied on the tertiary structure of $C3^{bot}$ or were based simply on the amino acid sequence. In this regard we observed promoting effects on axon outgrowth after application of nanomolar concentrations of $C3^{bot}$ peptide comprising the amino acids 154 to 182. Additionally, we further extended our investigations from the hippocampus to distinct neuronal subpopulations located in different areas of the CNS. In detail, we analyzed possible axonotrophic effects on cholinergic α-motoneurons of the spinal cord, GABAergic cerebellar Purkinje cells as well as on serotonergic neurons of the raphe nuclei. Data on the morphometrical analysis of the various neuronal subpopulations will be shown.

Institut für Toxikologie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany; Centrum für Anatomie (AG Funktionelle Zellbiologie), Charité-Universitätsmedizin Berlin, Philippstr. 12, D-10115 Berlin

339

CLOSTRIDIUM BOTULINUM EXOENZYME C3 ACTS AS NEUROTROPHIC FACTOR ON HUMAN NEURONS

C. Mühlenstädt, R. Brandt, F. Hofmann, I. Just

Clostridium botulinum exoenzyme C3 ($C3^{bot}$) is a member of the ADP-ribosyltransferase family to specifically inactivate RhoA, B and C. C3 exoenzymes are widely used as pharmacological tools to analyze the intracellular functions of Rho GTPases. Beside many other effects, Rho GTPases serve as key regulators in various aspects of neuronal development. This includes neurite outgrowth, differentiation and axon pathfinding. Recently, we demonstrated in murine hippocampal primary neurons that extracellular application of $C3^{bot}$ and its enzymatically inactive mutant $C3^{bot}$E174Q in nanomolar concentrations induced axonal growth and branching. In order to extend these findings we used the human teratocarcinoma cell line NT2, which can be differentiated by retinoic acid treatment into postmitotic, terminally differentiated and polarized neurons (NT2-N). Application of $C3^{bot}$ or $C3^{bot}$E174Q led to a significantly increased axonal growth in NT2-N cells compared to non-treated control cells. Further truncation of the holo-C3 (211 amino acids), revealed a small fragment consisting of 30 amino acids ($C3^{bot}$154-182) able to induce the same effects. In contrast, the fragment $C3^{bot}$181-211 did not induce axonal growth and was therefore used as a negative control. In combination with the observations in murine hippocampal primary neurons these findings suggest a novel neurotrophic function of $C3^{bot}$ not mediated by direct inactivation of RhoA due to ADP-ribosylation. Furthermore, the low concentration required to cause axonotrophic effects suggest a ligand-like interaction with a membrane receptor.

Institut für Toxikologie der Medizinischen Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover; Abteilung Neurobiologie der Universität Osnabrück, Barbarastr. 11, D-49076 Osnabrück

340

KNOCK-DOWN OF PORCINE α-1,3-GALACTOSYLTRANSFERASE USING LENTIVIRAL RNAI

D. Messow[1], A. Hofmann[1], B. Kessler[2], R. Schwinzer[3], E. Wolf[2], A. Pfeifer[1]

The critical shortage of human donor organs has generated growing interest for porcine xenotransplantation. Humans and old world monkeys carry natural occurring antibodies against galactosyl-α-1,3-galactose (αgal) epitopes present on the surface of pig cells which mediate hyperacute rejection (HAR). To overcome this most immediate barrier to successful xenotransplantation we used lentiviral vectors to induce RNA interference (RNAi). We generated a panel of different lentiviral shRNA vectors for silencing of the α-1,3-galactosyltransferase (αGT), the main enzyme generating the αgal epitopes. Porcine cells transduced with lentiviral vectors encoding shRNAs targeting αGT demonstrated efficient knock-down. From 18 lentiviral shRNAs tested, three constructs induced a knock-down of αGT mRNA below 19% of wild-type levels (ranging from 13.8% ± 0.87% to 18.9% ± 1.16%, n=3 ± SEM). Staining with FITC-conjugated lectin (GS-IB4) revealed an equivalent reduction of αgal epitopes, as demonstrated by immunocytochemistry and Fluorescence Activated Cell Sorting (FACS). To test the functional significance of lentiviral shRNA-mediated knock-down of αGT, we analyzed complement-mediated lysis. Most importantly, complement-mediated lysis of lentivector transduced porcine cells was decreased by 94.8%. In addition, analysis of cell clones with one to four viral shRNA integrants showed stable knock-down of αGT mRNA over six passages. Depending on the number of integrants complement-mediated lysis was suppressed in these clones. Taken together, lentiviral shRNA induced an efficient and stable knock-down of αGT. Induction of RNAi with lentiviral shRNA vectors is a promising step towards xenotransplantation. The combination of lentiviral transgenesis with RNAi might be a faster and cheaper method than generation of αgal knockout pigs produced by nuclear transfer using gene-targeted cells.

[1]Institute of Pharmacology and Toxicology, Friedrich-Wilhelms-University Bonn,
[2]Department of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-University Munich,
[3]Transplantation Laboratory, Department of Visceral and Transplantation Surgery, Hannover Medical School

341

CREB DIMERIZATION IS CRUCIAL FOR THE INTERACTION WITH TORC (TRANSDUCER OF REGULATED CREB)

A. Heinrich, U. Böer, W. Knepel

The CRE-binding protein (CREB) is a ubiquitously expressed transcription factor strongly implicated in adaptive brain function. CREB contains a transactivation domain, in which phosphorylation of serine 119 is promoting transactivation; it also contains a basic leucine zipper region (bZip) responsible for dimerization and DNA-binding to the cAMP-responsive element. Recently a new cofactor family of CREB has been identified: the transducer of regulated CREB activity (TORC). TORC has been reported to interact with the CREB bZip at arginine 300 (R300), since this interaction was interrupted when arginine 300 was mutated to alanine (R300A). Analysis of the crystal structure of bZip predicted the amino acids R300 and glutamate 304 (E304) to mediate the dimerization of CREB proteins. Regarding these facts converging on one amino acid, we focused on the question whether dimerization of CREB is necessary for an interaction with TORC. Therefore, we established a cell-free interaction assay using GST-fused full length CREB (wild type / R300A) and [^{35}S]-labelled CREB (wild type / R300A) in pull-down experiments. The necessity of CREB dimerization for an interaction with TORC was tested using GST-fused CREB (wild type / R300A) and [^{35}S]-labelled TORC. R300 was confirmed to be crucial in mediating dimerization of CREB, as the interaction between GST-CREB-R300A and [^{35}S]-CREB-R300A was reduced by ~85% with respect to the wild type proteins. All three TORC isoforms showed markedly reduced interactions with the mutant GST-CREB-R300A. Importantly, the interaction was decreased to the same extend as the CREB dimerization was reduced due to the mutation. Thus we confirmed the importance of R300 for the CREB-TORC interaction, but assume that the disruption of the CREB-TORC interaction is most likely not due to the exchange of one single amino acid, but rather to an impaired CREB dimerization. For the first time it was shown here, that R300 is indeed crucial for the formation of a CREB dimer, while the dimerization of CREB is necessary for an interaction with TORC.

Department of Molecular Pharmacology, University of Goettingen

342

IS IN-VITRO INDUCTION OF CYP2B IN RAT LIVER SLICES (RLS) BY PHENOBARBITAL (PB) INFLUENCED BY CYP3A INDUCING DEXAMETHASONE (DEX)?

R. Glöckner, S. Rohländer and D. Müller

Precision-cut liver slices are suitable to demonstrate *in vitro* induction of several CYP forms by model inducers. For slices prepared from human liver tissue marked interindividual differences have to be expected, which might be caused by quite different CYP expression states. With RLS we could demonstrate that *in vitro* inducibility of CYP2B1, in contrast to CYP1A1 and CYP3A1, is substantially influenced by preceding *in vivo* induction of the tissue donor with the same inducer. Now we demonstrate the influence of DEX on the inducibility by PB. RLS were exposed to PB (5×10^{-6} - 10^{-4} M) for 24h to induce CYP2B1. DEX treatment was performed either *in vivo* (3 x 4 mg/kg i.p.) with RLS preparation 24h after the last injection, or *in vitro* for 24h (0.01 or 1 µM) simultaneously with or before PB exposure. In RLS from DEX pretreated rats, CYP3A1 dependent activity was initially enhanced as expected, but had decreased within 24h of slice incubation. The concentration dependent CYP2B inducibility by PB *in vitro* was not disturbed, as shown at the level of both enzyme activity and CYP2B1-mRNA expression. If RLS of untreated animals were incubated simultaneously with PB and DEX for 24h, CYP2B1 induction was not disturbed either. 1 µM but not 0.01 µM DEX alone enhanced CYP activities, thus decreasing induction factors despite of higher absolute activities. A succesive *in vitro* exposure to DEX for 24h, followed by PB for 24h, improved increase of enzyme activities in some cases, although similar absolute biotransformation rates were reached. Induction of CYP2B1-mRNA expression seemed to be depressed by foregoing exposure to 1 µM DEX. We conclude that CYP2B induction can be detected *in vitro* in RLS independent of preceding or simultaneous exposure to DEX at the level of CYP activity. Preceding CYP3A1 induction *in vitro*, but not *in vivo*, seems to depress CYP2B1-mRNA induction.

Klinikum der Friedrich-Schiller-Universität, Institut für Pharmakologie und Toxikologie, 07740 Jena, Germany

343

ETHOXYRESORUFIN- AND METHOXYRESORUFIN-*O*-DEETHYLATION ACTIVITIES IN HUMAN LIVER SAMPLES: GENETIC AND OTHER DISPOSITION FACTORS

A. Lupp[1], U. D. Kuhn[2], J. Etzrodt[3], W. Daffner[3], M. Hommann[3], U. Settmacher[3], D. Müller[1]

Cytochrome P450 (CYP) 1A2 is involved in the biotransformation of many drugs and environmental chemicals. Several single nucleotide polymorphisms have been shown for the gene of this enzyme and known inducers are polycyclic hydrocarbons, found e.g. in tobacco smoke and charcoal-broiled meat. In the present study, the impact of five mutations in the CYP1A2 gene was investigated in comparison to the influence of other factors on the *O*-deethylation of two known probe drugs for CYP1A2, 7-ethoxyresorufin and 7-methoxyresorufin (EROD, MROD), in a total of 103 histologically tumour-free human liver samples obtained from surgical waste. 100 of these livers were genotyped using polymerase chain reaction amplification and restriction fragment length polymorphism analysis. EROD and MROD activities were determined in 9000*g* supernatants of the livers. From the 100 livers genotyped 4 were heterozygous for CYP1A2*1C (-3860G>A), 57 heterozygous and 5 homozygous for CYP1A2*1F (-163A>C), and 4 heterozygous for the CYP1A2*1E (-739T>G) mutation. No -729C>T and 558C>A (CYP1A2*11) mutations were found. EROD and MROD activities were significantly elevated in the few livers heterozygous for the -739T>G mutation. Smokers displayed distinctly higher enzyme activities than non-smokers when carrying the -163AA genotype only. No differences between smokers and non-smokers were seen with individuals having the -163AC or -163CC genotype or in the few cases with a -3860G>A or -739T>G mutation. EROD and MROD activities strongly correlated with each other. A weak association was also seen with the serum albumin concentration, the body mass index and the inflammation parameter CRP. No influence on enzyme activities could be demonstrated with respect to age, gender, alcohol consumption, diagnosis leading to surgical intervention (except for significantly lower values with cholangiocarcinomas), duration of the surgical procedure, time span until sample freezing, serum parameters indicating liver damage or function, blood count or blood glucose levels.

[1]Institute of Pharmacology and Toxicology, [3]Institute of Clinical Pharmacology, [3]Department of General and Visceral Surgery, Friedrich Schiller University, Jena, Germany

344

CHARACTERIZATION OF CYTOCHROME P450 EXPRESSION IN HUMAN UROTHELIAL CELLS

S. Tschirbs, A. Dörrenhaus, P.H. Roos

Exposure to polycyclic aromatic hydrocarbons or to arylamines such as benzo[a]pyrene and 4-aminobiphenyl are associated with elevated risk of bladder cancer in human. We could show that human exfoliated urothelial cells express several cytochromes P450 (CYP) which are involved in the metabolism of these compounds (Roos et al., 2006; Dörrenhaus et al., 2006) and, therefore can be considered as site-relevant mediators in the process of carcinogenesis. We would like to know whether urothelial derived cell lines are comparable to cultured human exfoliated cells with respect to expression and inducibility of CYPs and can therefore be considered as suitable model systems in toxicological studies.

Methods. Relative transcript levels of the following CYPs were determined by RT-PCR in 5637 cells (urothelial cell line): 1A1, 1B1, 2B6, 2E1, 2F1, 2J2, 2S1, 3A4, 3A5 and 4B1. For induction experiments, the cells were treated for 48h with several inducers: benzo[a]pyrene (5/10/20/30/40/50µM), a combination of benzo[a]pyrene (10µM)/4-aminobiphenyl (3/10µM), a combination of benzo[a]pyrene (10µM)/beta-naphthylamine (3/10µM) or left untreated. For detection of CYP1A1-protein cells were grown and fixed on glass slides, immunostained with anti-CYP1A1 and a TRITC-conjugated secondary antibody and examined by fluorescence microscopy.

Results. The following CYPs were found to be constitutively expressed on the mRNA-level in 5637 cells: 1A1, 1B1, 2E1, 2J2, 2S1, 3A5, 4B1 while CYP2B6, CYP2F1 and CYP3A4 could not be detected. Treatment of the cells with benzo[a]pyrene lead to induction of CYP1A1-mRNA, CYP1A1-protein and CYP2S1-mRNA while expression of CYP4B1-mRNA was suppressed. The mRNA-levels of CYP1B1, 2J2 and 3A5 were not altered by benzo[a]pyrene. Treatment with ethanol induced CYP2E1-mRNA expression whereas treatment with benzo[a]pyrene inhibited the expression of CYP2E1-mRNA.

Conclusions. We showed that a broad range of cytochromes P450 are expressed at least on the mRNA-level in the urothelial cell line 5637 and that CYP1A1, 2S1 and 2E1 are inducible by their respective inducers. Considering published data on human exfoliated urothelial cells, we found some differences compared to 5637 cells with respect to the constitutive CYP-expression pattern.
Institut für Arbeitsphysiologie, Ardeystr. 67, D-44139 Dortmund, Germany

345

EXPRESSION AND INDUCIBILITY OF CYP1A1 IN HUMAN EXFOLIATED UROTHELIAL CELLS

A. Dörrenhaus, S. Tschirbs and P.H. Roos

Xenobiotics such as polycyclic aromatic hydrocarbons or arylamines may induce carcinogenic processes in human urothelium. In exfoliated urothelial cells (EUC) from the human urinary sediment, transcripts of several cytochromes P450 (CYP) can be detected (Roos et al, 2006, *Arch Toxicol* 80, 45-52). Additionally, the presence of CYP1A1 protein has been shown by immuno fluorescence microscopy in these cells. Interestingly, the numbers of CYP1A1-positive cells differ between smokers (44%) and non-smokers (6%) (Dörrenhaus et al., 2006, *Arch Toxicol* 80, 45-52). On the background of this differential expression and on the fact that EUCs of some persons, either smokers or non-smokers, reproducibly did not show any CYP1A1-expression, we investigated the inducibility of CYP1A1 by benzo[*a*]pyrene (BaP) treatment in primary cultures of human exfoliated urothelial cells. In parallel, the effect of ethanol on the Ah receptor-independent induction of CYP2E1 was analysed. METHODS: Induction of CYP1A1 and CYP2E1 was followed by immuno fluorescence microscopy using enzyme-specific monoclonal antibodies. Identification of urothelial cells in the cell preparation was done by co-staining with FITC-conjugated Griffonia simplicifolia lectin, which binds to specific cell surface glycoconjugates of urothelial cells. RESULTS: BaP treatment of cultured EUCs obtained from persons exfoliating CYP1A1-positive cells leads to induction of CYP1A1 protein in these cells. In contrast, cells of persons exfoliating CYP1A1-negative cells exclusively, do not respond to BaP treatment (up to 40µM). Upon treatment with ethanol, however, CYP2E1 can be induced in these cells. CONCLUSIONS: In conclusion, human exfoliated urothelial cells maintain their CYP1A1-inducibility during cell culture. Within our study group of 40 persons two phenotypes with respect to basal expression and inducibility of CYP1A1 in EUCs can be distinguished. It appears that in 12% of the test persons CYP1A1 expression/ induction is affected. Currently, we investigate the molecular background of this phenomenon.
Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystraße 67, 44139 Dortmund, Germany

346

REGULATION OF CYTOCHROME P450 EXPRESSION BY β-CATENIN

A. Braeuning, C. Koehle, A. Buchmann, M. Schwarz

Cytochrome P450 (Cyp) isoenzymes constitute the most important group of xenobiotic-metabolizing enzymes in the liver, exhibiting inducible expression regulated by different exo- and endogenous compounds via a set of ligand-activated transcription factors. However, the mechanisms that regulate basal Cyp expression in liver are poorly understood. Recently, overexpression of several Cyp isoforms as well as of nuclear receptors mediating Cyp induction, e.g. aryl hydrocarbon receptor (AhR) and constitutive androstane receptor, in mouse liver tumors with mutations in the *Ctnnb1* (encoding β-catenin) proto-oncogene has been reported (Stahl et al., Hepatology 42, 353-61, 2005). To study Cyp induction by β-catenin, primary mouse hepatocytes were treated with inducers of the Wnt/β-catenin signaling pathway, leading to an upregulation of several Cyp mRNAs. Moreover, activation of the β-catenin pathway elevated the inducibility of Cyp1a mRNA by 2,3,7,8-tetrachloro-dibenzo-(p)-dioxin (TCDD). Depletion of β-catenin by siRNA reduced basal Cyp1a expression as well as attenuated induction of Cyp1a by TCDD. To test whether the induction of Cyp1a by β-catenin was caused by an increase in mRNA levels of the AhR, a known target gene of β-catenin signaling, luciferase reporter vectors for human Cyp1a1 were constructed containing either the wild-type promoter sequence or mutated versions lacking the transcription factor binding sites for AhR/Arnt or β-catenin/TCF. Luciferase activity was equally inducible in the wild-type and the AhR binding site-deficient vectors by stimulation of β-catenin signaling. Moreover, both basal and maximum TCDD-inducible luciferase activity was decreased by mutating the β-catenin/TCF binding site in the promoter. In BP8+ rat hepatoma cells with constitutive, non β-catenin-inducible expression of the AhR, activation of β-catenin by LiCl treatment led to an induction of Cyp1a mRNA comparable to that in the AhR wild-type parental cell line 5L. In conclusion, the data suggest a direct participation of β-catenin in transcriptional regulation of basal and inducible expression of Cyp1a.
Institute of Pharmacology and Toxicology, University of Tuebingen, Wilhelmstrasse 56, 72074 Tuebingen, Germany

347

REGULATION OF CYP2E1 AT THE PROTEIN- AND ACTIVITY-LEVEL IN MINIPIG-TISSUES

C.U. Koehler[1], P. Welge[2], P.H. Roos[1]

For cytochromes P450 (CYP), gene-activation in response to xenobiotics is a well-characterised phenomenon. In the case of CYP2E1, effects of xenobiotics also include processes downstream of transcription such as mRNA- and protein-turnover. To understand xenobiotic effects on enzyme-activity, the biosynthesis of the protein has to be monitored at its various developmental stages. We analysed CYP2E1-protein-expression and activity in minipig-tissues and studied its regulation in response to ethanol- and isoniazid-exposure.
Methods. Male Göttingen minipigs were treated with ethanol and isoniazid for seven days and sacrificed. CYP2E1-protein-levels were determined by immunoblotting. Chlorozoxazon-6-hydroxylation was measured. Its dependence on CYP2E1 was proven by applying the CYP2E1-specific inhibitor diethyldithiocarbamate.
Results. Liver, kidney, lung, forebrain and small intestine showed constitutive chlorozoxazon-6-hydroxylation. Except for the kidney, CYP2E1-protein was detected in all these tissues, too. 70 to 90 % of the chlorozoxazon-6-hydroxylase-activity could be inhibited by diethyldithiocarbamate, also in the kidney. Liver and kidney possess the highest metabolic capacity. Ethanol induced chlorozoxazon-6-hydroxylase-activity in the liver and suppressed it in the lung. Isoniazid repressed chlorozoxazon-6-hydroxylation in both tissues. Strongest effects were observed for the brain: Ethanol elevated the chlorozoxazon-6-hydroxylase-activity 17-fold, while isoniazid concomitantly increased activity (24-fold) and CYP2E1-protein-expression (11-fold). Within one organ, CYP2E1-expression and activity were rarely correlated. A positive correlation (R^2= 0.8331) could only be observed for the small intestine under isoniazid-exposure. Interestingly, we found a strong negative correlation (R^2= -0.9165) between CYP2E1-expression and activity in the lung of ethanol-treated minipigs.
Conclusions. Catalytically active CYP2E1 can be detected in all investigated minipig-tissues. Protein-expression and activity show tissue- and inducer-specific responses to ethanol- and isoniazid-treatment. As protein-expression and –activity are not necessarily modified concomitantly, post-translational events seem to determine CYP2E1-activity in most tissues. The strong inducibility of brain-CYP2E1 raises the question, if susceptibility to neurotoxic effects of some CYP2E1-substrates is dependent on the individual induction-situation (e.g. of alcohol abusers).
[1]Institut für Arbeitsphysiologie, Ardeystr. 67, D-44139 Dortmund, Germany
[2]Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin, Bürkle-de-la-Camp Platz 1 D-44789 Bochum, Germany

348

TRANSCRIPTIONAL REGULATION OF UDP-GLUCURONOSYLTRANSFERASE UGT2B1 EXPRESSION IN PRIMARY RAT HEPATOCYTE CULTURES

V. Ritz[1], C. Lass[1], K. Barnewitz[1], C. Ziemann[2], G. Rüdell[1], D. Bauer[1], C. Schmitz-Salue[1], and K. I. Hirsch-Ernst[1]

Rat UDP-glucuronosyltransferase UGT2B1 is involved in metabolism of endogenous and xenobiotic substrates, including opioids, testosterone and bisphenol A. UGT2B1 belongs to the spectrum of hepatic enzymes subject to induction by phenobarbital (PB). In primary rat hepatocyte cultures, UGT2B1 mRNA expression was maximally induced within 3 days by 1.5 mM phenobarbital (PB) or 100 µM permethrin, a pyrethroid acting as a PB-type inducer. Induction was repressed by the hepatotrophic growth factor EGF (16 nM) and by the prostaglandin E receptor agonist misoprostol (1-10 µg/ml), while induction was markedly enhanced in the presence of the antioxidant N-acetylcysteine (10 mM). Noteworthy, PB-dependent induction of cytochrome P450 CYP2B1 was also modulated by EGF, misoprostol or *N*-acetylcysteine, demonstrating coordinate regulation of UGT2B1 and CYP2B1 expression. To investigate molecular mechanisms of *UGT2B1* gene regulation, primary rat hepatocytes were transfected with *UGT2B1*-luciferase reporter gene constructs and luciferase expression observed following treatment with modulators of UGT2B1 expression. It was shown that a 1311 bp *UGT2B1*-promoter fragment was responsive to PB-type inducers, as well as to modulators of induction, indicating that transcriptional regulation greatly contributed to control of UGT2B1 mRNA expression. Systematic UGT2B1 promoter deletion analysis revealed that a proximal fragment containing 239 bp of the 5´-*UGT2B1* gene flank was sufficient in conferring responsiveness to PB-like inducers and modulators of induction. Comparison to the human *CYP3A4* proximal promoter (that contains designated sites involved in xenobiotic responsiveness), suggests that a sequence within the proximal *UGT2B1* promoter fragment, comprising a putative nuclear receptor binding site in close proximity to a C/EBP-α binding site, may be a target of PB-dependent activation. Striking parallels in regulation of UGT2B1 and CYP2B1 mRNA expression by PB-type inducers, cytokines, cellular redox status and prostaglandin E receptor stimulation, suggest that integrative molecular mechanisms for regulation of different xenobiotic metabolizing enzyme genes exist. (Supported by SFB 402, TPA2)

[1] Institute of Pharmacology and Toxicology, University of Göttingen, D-37075 Göttingen,
[2] Fraunhofer Institute of Toxicology and Experimental Medicine, D-30625 Hannover

349

DEVELOPMENT OF AN ANTIBODY TARGETED AGAINST HUMAN CYTOCHROME P450 2F1 AND ITS APPLICATION IN HUMAN LUNG MICROSOMES

U. Bernauer[1], B. Heinrich-Hirsch[1], M. Tönnies[2], P. Wolski[1] and U. Gundert-Remy[1]

Background: Target tissue toxicity can be related to local metabolic activation in the target tissue. The lung represents an important target tissue for airborne substances of which some require metabolic activation in order to be toxic. It could be demonstrated in animal (mainly mouse) models, that CYP2F is involved in the pulmonary activation of many compounds, which are toxic to the lung of animals (e.g. naphthalene, styrene, 3-methylindole, 4-ipomeanol). In humans, pulmonary CYP2F1 has been detected at the mRNA level, but there are no published results on the expression of CYP2F1 protein. In order to determine whether CYP2F1 protein is present in human lung, we developed a polyclonal antibody targeted against human CYP2F1 and investigated human lung microsomes from different human individuals by using this antibody.
Methods: Rabbits were immunised with a mix of two different coupled peptides representing characteristic fragments of the human CYP2F1 protein. Antisera (from different bleedings after immunisation) were affinity purified against each of the two peptides and checked against peptides by ELISA. Western Blotting used the serum yielding highest specificity in ELISA for analysing human lung microsomes: 10 µg of human microsomal lung protein from each individual was loaded onto SDS-polyacrylamide gels.
Results: 108 different individual lung samples were investigated. In all human samples investigated, a distinct single immunoreactive band could be determined, which had a molecular weight comparable to that described for CYP2F1 in the literature (55 kDa). The result points to the fact that human lung constitutively expresses CYP2F1 protein. Currently we seek to confirm our findings by mass spectrometric identification of the immunoreactive protein and by establishing a specific activity assay. From the results, it can be concluded that human lung is able to metabolise and to activate CYP2F1 substrates that enter the lung.
[1]Federal Institute for Risk Assessment /Bundesinstitut für Risikobewertung (BfR), Thielallee 88-92, D-14195 Berlin, Germany
[2]Zentralklinik Emil von Behring, Department Lungenklinik Heckeshorn, Zum Heckeshorn 33, D-14109 Berlin, Germany

350

CO-REGULATION OF CYP3A AND PXR GENE EXPRESSION IN RAT AND HUMAN HEPATOMA CELLS

Y. Fery[1], S.O.Mueller[2], D. Schrenk[1]

Nuclear receptors are ligand-activated transcription factors that respond to endogenous substances, food constituents, therapeutic drugs and other xenobiotics. They regulate metabolic pathways by alterations in gene expression of key metabolic enzymes. One of these receptors, the Pregnane X Receptor (PXR) was found to be efficiently activated by pregnanes, clinically used drugs, and by a broad spectrum of other compounds including plant constituents, pesticides, and environmental contaminants. PXR mediates the induction of several drug-metabolizing enzymes most notably of the CYP3A subfamily. Both PXR and CYP3A are co-expressed mainly in liver and intestine. As a heterodimer with the Retinoid X Receptor (RXR) PXR binds to CYP3A promoter sequences activating CYP3A transcription. To assess the ability of several xenobiotics to induce CYP3A enzymes via activation of PXR we examined the levels of PXR in human and rat hepatoma cell lines and its inducibility by the ligands dexamethasone and rifampicin. We assayed CYP3A4/1 and PXR protein levels by Western blotting and mRNA levels by real time PCR. Furthermore a PXR-dependent reporter gene assay was designed. Both cell lines expressed PXR being inducible by dexamethasone in the rat hepatoma cell line H4IIE and by rifampicin in the human hepatoma cell line HepG2. After activation of PXR we could observe a concentration-dependent increase of CYP3A1 mRNA and protein in rat cells and of CYP3A4 in human cells. Transient transfection assays with a XREM- reporter gene correlated with the results of Western blotting and PCR. In summary, our findings show that rat and human PXR expression is inducible in hepatoma cell lines in a species-selective way similar to regulation of CYP3A enzymes.

[1]Food Chemistry and Environmental Toxicology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany
[2]Institute of Toxicology, Molecular Toxicology, Merck KGaA, D-64293 Darmstadt, Germany

351

CRYSTAL STRUCTURE AND CHARACTERIZATION OF HUMAN CARBONYL REDUCTASE 3

Y. El-Hawari[1], F. Niesen[2], K.L. Kavanagh[2], Ewa Pilka[2], V. Wsol[3], H-J. Martin[1], U. Oppermann[2] and E. Maser[1]

Substances containing carbonyl groups are regularly found in food, drugs, cosmetics and many other articles of everyday life. Reduction of the carbonyl group to the corresponding alcohol is often a critical phase I reaction that ultimately leads to the excretion of xenobiotic compounds. Members of two enzyme superfamilies – short-chain dehydrogenases/reductases (SDR) and aldo-keto-reductases (AKR) – catalyze this reaction. AKRs and SDRs have vital functions in the synthesis and conversion of steroids, sugars, prostaglandins and other bioactive molecules. Human carbonyl reductase (CBR1) belongs to the SDR superfamily. CBR1 has a broad substrate specificity and its main physiological function is still uncertain. Recent findings suggest that CBR1 plays an important role in the neuro-protection against reactive oxygen species and is a general defense mechanism against harmful carbonyl compounds. Recently, another human isoform, CBR3, was identified based on sequence alignments. CBR3 shares 71 % sequence identity to CBR1. In order to assess its properties, we cloned and overexpressed CBR3 in *E. coli*. The crystal structure of CBR3 was determined at 1.9 Å resolution and compared to structural data of CBR1. CBR3 was found to reduce isatin, 1,2-naphthoquinone, 9,10-phenanthrenequinone and the anti-cancer drug oracin with NADPH as cofactor with low enzymatic efficiency in comparison to CBR1. A variety of steroid hormones was tested but none was reduced by CBR3. Surprisingly, CBR3 did not reduce menadione, a potent substrate of CBR1. Despite its ability to reduce di-ketones, the physiological role of CBR3 is still unknown.

[1]Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Germany; [2]Structural Genomics Consortium, University of Oxford, Botnar Research Centre, Oxford; [3]Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovskeho 1203, CZ-50005 Hradec Kralove

352

EXPOSURE TO BENZO[*A*]PYRENE IN THE HEPATIC CYTOCHROME P450 OXIDOREDUCTASE NULL MOUSE: DETOXIFICATION BY HEPATIC CYTOCHROME P450 IS MORE IMPORTANT THAN METABOLIC ACTIVATION

V.M. Arlt[1], M. Stiborova[2], C.J. Henderson[3], M. Thiemann[4], E. Frei[5], R. Singh[6], G.G. da Costa[1], O.J. Schmitz[4], P.B. Farmer[6], C.R. Wolf[3], D.H. Phillips[1]

Cytochrome P450 (CYP) enzymes such as CYP1A1 or CYP1B1 activate many polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene (BaP). As a result, it is widely accepted that CYP1 potentiates BaP genotoxicity. A mouse line, HRN™ (Hepatic Cytochrome P450 Reductase Null), has been developed in which cytochrome P450 oxidoreductase (POR), the unique electron donor to CYP enzymes is deleted specifically in the liver, resulting in the loss of essentially all hepatic P450 function. We used this *in vivo* model to evaluate the role of hepatic *versus* extrahepatic metabolism and disposition of BaP. HRN™ and wild-type (WT) littermates were treated with 125 mg/kg body weight (bw) BaP once daily for up to five days by intraperitoneal injection. DNA adduct levels measured by ^{32}P-postlabelling at day 1 and 5 were up to 10-fold higher in livers of HRN™ compared to WT mice. Formation of 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-N^2-BPDE) was confirmed by LC-MS/MS analysis. On day 1 sequential blood samples were obtained and analysed by HPLC with fluorescence detection for BaP clearance. The half-life of BaP in HRN™ was 4-fold higher compared to WT mice. When hepatic microsomal fractions from HRN™ and WT mice were incubated with BaP, DNA adduct formation was 7-fold higher in WT compared to HRN™ mice. Most of the hepatic microsomal activation of BaP *in vitro* was attributable to CYP1A enzyme activity. These data reveal an apparent paradox, whereby CYP enzyme activity appears to be more important for detoxification of BaP *in vivo*, despite being essential for its metabolic activation *in vitro*.

[1]Section of Molecular Carcinogenesis, Institute of Cancer Research, UK; [2]Department of Biochemistry, Charles University Prague, Czech Republic; [3]Cancer Research UK Molecular Pharmacology Unit, Biomedical Research Centre Dundee, UK; [4]Department of Analytical Chemistry, University of Wuppertal, Germany; [5]Division of Molecular Toxicology, German Cancer Research Center, Germany; [6]Cancer Biomarker and Prevention Group, University of Leicester, UK.

353

HUMAN CYP2E1 DEPENDENT FORMATION OF GLYCIDAMIDE FROM ACRYLAMIDE

E. Czech[1], U. Bernauer[2], R. Palavinskas[3], H.S. Klaffke[3], U. Gundert-Remy[2], K.E. Appel[1]

There is some concern about possible health risks from dietary exposure to acrylamide (AA). In experimental studies AA has been shown to be genotoxic and carcinogenic. It is also neurotoxic in humans. In laboratory animals AA is metabolized to glycidamide (GA) catalysed primarily by CYP2E1. AA and its metabolites are rapidly eliminated in urine, primarily as mercapturic acid conjugates of AA and GA. GA is suggested to be much more reactive than AA with DNA. In a study with primary rat hepatocytes no DNA damage was induced by AA despite substantial expression of CYP2E1 (Puppel et al., 2004). Therefore we addressed the question to which extent GA is formed from AA by human liver CYP2E1 by using monkey and human liver microsomes and genemodified V 79 cells expressing human CYP2E1. Special emphasis was laid on the analytical detection of GA. Microsomal incubations have been performed according to conventional procedures. ^{13}C-Glycidamide and D_3-Acrylamide were added as external standards. GA and AA were extracted and refined from microsomal suspensions by using organic solvents and by elution through activated carbon-aluminium oxide-columns. The detection of GA and AA was performed using a gas chromatograph masspectrometer (Finnigan Mat SSQ 710) equipment. The results show that AA is metabolized to GA in monkey and human liver microsomes. The activity in human liver is somewhat lower than in monkey liver. MAB-2E1 antibody (monoclonal, raised in mouse, human CYP2E1 selective) and diethyldithiocarbamate were used as inhibitors of CYP2E1. Generation of GA could be inhibited by MAB-2E1 by about 70 % in human liver microsomes and by about 80 % in monkey liver microsomes. Diethyldithiocarbamate led to an inhibition of about 95 %. While genemodified V 79 cells expressing human CYP2E1 were able to hydroxylate chlorzoxazone (CYP2E1 activity) formation of GA was below the detection limit when using the actual analytical procedure.

[1]Center for Experimental Toxicology, [2]Department of Safety of Substances and Preparations, [3]Center for Analytical Chemistry, Federal Institute for Risk Assessment, Thielallee 88-92, D-14191 Berlin

354

IN VIVO INVESTIGATION OF URINARY METABOLITES FROM DENTAL MATERIALS

M. Seiss[1], W. Marquardt[2], A. Oxynos[1], K. Kehe[1], M. Folwaczny[2], R.Hickel[2], F.X. Reichl[2]

Up to date little is known about the detailed metabolism of absorbed (co)monomers like TEGDMA (triethyleneglycoldimathacrylat) and BisGMA (Bisphenol A glycidyl methacrylate) and its related metabolic products in biological systems, e.g. human beings and animals, after uptake. As a consequence of this it is mostly unknown whether the applied substances and/or related metabolites are causing observed toxic effects. Recently it has been shown in the metabolism of radiolabeled TEGDMA (^{14}C methacrylic acid (^{14}C-MA)) that beside the exhalation via $^{14}CO_2$ (70-80 %) approx. 15 % of administrated TEGDMA activity is excreted from guinea pigs via urine. Whereas $^{14}CO_2$ formation is a consequence of e.g. metabolism of ^{14}C-MA via valine pathway and subsequently Krebs cycle up to date little is known about the composition of the remaining molecular moieties of dental materials in vivo. We now investigated analytes in urine of guinea pigs after administration of TEGDMA and BisGMA, respectively. For the detection solid phase micro extraction in combination with liquid chromatography-mass spectrometry was used. For the experiments each guinea pig (a total of 4 animals) received 6 ml of a solution containing 0.02 mmol/l TEGDMA and 0.02 mmol/l BisGMA, respectively, dissolved in 0.9 % NaCl solution. The urine of these animals was collected for 24 h. Spontaneous urine received before administration of TEGDMA and BisGMA, respectively, served as control. The following metabolites were identified (relative % of administered (co)monomer amount): in case of TEGDMA: 11±2 % unchanged TEGDMA, 1.5±0.6 % MA, and 35±3.5 % triethyleneglycol (TEG), in case of BisGMA: 10.5±1.4 % unchanged BisGMA, 2.2±0.6 % MA, and 60±4.5 % Bisphenol-A-bis(2,3-dihydroxypropyl)ether (BPDHE). These results demonstrate that in contrast to MA for which it is reported to be mainly metabolized via valine pathway the decomposition products TEG and BPDHE both remain unchanged and are excreted completely. Thus they are not of great concern for toxicological aspects.

[1] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, 80336 Munich, Germany
[2] Department of Operative Dentistry and Periodontology, Ludwig-Maximilians-University, 80336 Munich, Germany

355

INFLUENCE OF GLUTATHIONE CONCENTRATION ON THE *IN VITRO* FORMATION OF 2,3- EPOXYMETHACRYLIC ACID IN POOLED HUMAN LIVER MICROSOMES

A. Oxynos[1], M. Seiss[1], W. Marquardt[2], K. Kehe[1], J. Glas[2], F.X. Reichl[2], R. Hickel[2]

As demonstrated in former studies methacrylic acid (MA) is an intermediate in the metabolism of unpolymerised dental (co)monomers, which are released from dental restorative materials. It has recently been shown in an isolated system containing pooled human liver microsomes that MA can be converted via cytochrome P450 enzymes to its related epoxide 2,3-epoxymethacrylic acid (2,3-EMA) (Seiss *et al.*, Dent Mat, 2006 *in press*). It's a matter of common knowledge that many epoxides feature high toxicity. The conjugation of epoxides by glutathione is often described as an important key to the detoxification of epoxides in the organism (Guengerich FP, Chem Res Toxicol 14(6): 611-650, 2001). The presented study was performed in order to identify the influence of glutathione on the epoxides formation. Pooled human liver microsomes were incubated together with MA and increasing concentrations of glutathione (0-50 mg/l) at 37 °C. After ten minutes the content of 2,3-EMA was measured by headspace-gas chromatography-mass spectrometry. Each experiment was done in triplicate. The concentration of formed 2,3-EMA (5±1.3 mg/l), detected at a glutathione concentration of 0 mg/l declined to 3.2±1.1 mg/l at 10 mg/l glutathione, 0.4±0.4 mg/l at a glutathione level of 30 mg/l and 0.16±0.16 mg/l at 50 mg/l glutathione.These results reveal a dose depend decline of 2,3-EMA formation in the presence of glutathione but it has also to be underlined that at a stoichiometrical concentration of glutathione the generation of 2,3-EMA is not inhibited completely.

[1] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, 80336 Munich, Germany

[2] Department of Operative Dentistry and Periodontology, Ludwig-Maximilians-University, 80336 Munich, Germany

356

IN VITRO METABOLISM OF THE FUNGICIDE CARBENDAZIME: APPLICATION AND ASSESSMENT OF DIFFERENT IN VITRO METABOLISM SYSTEMS

E. Fabian[1], H. Kamp[1], G. Damm[2], S. Triebel[3], J. Döhmer[4], R. Landsiedel[1], B. van Ravenzwaay[1]

To assess different in vitro metabolism systems for their potential applicability as preincubation tools, the *in vitro*-metabolism of the fungicide Carbendazime (Methyl 3H-benzoimidazol-2-ylaminoformate) was investigated in these systems. The tested metabolism systems consisted of supersomes, rat liver in vitro-metabolism-systems (microsomes, S9-fraction, hepatocytes and liver slices) as well as in CYP 450 transfected V79 cells. Supersomes were obtained from BD Biosciences. Liver microsomes and liver S9 fraction were prepared and characeterized from Aroclor 1254 treated male Wistar rats. Rat hepatocytes were obtained from In Vitro-Technologies. Liver slices were prepared from Aroclor 1254 pretreated and untreated male Wistar rats. CYP 450 transfected V79 cells were obtained from Genpharmtox. The test systems were incubated with Carbendazime under appropriate conditions and the resulting incubates/lysates were analysed for Carbendazime and its metabolites by HPLC/UV and HPLC/MS. The main metabolite of Carbendazime in all investigated in vitro-systems was a hydroxy-metabolite, resulting from the hydroxylation of the benzimidazole-ring of the molecule. Based on systematic incubations with supersomes, containing different human CYP 450 isoenzymes (CYP profiling) it could be demonstrated, that the hydroxylated Carbendazime is formed by hCYP 450 1A2. At a substrate concentration of 100 µM, the metabolisation of the substrate in microsomes and S9-fraction of Aroclor 1254 treated rats were almost 100 %. The metabolic turn over was lower in rat hepatocytes and yielded in about 1 % and 40 % at substrate concentrations of 100 and 10 µM, respectively. In liver slices, the metabolic turn over was about 5 % and 56 % for substrate concentrations of 100 and 10 µM, respectively. It was possible to increase the metabolisation of Carbendazime in liver slices by a pre-treatment of rats with Aroclor 1254 and in induced liver slices, Carbendazime was almost completely metabolised at a substrate concentration of 10 µM. The HPLC/MS analysis of the lysate of hCYP 450 1A2 transfected V79 cells demonstrated that after an incubation of 18 h at a test substance concentration of 25 µM, the metabolic profile consisted of Carbendazime and its hydroxy-metabolite in a ratio of about 1:1. Taken together, these investigations demonstrated both similarities as well as differences of the investigated in vitro metabolism systems taking Carbendazime as a model substrate. The obtained data will be used to select appropriate in vitro-metabolism options for incorporation of metabolic competence into in vitro toxicity tests.

[1]Experimental Toxicology and Ecology, BASF AG, D-67056 Ludwigshafen
[2]Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, University of Karlsruhe (TH), Post Box 6980, D-76128 Karlsruhe
[3]Chemisches und Veterinäruntersuchungsamt Karlsruhe. Weißenburgerstraße 3. D-76187 Karlsruhe (current address)
[4]GenPharmTox Bio Tech AG, Fraunhoferstrasse 9, D-82152 Martinsried / Planegg

357

EFFECT OF IN VITRO METABOLISM ON THE ENDOCRINE ACTIVITY OF TESTOSTERONE IN A YEAST-BASED REPORTERGENE ASSAY

H.G. Kamp[1], E. Fabian[1], G. Damm[2], H.-A. Huener[1], A. Verlohner[1], R. Landsiedel[1], B. van Ravenzwaay[1]

Test substances can be screened for possible endocrine activities using in vitro reportergene assays. To this end, BASF is using yeast-based reportergene assays. Yeast cells, however, lack of the capability of mammalian xenobiotic metabolism. Using testosterone as a reference compound we evaluated whether rat liver microsomes could compensate this deficit of the YAS assay (yeast androgen screen). Rat liver microsomes were prepared and characterized from Aroclor 1254 treated male Wistar rats. Testosterone, incubated at a concentration of 200 µM, was almost completely metabolised by the microsomal preparations. The metabolites were verified by HPLC. Incubations of Testosterone with active microsomes (active incubation, AI) as well as heat-desactivated microsomes (matrix control, HDC) were introduced into the YAS assay. The yeast cells were incubated with the samples for 48 h in 96 well plates and the androgenic activity was determined measuring a reporter protein-dependent colour conversion in the culture medium. Cytotoxicity was assessed simultaneously via the optical density in the respective wells of the 96 well plates. In the YAS assay, testosterone showed the well-established androgenic activity. Adding the HDC did neither change the measured androgenic activity nor the cytotoxicity, indicating no impact of the microsomes on the performance of the yeast cells. The incubation of the yeast cells with the AI of testosterone reduced the androgenic activity almost completely; without any impact on the cytotoxicity. Since oxidative testosterone metabolism is believed to massively decrease of the androgenic activity of the hormone, this result shows the validity of the combination of metabolizing in vitro systems and the YAS assay. In conclusion, these investigations demonstrate that it is possible to assess the influence of metabolism on the endocrine potential of test substances. This gives further evidence that combining in vitro methodologies with in vitro metoblism systems is possible and can increase their significance. However, this, as well as other combinations of in vitro metabolism and cell assays, needs further validation.

[1]Experimental Toxicology and Ecology, BASF Aktiengesellschaft, D-67056 Ludwigshafen
[2]Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, University of Karlsruhe (TH), Post Box 6980, D-76128 Karlsruhe

358

METHYLATION OF CATECHOL ESTROGENS IN HUMAN CANCER CELLS

S. Gerstner, E. Pfeiffer and M. Metzler

The endogenous steroid hormone 17ß-estradiol (E2) plays a major role in the etiology of breast and endometrial cancer. For the tissue and plasma levels of E2, its phase I and phase II metabolism are very important. Major phase I metabolites are estrone (E1) and the four catechol estrogens 2-HO-E2/-E1 and 4-HO-E2/-E1. These catechols undergo metabolic redox-cycling to generate reactive oxygen species and semiquinone/quinone intermediates which may damage DNA. 4-HO-E2 is a carcinogen, whereas the methylation product of 2-HO-E2, i.e. 2-MeO-E2, is believed to protect against cancer. Methylation and glucuronidation are part of phase II metabolism, but only glucuronidation leads to the complete inactivation and excretion of E2 and its metabolites.

The aim of the present study was to elucidate the methylation of the catechol estrogens in HepG2 liver cancer cells, which show active UDP-glucuronosyltransferases, and to compare these results with earlier studies in MCF-7 breast cancer cells. HepG2 cells were incubated with each of the four catechol estrogens, the media was extracted with ethyl acetate and the metabolites were analyzed by HPLC. Whereas 2-HO-E2/-E1 gave rise to two methylation products each, only one product was detected with 4-HO-E2 and 4-HO-E1. The formation of the products was observed for 24 h. Moreover, coincubations of the different catechol estrogens were conducted in order to determine the mutual influence on their methylation. The results of this study are consistent with those obtained in MCF-7 cells, indicating that 2-HO-E2/-E1 but not the carcinogenic 4-HO-E2/-E1 are good substrates of COMT. Therefore, 2-HO-E2/-E1 are first methylated and their methylation products then eliminated by glucuronidation. For 4-HO-E2/-E1, glucuronidation appears to be more important for inactivation than methylation.

Supported by the Deutsche Forschungsgemeinschaft (Grant ME 574/26-1).

Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe (TH), P.O. Box 6980, 76128 Karlsruhe

359

METABOLISM OF 1,3-BUTADIENE TO EPOXIDES AND TO 3-BUTENE-1,2-DIOL IN SPRAGUE-DAWLEY RATS AND B6C3F1 MICE

C. Hutzler, W. Kessler, J.G. Filser

1,3-Butadiene (BD) is carcinogenic in rodents, an effect related to reactive metabolites such as 1,2-epoxy-3-butene (EB), 3,4-epoxy-1,2-butanediol (EBD), and, especially, 1,2:3,4-diepoxybutane (DEB). Tissue burdens by DEB are reported to be at least 20 times higher in mice than in rats. EBD can be formed from DEB and from 3-butene-1,2-diol (B-diol), the hydrolysis product of EB. In order to clarify the kinetic interactions among BD metabolites, we studied the formation of EBD from DEB and from B-diol in once-through perfused male rat livers and determined the steady state concentrations of EB, B-diol, EBD, and DEB in blood of male rats and mice which were exposed over 6-8 h to constant atmospheric concentrations of BD. In perfused rat livers, both DEB (0.3-0.5 µmol/l) and B-diol (14-28 µmol/l) were metabolised to EBD. The EBD yield of the DEB metabolism was 40-50%, that of the B-diol metabolism only 0.35%. In BD exposed animals, EB in blood increased with the BD concentration, amounting to 2.6 µmol/l in rats and to 23.5 µmol/l in mice at 2000 ppm BD and to 4.6 µmol/l in rats at 10000 ppm BD. DEB (detection limit 0.01 µmol/l) was found only in blood of mice rising to 3.2 µmol/l at 1280 ppm BD. B-diol and EBD were the predominant metabolites. B-diol in blood increased in both rats and mice with the BD concentration, reaching 60 µmol/l at 1200 ppm BD. Maximum EBD concentrations were 9.5 µmol/l at 150 ppm BD (rat) and 42 µmol/l at 300 ppm BD (mouse). At higher exposure concentrations, EBD concentrations in blood decreased in both species. Obviously, the metabolites are not only formed after re-uptake of their actual parent compounds into the liver from blood, but almost simultaneously in the endoplasmic reticulum as first step BD metabolites. Furthermore, in rats as well as in mice BD probably inhibits competitively the CYP450 species that oxidizes EB and derivatives thereof.

Supported by the American Chemistry Council.

GSF-Institut für Toxikologie, Ingolstädter Landstraße 1, D-85764 Neuherberg

360

MUTUAL KINETIC INTERACTIONS OF ETHYLENE AND ETHYLENE OXIDE IN ETHYLENE EXPOSED RATS

E. Erbach, Gy.A. Csanády, J.G. Filser

Ethylene (ET) is ubiquitous in the environment and is an important industrial chemical. In mammals, ET is metabolised via CYP450 to ethylene oxide (EO). In rats exposed to constant high ET concentrations, the resulting EO concentrations in blood showed a complex concentration-time course: an initial peak followed by a decline approaching a plateau. This signifies that the health risk by the metabolite EO is not only dependent on the ET dose but additionally on the exposure duration. In order to clarify the mechanism underlying this behaviour, we investigated whether (A) EO inhibits its formation from ET or (B) enhances its detoxification by influencing epoxide hydrolase or glutathione S-transferase.

A: Rats were exposed in closed chambers to constant ET concentrations (30-3000 ppm). At selected time points CYP2E1 activity in liver was determined using chlorzoxazone.

B: Different concentrations of EO were administered over 3 h to anesthetised rats via one vena femoralis at a constant infusion rate and EO concentrations in blood between 0.4 and 10 nmol/ml were established. The EO concentration-time courses in blood taken from the other vena femoralis were monitored using GC/MS. S-(2-hydroxyethyl)-glutathione levels in livers were determined by LC/MS/MS.

A: In rats exposed to 1000 ppm ET, CYP2E1 activity showed a time dependent decline reaching a plateau of about 20% after 2 hours of exposures. Activity did not decrease at an ET concentration of 30 ppm resulting in a relatively high EO burden compared to that at high ET concentrations.

B: Plateau levels of EO in blood were reached after 2 hours of exposure indicating a constant EO elimination rate over time. Also, S-(2-hydroxyethyl)-glutathione did not show deviation from linearity.

We conclude that the complex concentration-time courses of EO in blood observed at high ET concentrations results from inhibition of CYP2E1 and not from induction of the EO metabolism.

Financially supported by the American Chemistry Council and the Lower Olefins Sector Group of the European Chemical Industry Council.

GSF-Institut für Toxikologie, Ingolstädter Landstraße 1, D-85764 Neuherberg

361

PERFORMANCE OF THE HERSHBERGER ASSAY USING INTACT JUVENILE RATS – A PILOT STUDY

A. Freyberger, L. Schladt, A. Brockes, F. Krötlinger

Under the auspices of OECD the Hershberger assay (HA) on peripubertal castrated rats is being validated as an in vivo screen for compounds with anti-androgenic potential. Recently, intact juvenile male rats have been proposed as alternate animal model for the HA. In a pilot study we investigated the performance of the HA using intact juvenile male rats and compared it with the classical animal model using castrated rats. Administration of testosterone propionate (TP) for ten days - the necessary stimulus when testing for an anti-androgenic potential - in recommended doses (castrated rat: 0.4 mg/kg, juvenile intact rat: 1 mg/kg) resulted in similar weight increases of levator ani and bulbocavernosus muscles (LABC) and Cowper's glands (CG) in both animal models, whereas the weight increase of seminal vesicles (SV) was slightly, of the ventral prostate markedly more pronounced in castrated animals. In addition, in intact animals a decrease of testicular weight and a moderate increase of epididymal weight were observed. Compared to TP-treated animals, co-administration of either the anti-androgen flutamide (3 mg/kg) or challenging compounds with weak anti-androgenic potential, namely p, p'-DDE (160 mg/kg) and linuron (100 mg/kg), resulted in significant decreases of ventral prostate (VP), SV, LABC and CG weights in castrated animals. Qualitatively similar changes were observed in intact rats for flutamide and p, p'-DDE, whereas linuron only moderately decreased SV, LABC and CG weights. All three compounds clearly reduced epididymal weight in intact animals. These findings indicate that the juvenile intact rat has the potential to replace the peripubertal castrated rat in the HA thereby contributing to a refinement of the assay in terms of animal welfare. Further testing in a world wide effort will provide comprehensive information on the sensitivity of this new animal model. This study was performed in collaboration with OECD and was sponsored by CEFIC-EMSG.

Bayer HealthCare AG, Pharmaceuticals, GDD, Toxicology, D-42096 Wuppertal, Germany

362

METABOLISM OF THE PHENION® FULL THICKNESS SKIN MODEL

C. Wiegand, K. Reisinger, D. Eschrich, K. Schroeder

The skin functions as a barrier between organism and environment protecting humans against physical and chemical perturbances. Although it is an easy accessible tissue less is known about xenobiotic metabolism. Phase I and/or phase II enzymes are involved in metabolism of a wide range of exogenous compounds. Like in other organs CYP P-450 are the most important phase I enzymes. They play a major role in many detoxifying reactions, but the regulation and physiological functions of CYPs in human skin are not well understood. Commercial available skin models (Skinetik/L´Oreal, MatTek, Cellsystems) mimic native human skin and are increasingly used for toxicological testing. So far, only epidermis models are in use for testing skin corrosion or irritations. For several toxicological endpoints skin metabolism play a major role and it is not clear whether epidermal models provide a full metabolic competence comparable to native skin. In contrast, the Phenion® Full Thickness Skin Model (Phe®-FT-SM) consists of a dermal and epidermal layer. Therefore it provides a better system for studying drug metabolism in human skin because the skin interactions between both compartments are essential for differentiation, regeneration as well as xenobiotic metabolism e.g. of retinoic acid and 4-oxometabolites. In order to evaluate the suitability of the Phe®-FT-SM for analyzing skin metabolism *in vitro* we first analyzed gene expression of phase I and II enzymes with quantitative realtime PCR. Several Phase I and II enzymes were expressed in the Phe®-FT-SM similar to human skin like CYP2A6, 2B6, 2D6, and glutathione S-transferase p1. Furthermore, CYP1A1 could be clearly induced by β-naphthoflavone as it was seen in human native skin. Further approaches will comprise the analysis of additional phase I and II enzymes and the measuring of enzyme activities. The results will be compared with native skin and liver showing whether the Phe®-FT-SM is a suitable model for *in vitro* toxicological and metabolic studies.

Phenion GmbH & Co. KG, Düsseldorf, Germany
christian.wiegand@henkel.com

363

EXPRESSION OF MATRIX METALLOPROTEASE 1 (MMP1) IN HUMAN SKIN IS DEPENDING ON A MOLECULAR CROSSTALK OF ULTRAVIOLET (UV) A- AND UVB-INDUCED SIGNALING

K.Ruwiedel*, S.M.Schieke#, H.Brenden*, S.Bruhnke*, C.Schäfer*, P.Schroeder*, E.Fritsche*, J.Abel*, J.Krutmann* and S.Grether-Beck*

In previous years, UVA- and UVB-induced stress response has been characterized in great detail and multiple wavelength-specific distinctions have been identified. Since human skin is mainly exposed to UV radiation from natural sunlight consisting of both UVA and UVB radiation, we determined whether these two stress responses occur independently of each other or whether the involved signaling pathways interact. Here we demonstrate that sequential exposure of primary human epidermal keratinocytes to UVA and UVB produces a MAPK (mitogen-activated protein kinase) activation pattern differing from that generated in response to either one of them alone: ERK1/2-activation (extracellular related kinase1/2) in UVB-irradiated cells is reduced after preirradiation with UVA whereas UVB-induced activation of p38 and JNK (c-Jun amino-terminal kinase) was significantly enhanced. This mechanism is also found in primary human dermal fibroblasts. The observed crosstalk between UVB and UVA-induced signaling was most likely mediated by second-messenger ceramide, since pretreatment of cells with C_2-ceramide was capable of mimicking the UVA effect on the UVB response, i.e. attenuation of ERK1/2 activation and stimulation of p38 and JNK activation. Furthermore MMP1-expression is subject to this crosstalk of UVA- and UVB-induced signaling. UVA, a powerful inducer of MMP1-expression in the human dermis, is mainly responsible for degradation of type 1 collagen. UVB irradiation is able to inhibit the UVA-induced MMP1-expression, which could be observed on mRNA-level and active secreted MMP1 detected by gelatin zymography. These findings were verified in *in vitro* systems and in an *in vivo* study done with human buttock skin. Further characterization of this crosstalk with MMP1 as an endpoint may provide new insights into the mechanisms of skin aging induced by sunlight and artificial UV sources like sunbeds.

* Institute for Environmental Health Research (IUF gGmbH) at the Heinrich Heine University Duesseldorf, Auf'm Hennekamp 50, 40225 Duesseldorf, Germany
# Cardiovascular Branch, National Heart, Lung and Blood Institute, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892-1622, USA

364

IMPACT OF THE ARYLHYDROCARBON RECEPTOR ON THE EUGENOL AND ISOEUGENOL INDUCED CELL CYCLE ARREST IN HUMAN IMMORTALIZED KERATINOCYTES (HACAT)

M Kalmes[1], A Neumeyer[1], P Rio[2], H Hanenberg[3], E Fritsche[4], B Blömeke[1]

Fragrances such as eugenol (4-allyl-2-methoxyphenol) and isoeugenol (2-methoxy-4-propenylphenol), naturally found in reasonable quantities in the essential oils of different spices, are not only common causes of contact dermatitis but also known for their antiproliferative actions. Previously, we found a cell cycle arrest and a arylhydrocarbon receptor (AhR)-mediated activation of CYPs in immortalized keratinocytes (HaCaT) by both compounds. Now, we investigated if the cell cycle arrest of eugenol and isoeugenol is mediated by the AhR in HaCaT cells. Analysis of the cell cycle status by fluorescence-activated cell sorting (FACS) revealed an arrest of cells (32-34%) in the G_0/G_1 phase by both compounds. This arrest was associated with reduced proliferation and increased expression of p27kip1. This was found in synchronized HaCaT cells, natural HaCaT, and siRNA AhR transfected HaCaT. The induced G_0/G_1 arrests were reduced in the presence of the highly selective AhR antagonist 3´-methoxy-4´-nitroflavone (MNF). In summary, these results together with our previous findings that both compounds induced translocation of the AhR into the nucleus, provide good evidences that the effects of eugenol and isoeugenol in skin and keratinocytes are mediated by the AhR. Furthermore, these data suggest that the known growth suppressive effects of these compounds in some skin cells may be mediated by AhR interactions.

[1]Department for Ecotoxicology and Toxicology, University of Trier, 54296 Trier, Germany
[2]Department of Pediatric Oncology, Hematology & Immunology, Children's Hospital, Heinrich-Heine-University Medical Center, 40225 Düsseldorf, Germany, current address: CIEMAT, Madrid, Spain
[3]Department of Pediatric Oncology, Hematology & Immunology, Children's Hospital, Heinrich-Heine-University Medical Center, 40225 Düsseldorf, Germany
[4]Group of Toxicology, Institut für umweltmedizinische Forschung (IUF) gGmbH an der Heinrich-Heine Universität, 40225 Düsseldorf, Germany

365

EFFECT OF THE DENTAL RESIN MONOMER TEGDMA ON THE SURFACE ANTIGEN EXPRESSION OF MURINE RAW264.7 MACROPHAGES

A. Eckhardt , J. Limtanyakul, T. Harorli, K.-A. Hiller, G. Schmalz, H. Schweikl

A well known fact is that dental resin monomers like triethylene glycol dimethacrylate (TEGDMA) have cytotoxic effects on oral cells. These effects are characterised by a rise in reactive oxygen species (ROS), DNA damage and cell cycle arrest. Here, we scrutinized the effect of TEGDMA on macrophages, which are a major component of the innate immune system and, therefore, part of the acute defence against various antigens. Thus, we studied the effects of TEGDMA in murine RAW macrophages, a well characterised model system. The surface expressions of CD40 (T-cell interaction), CD80 (costimulation), CD14 (LPS binding) and CD54 (cell adhesion) were measured after 6, 24 and 48h stimulation. Lipopolysaccharide (LPS) from *E. coli*, which is a strong stimulus for immune cells served as a positive control. Stimulation with TEGDMA alone did not result in significant changes as compared to medium only. TEGDMA concentrations of 4 and 8mmo/L induced strong cytotoxic effects. Next we stimulated with LPS and various concentrations of TEGDMA, which resulted in a concentration dependent decrease in surface marker expression. Compared to stimulation with LPS only, the combined stimulation with LPS and 0.125mM TEGDMA resulted in 27% decrease of CD40 expression after 24h, whereas 2mM TEGDMA induced a 98% reduction. Similar effects were measured in the expression of CD80: 0.125mM reduced the expression by 9%; 2mM reduced it by 74% after 24h of stimulation. In contrast, low concentrations of TEGDMA (0.125 and 0.25mM) increased the surface expression of CD14 by 9 and 27%. Higher concentrations (0.5 to 2mM) again resulted in a decrease of CD14 expression. After stimulation with LPS and 2mM TEGDMA dropped to 38% compared to LPS alone. CD54 expression increased in a concentration dependent manner. Stimulation with LPS and 2mM TEGDMA resulted in an 80% increase. These data strongly indicate that TEGDMA interferes in a concentration dependent manner with the stimulatory potential of innate immune cells.

Dept. Oper. Dent. and Periodontology, Univ. of Regensburg, D-93042 Regensburg, Germany

366

***PARA*-PHENYLENEDIAMINE AND ALLERGIC SENSITIZATION: RISK MODIFICATION BY *N*-ACETYLATION**

B Blömeke[1], R Brans[2], T Pietzsch[1], PJ Coenraads[3], H Dickel[4], T Bruckner[5], M Heesen[6], DW Hein[7], HF Merk[8], Y Kawakubo[9]

Para-phenylenediamine (PPD) and related compounds are known contact sensitizers in man. We and others reported that PPD is acetylated to monoacetyl-PPD (MAPPD) and to *N,N'*-diacetyl-PPD (DAPPD) in human skin, keratinocytes and hepatocytes. In addition, we found *in vitro* that the acetylated compounds have no or only limited capacities to reactivate T cells of sensitized persons. We found that acetylation of PPD can be catalyzed by the *N*-acetyltransferase 1 (NAT1) and by *N*-acetyltransferase 2 (NAT2) using a recombinant yeast system. Studying the capacities of MAPPD, DAPPD and PPD *in vivo* by performing epicutaneous skin tests in individuals (n=455, 182 males and 273 females) who underwent routine patch testing. Patch-test readings after 72 or 96h revealed 20 PPD-sensitized persons (4.4%) and one person reacted to DAPPD (0.2%). From these results, assuming that *N*-acetylation of PPD is a detoxification reaction with respect to sensitization to PPD, we studied the *NAT1* and *NAT2* genotypes in PPD-sensitized individuals. The study was performed in 145 PPD-sensitized cases and age- and gender-matched healthy controls (n=200). Genotyping for the *NAT1* haplotypes *NAT1*3*, *NAT1*4*, *NAT1*10*, *NAT1*11* and *NAT1*14* did not reveal

statistically significant differences but the rapid-acetylator NAT1*4*10 genotype was underrepresented in PPD-sensitized cases (OR=0.7, 95% CI: 0.4-1.10). For *NAT2*, we genotyped successfully *NAT2*4, NAT2*5AB*, *NAT2*5C*, *NAT2*6A*, and *NAT2*7B* in 145 cases and 190 controls. Again, individuals homozygous for *NAT2* allele *NAT2*4* were underrepresentated in cases compared to controls (4.8 vs 10.0%, p=0.06). The slow NAT2 acetylator genotypes were not significantly increased in cases compared to controls but the subgroup of *NAT2*5AB*5AB* carriers was increased in cases (21.4% *vs* 11.6%) and conferred increased risk compared to *NAT2*4*4* carriers (OR=3.8, 95% CI: 1.4-10.7, p=0.009). Overall, our *in vivo* results suggest that acetylated products of PPD are unable to reactivate T cells and probably incapable of eliciting allergic contact dermatitis. Our studies found that the "fast" acetylator genotype is not associated with susceptibility to sensitization.

[1]Department of Ecotoxicology and Toxicology, University Trier, Am Wissenschaftspark 25-27, 54296 Trier, Germany
[2]Division of Plastic Surgery, Dr. Eriksson's Lab, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA
[3]Department of Dermatology, University Medical Center Groningen, University of Groningen, 9700 RB Groningen, The Netherlands
[4]Department of Dermatology and Allergology, Ruhr-University Bochum, Gudrunstrasse 56, 44791 Bochum, Germany
[5]Department of Social Medicine, Occupational and Environmental Dermatology, University Hospital of Heidelberg, 69115 Heidelberg, Germany
[6]Department of Anesthesia, Klinikum Bamberg, Burger Str. 80, 96049 Bamberg, Germany
[7]Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, USA
[8]Department of Dermatology and Allergology, University Hospital of the RWTH Aachen, Pauwelsstr. 30, 52057 Aachen, Germany
[9]Department of Dermatology, Teikyo University School of Medicine, Ichihara Hospital, Anesaki 3426-3, 299-0111 Ichihara, Japan

367

CONCENTRATION OF THE MAJOR BIRCH POLLEN ALLERGEN BET V 1 IN AMBIENT AIR DEVIATES FROM POLLEN COUNTS IN MUNICH, GERMANY

H. Behrendt[1], I. Weichenmeier[1], S. Ochs[1], W. Kreyling[2], A. Boere[3], W. Schober[1], J.T.M. Buters[1]

Background Exposure to allergens like Bet v 1, the major birch pollen allergen, is generally deduced from birch pollen counts in ambient air. Proof is lacking that pollen counts reflect exposure to this allergen.

Methods At the hospital campus in Munich, Germany, 562 m above sea level, 1.8 m above ground, we monitored simultaneously birch pollen counts with a standard Burkard pollen trap, and Bet v 1 in ambient air with a Chemvol® High-Volume sampler (900l/min). Bet v 1 was extracted from the filters with 0.1 M NH_4HCO_3 and quantitated with a Bet v 1 specific ELISA.

Results Peak birch pollen count in 2004, 2005, and 2006 were 729, 3522 and 1681 pollen/m^3/24h, respectively. At the same time peak Bet v 1 concentration was 148, 364 and 1390 pg Bet v 1/m^3, respectively. According to pollen count, 2005 showed the strongest exposure to allergens. In 2006, when pollen counts were half of 2005, Bet v 1 exposure was about 4 times higher than 2005, indicating that the content of Bet v 1 per pollen was 8-times higher than in 2005. Also, later in the season when pollen counts were low, Bet v 1 persisted in ambient air.

Conclusion Exposure to birch pollen allergens is dependent on the amount of pollen released in a particular year (pollen flight). On top of that is also the difference in allergen content of that pollen between years. This difference in allergen content of pollen between years can be 8-fold. Thus birch pollen counts do not necessarily reflect birch allergen exposure, especially later in the season.

[1]Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM-Center for Allergy and Environment, Technical University Munich, Munich, Germany, [2]GSF-Focus: Aerosols and Health, Institute for Inhalation Biology, GSF - National Research Center for Environment and Health, Neuherberg, Germany; [3]National Institute for Public Health and the Environment, Centre for Environmental Health Research, Dept. Toxic Effects of Air Pollution, Bilthoven, Netherlands.

368

ENVIRONMENTAL POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) DRIVE ALLERGIC INFLAMMATION IN HUMAN ATOPICS

W. Schober, S. Lubitz, B. Belloni, G. Gebauer, B. Eberlein, H. Behrendt , J. Buters

Background: Diesel exhaust particles (DEPs) have been implicated in the worldwide increased incidence of asthma and allergic rhinoconjunctivitis over the past century. There is growing evidence that PAHs from road side emissions participate in the development and maintenance of IgE-mediated allergic diseases. To address this issue we investigated the impact of single U.S. EPA priority PAHs and PAH derivatives on human basophils, which markedly contribute to allergic inflammation through the release of preformed and granule-derived mediators.

Methods: Purified basophils from 12 birch pollen allergic subjects (7 males, 5 females; mean age 26.8 ± 3.5) and 9 non-atopic donors (1 male, 8 females; mean age 31.7 ± 11.4) were incubated with phenanthrene (Phe), benzo[*a*]pyrene (B[*a*]P) or their redox-active derivatives (Phe-9,10-hydroquinone, B[*a*]P-1,6-quinone) for 2 hours prior to rBet v 1 stimulation. Supernatants were assayed for IL-4, IL-8 and histamine by means of ELISA.

Results: B[*a*]P and Phe at concentrations of 0.1 – 5 µM drove IL-8 secretion from sensitized basophils in a dose-dependent manner. The effect was more significant for B[*a*]P (*p = 0.0004*) than for Phe, but could be observed with and without antigen added. Both PAHs significantly enhanced IL-4 secretion (*p = 0.02*) from purified basophils in the absence of antigen suggesting an adjuvant role of DEP-PAHs in allergic sensitization. No relevant further increase in IL-4 secretion was seen in the presence of specific antigen. Phe but not B[*a*]P significantly enhanced histamine release (*p = 0.04*) from basophils of birch pollen allergic individuals in absence of rBet v 1. In contrast, stimulation with PAH derivatives had virtually no influence on mediator release. None of basophil samples from healthy controls showed any PAH effect on cytokine or histamine secretion.

Conclusion: Our data suggest that diesel soot associated PAHs can not only drive proallergic processes through enhanced FcεRI-coupled mediator release from human basophils, but also initiate a proinflammatory Th2 dominated immune response via direct action of single PAHs on sensitized basophils.

Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM – Center for Allergy and Environment, Technische Universität München, Germany

369

TOXICITY OF NANOPARTICLES: DEVELOPING A STANDARD SHORT-TERM INHALATION TEST

R.Landsiedel, L. Ma-Hock, S. Burkhardt, V. Strauss, A. Gamer, K. Wiench and B. van Ravenzwaay

The engineering of materials to nano-scale establishes new, fascinating properties, which are not found with larger-scaled structures (micrometer and above) of the same material. These properties promise great technological advances. However, they also raise questions about possible adverse effects on humans. The nanotechnology industry, together with academia, is investigating the biological effects of nanomaterials to answer questions on possible health risks and ensure adequate and safe handling of nanomaterials. Among the possible effects on human health, inhalation of aerosols containing nanoparticles, is of the highest concern (based on existing experience with fine and ultra-fine dusts). Germany, as one of the leading countries in developing nano-technology, has set-up the *NanoCare* project to investigate and characterise the exposure and the effects of nanoparticles on humans. In this project, biological effects of nanoparticles are studied on cellular and whole organism levels. The *in vitro* models will provide mechanistic information and may eventually offer fast and relatively simple test systems. However, an aerosol from nanoparticles is a complex system and so is the process of its inhalation. Thus any *in vitro* model will require validation by *in vivo* inhalation studies. A realistic assessment of possible effects may be provided by modified standard inhalation test methods. To develop such a test method we generated and characterised atmospheres from ten different nanoparticles (see poster of Ma-Hock et al.: Characterization of Aerosolized Nano-Materials) and exposed rats to various concentrations of nano-TiO_2 and nano-ZnO for five days. Directly at the end of the exposure period, and two and twelve days thereafter, animals were examined for multiple parameters: Organ burdens were estimated in seven tissues. An extensive set of over seventy parameters (cytokine profiles, indicators of oxidative stress and indicators of complement activation, as well as standard clinical pathology parameters) was analyzed in lung lavage fluid and in blood. The wide range of examined parameters in this studies, gives a differentiated picture of the effects in the lung. It also allows the selection of a number of most predictive and selective early indicators of toxicity for future *in vivo* and *in vitro* investigations. The findings observed in these inhalation studies will be compared to intra-tracheal instillation studies as well as tests with cell cultures.

This work is part of the *NanoCare* project of the Federal Ministry of Education and Research in Germany *(BMBF)*

BASF, Product Safety, 67056 Ludwigshafen, Germany

370

CHARACTERIZATION OF AEROSOLIZED NANO-MATERIALS FOR INHALTION TOXICITY STUDIES

L. Ma-Hock, A. Gamer, R. Landsiedel, E. Leibold, T. Frechen, B. Sens, G. Huber and B. van Ravenzwaay

The inhalation toxicity of nanomaterials is currently investigated by various methods in many laboratories. It is the common understanding, that the specific physical properties of nanomaterials and the complex nature of ultrafine aerosols dictate a thorough characterization of the test material and the aerosols generated thereof. As the first step of BASF's program to evaluate the inhalation toxicity of nano-materials the potential for aerosolisation of ten different nanomaterials was examined. The following manufactured nano-materials have been evaluated for their potential to releases submicrometer-sized particles using aerosolization systems designed for inhalation toxicology studies: Titandioxide, Carbon black, Aluminum oxide, Copper(II) oxide, three amorphous Silicon dioxide, Zinc oxide, and Zirconium(IV) oxide. Prior to aerosolisation, the particle size, specific surface area, zeta potential and morphology of each of the test substances were determined. Aerosols of each material were generated using a dry powder aerosol generator and a spray system (nebulization of suspended powders). The mass concentration of the test substance in the inhalation atmosphere was determined gravimetrically and the particle size was determined using a cascade impactor; an optical particle counter and a scanning mobility particle sizer. Although the aersosol generation used relatively high energy, most of the particles in the atmospheres were found to be agglomerates of nano-particles and no more than a few mass percent of particles below 100 nm were present in the aerosols. To evaluate whether the agglomerates are stable in the lung or will get dispersed by inhalation or deposition in the lung, a short-term inhalation study with nano-TiO_2 was carried out in rats. The particles deposited in the lung were examined by electron microscopy. We found virtually the same particle sizes in the lung as we analysed in the inhaled aerosol: mostly large agglomerates of up to 2.5 µm in diameter. The particles were located extracellularly and inside the macrophages. Based on these observations a stepwise testing approach is recommended: Characterizing the agglomerated particles in the aerosols from different nanomaterials may allow grouping and cross-reading of results from inhalation studies and could therefore reduce the number of animals used in testing.

BASF Aktiengesellschaft, Product Safety, 67056 Ludwigshafen, Germany

371

SILIBININ PROTECTS OTA-MEDIATED TNF-α RELEASE FROM PERFUSED RAT LIVERS AND ISOLATED RAT KUPFFER CELLS

L. AL-Anati, E. Petzinger

OTA causes necrotic and apoptotic changes in the liver, single-chain breakdown of DNA in hepatocytes, and liver tumors in mice. New acute effects of OTA on the liver are the release of TNF-α from blood-free perfused rat livers, in particularly from Kupffer cells. Since silibinin has strong hepatoprotective activity against several liver toxin including phalloidin and CCl_4, we investigated its role on OTA acute effects namely on TNF-α release and the leakage of cytotoxic markers (GLDH, LDH) and compared them with LPS-induced cytokine release. For this purpose, isolated blood free rat livers were perfused with Krebs-Henseleit buffer containing 2% dextran for 90 min, and isolated pure Kupffer cells were incubated for 24 hrs under standard cultural conditions. In the recirculation perfusion model OTA induced the release of TNF-α in a time- and dose dependent fashion. At 2.5µmol/L OTA, 2600pg/ml TNF-α occurred in the perfusion medium after 90 min without significant increase of cytotoxicity markers (LDH, GLDH). However, a toxic dose of OTA (12.5µmol/L) released about 120% higher amounts of TNF-α, but

also 2 fold higher LDH and GLDH. LPS at 0.1 µg/ml induced 3000 pg TNF-α/ml at the end point, but caused also significant leakage of GLDH and LDH. Under similar experimental conditions, the addition of 2.5 µg/ml silibinin 10 min prior to low-dose of OTA and LPS reduced TNF-α by 50% and restored the basal levels of LDH and GLDH. At 12.5µg/ml silibinin had a stronger effect and levels amounted to only 20%, 30% of OTA and LPS induced levels, respectively, but were still higher than the control levels (without treatment). At high-dose silibinin (12.5µg/ml) GLDH and LDH levels in the perfusate were completely restored. Further experiments were done to determine the effects of silibinin on TNF-α release from isolated Kupffer cells. Silibinin at different doses (0.5µg/L, 2.5µg/L, 12.5µg/L) was added 1 hr before OTA or LPS into the culture medium. Even at the lowest concentration silibinin reduced basal and abrogated any OTA-mediated TNF-α release, whereas much weaker protection was observed on LPS-treated Kupffer cells. In conclusion, the protection by silibinin on OTA- or LPS-mediated release of TNF-α and of cytoxicity markers indicates that silibinin interacts with trigger signalling pathways of pro-inflammatory cytokine release.

Faculty of Veterinary Medicine, Institute of Pharmacology and Toxicology, Justus-Liebig-University Gießen, Frankfurter Street 107, D-35392 Gießen, Germany.

372

MATHEMATICAL MODELLING OF LIVER REGENERATION AFTER INTOXICATION WITH CCl_4

J.G. Hengstler, M. Brulport, M. Hermes, A. Bauer, W. Schormann, D. Drasdo, E. Bockamp[1]

Liver regeneration is a complex process, having evolved to protect animals from the consequences of liver loss caused by food toxins. In this study we established a mathematical spatial-temporal model of the liver lobule regenerating after CCl_4 intoxication. The aim of modelling the regeneration process by matching experimental observations with those from a mathematical model is to gain a better understanding of the process and to recognize which parameters are relevant for specific phenomena. In order to set up a realistic minimal model we first reconstructed a schematised liver lobule. In a next step we determined the time course of cell death and BrdU incorporation after intoxication of male Sprague Dawley rats with CCl_4, thereby differentiating between inner, midzonal and peripheral hepatocytes. These parameters were used to construct a model. The basic unit of this model is the individual cell. The detailed behaviour of the cells is studied, controlled by the model parameters: (1) probability of cell division at defined positions of the lobule at a given time, (2) ''coordinated cell orientation'', i.e., the ability of the cells to align during the regeneration process into columns towards the central vein of a liver lobule (3) cell cycle duration, and the (4) migration activity. This model was used to systematically analyze the influence of parameters 1-4. Interestingly, coordinated cell orientation was identified to be the most critical parameter. Elimination led to destruction of the characteristic micro-architecture of the lobule and to a high degree of disorder characterized by hexagonal cell structures. Our model suggests that the ability of hepatocytes to realign after cell division by a process of coordinated cell orientation and migration (model parameters 2 and 4) may be at least as critical as hepatocyte proliferation (model parameter 1) itself.

Center for Toxicology, University of Leipzig and Institute of Toxicology, University of Mainz[1]

373

THE DEMYELINATING COPPER CHELATOR CUPRIZONE (BISCYCLOHEXANONE OXALYLDIHYDRAZONE) CAUSES EPILEPTIC SEIZURES AND HIPPOCAMPAL DAMAGE IN MICE

K. Hoffmann[1], M. Lindner[2,3], M. Stangel[2,3], W. Löscher[1,3]

The cuprizone model of demyelination in mice resembles myelin deficiency in multiple sclerosis (MS). Spontaneously occurring seizures have been described early in this model (Kesterson & Carlton, 1970), but have not been characterized in any detail yet. A causal relationship between MS and seizures was assumed, since the prevalence of seizures in MS is about three to six times that in the general adult population (Poser & Brinar, 2003). The exact mechanisms why myelin deficiency can lead to seizures are not known, but may involve axonal pathology and resultant alterations in neuronal excitability. In the present study, we used continuous EEG/video monitoring to record seizures occurring after chronic demyelination in C57BL/6J mice. Furthermore, we histologically examined demyelination patterns in the brain and especially in the hippocampal formation. In search for morphological correlates of seizures, we quantitfied neuronal density and area of the dentate hilus. We observed three kinds of epileptiformic discharges in cuprizone-treated mice: in all mice we recorded epileptiform spike-wave formations in the EEG. Furthermore, most of the animals developed generalized, tonic-clonic seizures upon stress-inducing stimuli. Only one animal showed spontaneous ocurring, generalized seizures. Histological studies demonstrated - apart from the known demyelination of the corpus callosum - massively affected hippocampal structures. Hippocampal alterations were associated with decreased neuronal density in the hilus of the dentate gyrus and neuronal damage in the pyramidal cell layer, the granula cell layer and the hilus. Our study suggests that the seizures occurring in the cuprizone model are a consequence of neuronal degeneration in the hippocampal formation caused by demyelination in this region. Based on the present data, the potential role of the hippocampal formation for seizures occurring in different models of CNS demyelination should be examined.

Supported by the Deutsche Forschungsgemeinschaft and by internal grants of the MHH (HiLF).

[1]Department of Pharmacology, Toxicology, and Pharmacy, University of Veterinary Medicine, Hannover, Germany
[2]Department of Neurology, Medical School Hannover, Germany, and [3]Center for Systems Neuroscience, Hannover, Germany

374

EFFECTS OF MACROLIDE ANTIBIOTICS ON PROINFLAMMATORY SURFACE ANTIGENS ON HUMAN ENDOTHELIAL CELLS *IN VITRO*

M. Millrose, N. Rösener, J. Webb and R. Stahlmann

Infusion-related phlebitis is a common clinical problem following the i.v.-application of antibiotics. The pathomechanism of this adverse effect is not quite understood, but it is assumed that a chemical irritation of the endothelium causes an inflammatory response. Endothelial cells express surface antigens such as CD 34, E-Selectin (CD 62E), ICAM-1 (CD 54) and VCAM-1 (CD 106) which are essential for the inflammatory response. We studied the effects of three antibiotics on the human endothelial cell line EA.hy 926 by flow cytometry. The antigens were stained with monoclonal IgG mouse-antibodies for the surface antigens mentioned above. The cells were incubated with the antibiotics azithromycin (100-800 mg/l), clarithromycin (100-800 mg/l) and erythromycin (200-1400 mg/l). Such concentrations occur under therapeutic conditions at the site of infusion. The cells were incubated for 2 h and analysis was carried out after an additional time period of 22 h. Control cells expressed the antigens at the following levels: CD 34 (2%), E-Selectin (4%), ICAM-1 (14%) and VCAM-1 (2%). The maximum effect on the antigens was detected after incubation with 400 mg/l clarithromycin, 600 mg/l, azithromycin and 800 mg/l erythromycin. We observed a 2 to 3-fold enhanced expression of all analysed surface antigens after the incubation with 800 mg/l erythromycin (CD 34: 7%, E-Selectin: 9%, ICAM-1: 28% and VCAM-1: 6%). At a concentration of 600 mg/l azithromycin provoked an even stronger upregulation of the proinflammatory antigens (CD 34: 19%, E-Selectin: 23%, ICAM-1: 42% and VCAM-1: 20%). At 400 mg of clarithromycin/l medium we observed a comparable effect to 800 mg erythromycin/l medium (CD 34: 7%, E-Selectin: 11%, ICAM-1: 37% and VCAM-1: 5%). Under our *in-vitro* conditions we could show that azithromycin has the greatest potency of stimulating the expression of proinflammatory antigens. The investigation of the expression of cell surface markers involved in cell-cell-interactions proved to be a suitable approach to further study the mechanisms of infusion phlebitis.

Institute of Clinical Pharmacology and Toxicology, Charité – Campus Benjamin Franklin, Garystrasse 5, 14195 Berlin, Germany

375

MITOCHONDRIAL TOXICITY OF COMBINATIONS OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIS) – INVESTIGATION OF POTENTIAL ADDITIVE OR SYNERGISTIC TOXIC EFFECTS IN HEPG2 CELLS

D. Höschele, M. Wiertz, I. Garcia Moreno

Treatment of HIV infection is a combination therapy with dual NRTI-combinations being currently the backbone. Recently, there has been raised concern about potential additive or synergistic mitochondrial toxicity of NRTI-combinations, which is a major toxicity of NRTIs.

The aim of this study was to investigate the mitochondrial toxicity of dual combinations of the NRTIs didanosine (ddI), stavudine (d4T), zidovudine (AZT), and lamivudine (3TC) in relation to single agents in HepG2 cells. Cells were exposed to the NRTI-combinations ddI/d4T, AZT/ddI, AZT/d4T, AZT/3TC, 3TC/d4T, and ddI/3TC for 14 days in concentrations of 50 µM for each NRTI. The respective single NRTIs were investigated in parallel in each experiment at concentrations of 50 µM and 100 µM. Zalcitabine (ddC), which mitochondrial toxic effects are well characterized, was used as positive control. On day 14 of incubation mitochondrial toxicity was investigated by spectrometrical measurement of cellular lactate production and quantification of mtDNA using a one tube real-time PCR assay. In addition, cell number was analysed. Treatment with the positive control ddC induced a decrease in cell number, an increase in cellular lactate production, and a decrease in mtDNA content. Incubation of HepG2 cells with single NRTIs led to increase in cellular lactate production and decrease in cell number in the rank order: AZT> ddI > d4T > 3TC. These effects were accompanied by changes in mtDNA content. Whereas ddI and d4T decreased mtDNA content in HepG2 cells, an increase was observed for AZT. 3TC did not induce significant changes of mtDNA content. No additive or synergistic mitochondrial toxicity was observed with any NRTI-combination tested. The degree of changes of cell number, cellular lactate production, and mtDNA content was comparable to the effects observed with the most potent combination partner. In conclusion, our study demonstrates that the potential to induce mitochondrial toxicity of the NRTI-combinations tested was predictable from studies with single agents.

Federal Institute for Drugs and Medical Devices, Kurt-Georg-Kiesinger Allee 3, D-53175 Bonn, Germany

376

PREDNISOLONE ENHANCES APOPTOTIC EFFECTS OF LEVOFLOXACIN IN HUMAN-DERIVED TENOCYTES *IN VITRO*

A. Fagh, J. Sendzik, J. Webb, I. Baumann-Wilschke and R. Stahlmann

Several studies and case reports indicate that quinolones induce tendon disorders. Furthermore, clinical observations have shown that steroids in combination with quinolones increase the risk of this adverse drug reaction. We conducted *in vitro* experiments with human-derived tenocytes. Monolayer cultures (n = 6 per group) were incubated for 24 hours with (a) levofloxacin at concentrations of 0, 0.3, 1, 3, 10, and 30 mg/l medium, (b) 100 µg prednisolone/l medium, and (c) levofloxacin at the same range of concentrations in combination with prednisolone (100 µg/l). Using flow cytometry and annexin V staining, we analysed the early apoptotic response of the cells. In addition, we studied the apoptosis marker "activated caspase-3" (casp-3) in tenocytes incubated with 0, 3, 10, 30 mg levofloxacin/l medium for 1 to 4 days using immunoblotting. The annexin V staining showed a concentration-dependent increase of apoptosis in cells incubated with (a) levofloxacin alone. We observed a significant difference in comparison to controls at concentrations of 1 mg/l and higher (control: 8.2 % $\pm$ 1.5 % positively stained cells, 1 mg/l: 13.2 % $\pm$ 2.7 %, $p < 0.05$, t-test). Tenocytes incubated with (b) 100 µg prednisolone/l medium alone showed a significant increase of apoptotic cells as well. Furthermore, a combined incubation with (c) levofloxacin and prednisolone resulted in an enhanced response revealing a significant difference already at a concentration of 0.3 mg/l levofloxacin. The immunoblotting of activated casp-3 as a key enzyme in the apoptotic pathway showed time- and concentration-dependency in comparison to the untreated control. A first pronounced increase was seen after 10 mg levofloxacin/l medium on the fourth day of incubation (control: 0.01 $\pm$ 0.015 densitometric units, 10 mg/l: 0.1 $\pm$ 0.006; $p < 0.05$). The evidence of these apoptotic responses suggests that programmed cell death plays a key role in the pathomechanism of this adverse reaction. Furthermore, the clinical observations that prednisolone enhances quinolone-induced effects are supported by those data.

Institute of Clinical Pharmacology and Toxicology, Charité - Campus Benjamin Franklin, Garystr. 5, 14195 Berlin, Germany

377

DOES METHYLPHENIDATE CAUSE GENOMIC DAMAGE IN CHILDREN WITH ATTENTION DEFICIT HYPERACTIVITY DISORDER?

H. Stopper[1], B. Werner[1], M. Romanos[2], A. Warnke[2], M. Gerlach[2], S. Walitza[2]

Attention-deficit/hyperactivity disorder (ADHD) is the most frequent psychiatric disorder in children and adolescents (6-12 % affected). Methylphenidate (for example Ritalin®; MPH) is the most commonly prescribed treatment (psychostimulant) for ADHD and is therefore the most widely used psychiatric drug in children. There are so far no good epidemiological data concerning potential carcinogenic effects of this medication. A 2005 study by El-Zein and coworkers reporting a 3-fold increase in genomic damage in all 12 analyzed children after 3 months of therapy with MPH resulted in much concern about potential carcinogenic effects of this medication. In a prospective study, we analyzed the genomic damage in children with ADHD before and one (30 children), three (21 children) and six (8 children) month after initiation of MPH therapy. Patients were recruited within a study of our Clinical Research Group on ADHD in the Department of Child and Adolescent Psychiatry and Psychotherapy of the University of Wuerzburg. Assessment and treatment of the patients were performed during inpatient or outpatient health care. The measure for assessment of genomic damage was the frequency of micronuclei, a subset of chromosomal aberrations, in peripheral lymphocytes. In our investigation, MPH treatment resulted in no significant alteration in the micronucleus frequency. Thus, the findings published in 2005 by El-Zein and coworkers could not be replicated by us and the resulting concern regarding a potential increase in the risk of developing cancer later in life following long-term MPH treatment is not supported by our data.

[1] Department of Child and Adolescent Psychiatry and Psychotherapy, University of Wuerzburg, Fuechsleinstr. 15, 97080 Wuerzburg, Germany
[2] Department of Toxicology, University of Wuerzburg, Versbacherstr. 9, 97078 Wuerzburg, Germany

378

INTERACTION OF ARSENIC TRIOXIDE ON TUMOR AND NON-TUMOR CELL MODELS

A.-M. Florea, F. Splettstoesser, and D. Büsselberg

Some metals are essential for normal cell function (e.g. Zn), others have no known function or are highly toxic (lead, mercury). Thus, when exposure to low concentrations of metals occurs over long time, deregulation in cell function might occur, leading to toxicity. Arsenic trioxide is used for therapeutic purposes (e.g. for the treatment of chronic myelogenous leukaemia). Thus, it has been also shown that intake of arsenic compounds is toxic in humans. Today, there is a debate concerning arsenic's differential mechanisms of action in tumor and non-tumor cells. However, understanding the mechanisms by which arsenic interacts with normal and tumor cell models will open the possibility of improving the treatments against cancer and reduce secondary effects that result upon medication. In this work we approached the As_2O_3 influence on cytotoxicity and intracellular calcium ($[Ca^{2+}]_i$) homeostasis of human neuroblastoma SY-5Y and embryonic kidney cells (HEK 293). The concentrations applied have clinical as well as environmental relevance ranging between 1µM and 100pM. We performed cytotoxicity tests (trypan blue dye exclusion assay, MTT assay) where As_2O_3 significantly reduced cell viability in both cell types within hours. Staining of cell nuclei with Hoechst 33342 showed occurrence of apoptosis and DNA damage that was time and dose dependent after incubation with As_2O_3 for 24h, 48h, and 72h, respectively. For calcium measurements we used Ca^{2+} sensitive dyes (fluo-4) combined with laser scanning microscopy in order to measure $[Ca^{2+}]_i$ changes during the application of As_2O_3. As_2O_3 increased $[Ca^{2+}]_i$ in SY-5Y and HEK 293 cells even with the lowest concentration tested (100pM). Three forms of $[Ca^{2+}]_i$ elevations were found: (1) steady-state increases, (2) transient $[Ca^{2+}]_i$–elevations and (3) Ca^{2+}-spikes. Our data suggest that $[Ca^{2+}]_i$ is an important messenger in As_2O_3 induced cell death.

Institut für Physiologie, Universitätsklinikum Essen, Germany

379

THE CISPLATIN AND ARSENIC TRIOXIDE INDUCED INCREASE OF $[CA^{2+}]_I$ IS INDEPENDENT OF EACH OTHER AND A CO-APPLICATION RESULTS IN A SYNERGISTIC INCREASE OF $[CA^{2+}]_I$

D.A. Günes, A.-M. Florea, F. Splettstoesser, D. Büsselberg

Introduction: The pharmacodynamic of some chemotherapeutics (e.g. cisplatin (CDDP) and arsenic trioxide (As_2O_3)) is not fully understood. The goal of chemotherapy is the induction of apoptosis in specific malign cells. It is known that the induction of apoptosis could occur by an intracellular calcium ($[Ca^{2+}]_i$) signal. We have seen that As_2O_3 and CDDP induce an increase of $[Ca^{2+}]_i$ which results in apoptosis of specific cell-lines, and moreover that this increase is triggered by different mechanisms (As_2O_3: depletion of intracellular calcium stores; CDDP: influx of calcium ions; see Florea et al.; & Splettstoesser et al.). Intention of this study was to prove whether these mechanisms are independent and whether a co-application could result in a synergistic increase of $[Ca^{2+}]_i$.

Material & Methods: As_2O_3 and/or CDDP were single-/co-applied in human SY-5Y neuroblastoma cells and in mouse hippocampus cells (MHC) (1µM for 60min) using a flow-system at room-temperature. Cells were loaded by calcium-sensitive dye Fluo-4 and measurements were taken by confocal laser scanning microscopy.

Results: Application of these substances triggers different calcium signals: A) sustained increase and B) transient elevations. Single-application of As_2O_3/CDDP induces a sustained-increase of 37±31%/42±15% in SY-5Y and 74±50%/21±20% in MHC, while co-application of CDDP to As_2O_3 or As_2O_3 to CDDP increases $[Ca^{2+}]_i$ by 70±57%/114±25% in SY-5Y 324±198%/137±113% in MHC. This increase could not been reached by rising the concentration of As_2O_3/CDDP to 2µM. The number of fast transient elevations was increased by 40% (As_2O_3), 74% (CDDP) and 60% (As_2O_3 + CDDP) in SY-5Y and 30% (As_2O_3) 36% (CDDP) and 80% (co-application) in MHC.

Conclusions: The As_2O_3 and CDDP induced sustained $[Ca^{2+}]_i$ increase is independent and therefore a co-application results in a synergistic increase of $[Ca^{2+}]_i$. Using synergistic effects on $[Ca^{2+}]_i$ could rise the efficiency of chemotherapy and probably reduce side-effects.

University Hospital Essen, Institute of Physiology, Hufelandstrasse 55, 45122 Essen

380

QUALITATIVE AND QUANTITATIVE ANALYSIS OF ELUTED COMPOUNDS FROM DENTAL PRIMERS

F.-X. Reichl[1], M. Seiss[2], W. Marquardt[1], A. Oxynos[2], M. Folwaczny[1], J. Glas[1], K. Kehe[2], R. Hickel[1]

Primers are used as dental restorative materials in the dental adhesive bonding technique. Unconverted comonomers, e.g. 2-hydroxyethylmethacrylate (HEMA), can be released from restorative dental materials and can enter the body in humans. This study was evaluated to qualify and to quantify eluted compounds from various dental primers. Following primers were tested: Bioactiv Primer A (Saremco), OptiBond Solo Plus Etch Primer (Kerr), OneCoat Self Etching Bond Primer 1 (Coltène), and Clearfil SE Bond Primer (Kuraray Dental).

Unpolymerized primers (20 mg) were incubated in GC vials with 1 ml dest. water or 1 ml methanol, each at 37 °C for 24 hours. Aliquots were taken, and eluted compounds were analyzed with the method of gas chromatography - mass spectrometry (GC-MS) and liquid chromatography - mass spectrometry (LC-MS).

Following eluted comonomers were found and quantified (µg/ml; mean±sem; n=3):

	detected comonomers		detected comonomers	
Primer	dest. water		methanol	
	HEMA	EGDMA	HEMA	EGDMA
Bioactiv Primer A	n.d.*	n.d.*	n.d.*	n.d.*
OptiBond Solo Plus Etch Primer	6.0 ± 0.3	n.d.*	13.2±0.5	1.8 ± 0.1
OneCoat Self Etching Bond Primer 1	7.8 ± 1.3	n.d.*	8.6 ± 0.4	4.8 ± 0.6
Clearfil SE Bond Primer	12.7 ± 0.5	n.d.*	5.2 ± 0.5	n.d.*

* n.d. = not detectable

Following range of the eluted and detected comonomers from dental primers was found in dest. water (decreasing elution): Clearfil SE Bond Primer (Kuraray Dental) > OneCoat Self Etching Bond Primer 1 (Coltène) > OptiBond Solo Plus Etch Primer (Kerr) >>> Bioactiv Primer A (Saremco)(HEMA n.d.).

[1] Department of Operative Dentistry and Periodontology, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany
[2] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Goethestr. 31, 80336 Munich, Germany

381

SUPPORTED GLUTATHIONE SYNTHESIS DECREASES ZINC-MEDIATED TOXICITY IN LUNG CELL LINES

U.I. Walther and S.C. Walther

Zinc toxicity in vivo is caused mostly by inhalational exposure, while mechanism of zinc-mediated toxicity is not well understood. Zinc mediated toxicity in lung cell lines is enhanced after cellular glutathione has been decreased. Correspondingly, administration of glutathione esters decreases toxicity by zinc. In addition, zinc itself decreases cellular glutathione, therefore glutathione synthesis should be stimulated by zinc. The question arises whether supporting glutathione synthesis might be able to antagonise zinc toxicity. In previous experiments no effect by 200 µmol/l N-acetylcysteine (N-AC) was found, while no changes in glutathione contents occurred by this concentration (Toxicol in Vitro, 2006, in press). In this work cells were incubated with zinc in the presence of 2 mmol/l N-AC in order to support glutathione synthesis. Controls were administered the D-derivative N-A(D)C to assess a chelational and antioxidative effect of N-AC as well. Additionally, in order to stimulate glutathione synthesis normal cystein content of medium was decreased to 10, 1 or 0 µmol/l (L2 cells: 25, 5 or 0 µmol/l). Cellular glutathione content and methione incorporation inhibition were measured to assess zinc-mediated toxicity. In the lung cell lines an increase in cellular glutathione was found by N-AC as compared to N-A(D)C for 0 and 1 µmol/l Cys (L2: 0 and 5 µmol/l), but not for 10 µmol/l. In accordance after incubation with zinc a decrease in the zinc-mediated methionine incorporation inhibition was found only with 1 (L2: 5) or 0 µmol/l Cys in the medium, while glutathione depletion was decreased at all Cys concentrations. We conclude that enhancing glutathione synthesis rates might be beneficial in zinc-mediated oxidative stress. Suprisingly, in cell culture only milimolar concentrations of N-AC increase cellular glutathione and this only occurs when medium Cys is decreased to one tenth or less. Furthermore in two hepatic cell lines (HepG2, HTC) additionally tested neither an enhanced glutathione was found nor a decrease in zinc mediated toxicity occurred in these cell lines by 2 mmol/l N-AC.

Walther-Straub-Institut für Pharmakologie und Toxikologie der Ludwig-Maximilians-Universität München, Goethestr. 33, 80336 München, Germany

382

CELLULAR UPTAKE AND TOXICITY OF INHALABLE ARSENOPYRITE PARTICLES AND OF ITS NATURAL ANALOGUE HEMATITE.

K. Bhattacharya [1], E. Hoffmann [2], L.M. Hartmann [3], C. Albrecht [4], A.W. Rettenmeier [1], E. Dopp[1]

Arsenopyrite (FeAsS) is used by sulfur industries to commercially produce sulfur-dioxide via a high temperature roasting process. The remaining arsenopyrite cinder still contains high amounts of arsenic trioxide (As_2O_3). An arsenopyrite sample was tested for uptake and toxicity in human lung epithelial cells (BEAS-2B) and was compared to hematite and As_2O_3. Scanning electron microscopy revealed that the sample is a mixture of fine and ultrafine particles and EDX study showed a high percentage of Fe_2O_3 with an As_2O_3 content of about 16 ng/mg. In uptake studies arsenopyrite and hematite particles were found near the nuclear membrane and surrounded by mitochondrias. The cytotoxicity study revealed that arsenopyrite cinder caused 50 % cell death at a concentration of 50 µg/cm² whereas fine and nanosized hematite caused the same effect at 100 µg/cm². The acellular ROS measurement performed by electron spin resonance (ESR) revealed that ROS production is dependent upon particle size. Fine hematite particles (< 5 µm) induced the highest ROS formation, followed by the arsenopyrite particles, whereas nanosized hematite (< 1 µM) showed negligible ROS production. Intracellular radical formation was measured for short and long term exposures and the results were found to be comparable to those of the ESR studies. Taken together, it can be concluded that arsenopyrite particles are more toxic than the

naturally occuring form (hematite). However, acellular ROS production is more pronounced with fine hematite particles as compared to the nanoparticles, which produced just negligible amounts of ROS. Additional studies are required to analyse the cause for particle induced ROS formation and possible genomic damage in human lung epithelial cells.

[1] Institute of Hygiene and Occupational Medicine, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany.
[2] Department of Cell Biology and Biosystems Technology, Institute of Biological Sciences, University of Rostock, Albert-Einstein-Str. 3, 18051, Rostock, Germany.
[3] Institute of Environmental Analysis, University of Duisburg-Essen, Universitätsstrasse 3-4, 45141 Essen, Germany.
[4] Institut für Umweltmedizinische Forschung (IUF) an der Heinrich-Heine-Universität Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany.

383

EFFECTS OF *IN UTERO* AND LACTATIONAL EXPOSURE TO TRIPHENYLTIN CHLORIDE ON PREGNANCY OUTCOME AND POSTNATAL DEVELOPMENT IN RAT OFFSPRING

Grote, K. [a], Hobler, C. [a], Andrade, A.J.M. [a], Wichert Grande, S. [a], Gericke, C. [c], Appel, K.E. [b], Chahoud, I. [a]*

Introduction: The organotin compound (OTC) triphenyltin (TPT) is used extensively as a herbicide, pesticide and fungicide in agriculture as well as, together with tributyltin (TBT), in marine antifouling paints. We studied the effects of *in utero* and lactational exposure to triphenyltinchloride (TPTCl)/kg b.w. on pregnancy outcome and postnatal development in rat offspring. Materials and methods: Gravid Wistar rats were treated with 2 or 6 mg TPTCl per gavage from gestational days 6 until the end of lactation. The control group received peanut oil at a volume of 5ml/kg b.w. Results: In the 6 mg TPTCl dose group gestational mortality in dams as well as an increased incidence of anticipated and delayed parturition was observed. Furthermore, treatment resulted in a significant increase in perinatal mortality, a decrease in lactational body weight gain as well as in delayed physical maturation of offspring. Exposure to 2 mg TPTCl/kg b.w. resulted in a significant increase in perinatal mortality and in delayed eye opening. Lactational body weight gain and other landmarks of physical maturation were unaffected in the 2 mg TPTCl dose group. Conclusion: We conclude that in utero exposure to TPTCl at the described dose levels severely affected pregnancy outcome and perinatal survival of offspring. These results were unexpected, as in two earlier studies with pubertal rats at the same dose levels of TPTCl no signs of general toxicity were observed.

[a]Inst. of Clinical Pharmacology and Toxicology, Charité University Medical School, Campus Benjamin Franklin, 14195 Berlin, Germany; [b]Federal Institute for Risk Assessment, 14195 Berlin, Germany; [c]Inst. of Biometry and Clinical Epidemiology, Charité University Medical School, Campus Benjamin Franklin, 14195 Berlin, Germany

384

Withdrawn

385

PHARMACOLOGICAL ACTIVITY OF PHENOLIC COMPOUNDS ISOLATED FROM *PSORALEA CORYLIFOLIA*

[1,3]Ruhl S., [1]Wang Y., [2]Wang Z., [3]Rohrig R., [3]Kulawik A., [1]Proksch P., [2]Lou Y., [3]Wätjen W.

Psoralea corylifolia (Fabaceae) is a well known plant distributed in the tropical and subtropical regions of the world. *Psoralea corylifolia* has traditionally been used for treatment of various symptoms associated with aging in China like vitiligo and alopecia but also exerts a laxative, diuretic and diaphoretic effect. In spite of these therapeutic actions of *Psoralea species*, little is known about the pharmacologically active substances and the corresponding anticancer mechanisms. Therefore the seeds of the Chinese medicinal plant *Psoralea corylifolia* were extracted and analyzed for cytotoxicity and antioxidant properties. The isolation of the crude extract yields seven compounds: bakuchiol, psoralidin isobavachalcone, corylin, bavachin, neobavaisoflavone and 8-prenyldaidzein which was not isolated from this plant so far. We first analyzed the antioxidative activities of the substances using the TEAC- Assay. Furthermore the cytotoxic potential of the compounds was analyzed in metabolically active H4IIE rat hepatoma cells. Cytotoxicity was determined by a MTT-Assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). Isobavachalcone shows weak cytotoxic effects (24 h) whereas psoraldin and bakuchiol exerted moderate to prominent toxic effects: The IC_{50}-value of bakuchiol was determined to be 50 µmol/L. Further analysis on the mode of cell death were performed (caspase 3/7 activation, nuclear fragmentation). The isolated compounds may possess an interesting pharmaceutical potential e.g. for a use in cancer therapy.

[1]Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitätsstrasse 1, Building 26.23, 40225 Düsseldorf, Germany
[2]Institute of Pharmaceutical Science, Zheijing University, 310031 Hangzhou, 353 Yan'an Road, China
[3]Institute of Toxicology, Heinrich-Heine-University, P.O. Box 101007, 40001 Düsseldorf, Germany

386

CYTOTOXIC PROPERTIES OF 2'',3''-DI-OH-ABYSSINON IV AND 2'',3''-EPOXI-ABYSSINON V FROM *ERYTHRINA ADDISONIAE*

Kulawik A.[1], Rohrig R.[1], Ruhl S.[1], Chovolou Y.[1], Kampkötter A.[1], Suckow-Schnitker A.K.[2], Passreiter C.[2], Kahl, R.[1], Wätjen W.[1]

The genus *Erythrina* (Fabaceae) contains more than 100 different species, which are distributed in all tropical areas of the world. In traditional medicine several *Erythrina species* are used to defeat cancer. Beside these therapeutical effects of *Erythrina species*, little is known about pharmacological active substances and the corresponding anticancer mechanisms. Therefore the ground root bark of the Ghanaian medicinal plant *Erythrina addisoniae* was extracted in a Soxhlet apparatus and different prenylated flavonoids were purified. Within the great class of flavonoids, the sub-class of prenylated flavonoids has attractive pharmacological properties e.g. inhibitory effects on multidrug resistance mediated by P-glycoprotein or phytoestrogenic properties. In our studies we focussed on toxicological effects and involved mechanisms which may be relevant for anticarcinogenic properties.We analyzed the cytotoxic potential in rat hepatoma cells (H4IIE) of two prenylated flavonoids isolated from *Erythrina addisoniae:* 2'',3''-Di-OH-Abyssinon IV and 2'',3''-Epoxi-Abyssinon V. These substances showed a high cytotoxicity with EC_{50}-values of 5µM to 10µM measured via MTT-assay. To investigate whether the cell death was mediated by induction of apoptosis, we analyzed caspase-3/7 activation (Apo-ONE-Assay). In H4IIE cells the prenylated flavonoids effects an increasement of caspase-3/7 activation by a factor of 10. As a further feature of apoptosis nuclear fragmentation was observed after treatment and bis-benzimide staining. We conclude that the ground bark of *Erythrina addisoniae* contains several pharmacological active compounds. Prenylated flavonoids exhibited a prominent toxicity in H4IIE cells inducing apoptotic cell death. These substances may be active components and responsible for anticarcinogenic action of the plant extract.

[1]Institut für Toxikologie, Heinrich-Heine-Universität, P.O. Box 101007, 40001 Düsseldorf, Germany
[2]Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Universitätsstraße 1, 40225 Düsseldorf, Germany

387

PROAPOPTOTIC PROPERTIES OF PRENYLATED FLAVONOIDS FROM *ERYTHRINA ADDISONIAE*

Rohrig R.[1], Kulawik A.[1], Ruhl S.[1], Chovolou Y.[1], Kampkötter A.[1], Suckow-Schnitker A.K.[2], Passreiter C.[2], Kahl, R.[1], Wätjen W.[1]

The genus *Erythrina* (Fabaceae) contains more than 100 different species, which are distributed in all tropical areas of the world. Diverse *Erythrina* species are used against cancer, e.g. stomach cancer in traditional medicine. In spite of these therapeutic effects of *Erythrina species*, little is known about pharmacologically active substances and the corresponding anticancer mechanisms. Therefore the ground root bark of the Ghanaian medicinal plant *Erythrina addisoniae* was extracted in a Soxhlet apparatus and different prenylated flavonoids were purified. Within the great class of flavonoids, the sub-class of prenylated flavonoids has attractive pharmacological properties e.g. inhibitory effects on multidrug resistance mediated by P-glycoprotein or phytoestrogenic properties. On the other hand, little is known about the cytotoxicity of prenylated flavonoids and the corresponding mechanisms which may be relevant for anticarcinogenic action. We analyzed the cytotoxic potential of prenylated flavonoid compounds isolated from *Erythrina addisoniae* in hepatoma cells (human: HepG2, rat: H4IIE): The prenylated flavonoids 4´-OCH_3-Abyssinon; Abyssinon IV and Abyssinon V showed higher toxicity compared to the nonprenylated substances naringenin, isosakuranetin and liquiritigenin (MTT-assay). In H4IIE cells the prenylated substances had higher cytotoxic effects than in HepG2-cells (IC_{50}-value of 4´-OCH_3-Abyssinon: 5 µM and >25 µM, respectively). We further investigated whether the cell death was mediated via apoptosis analyzing caspase-3/7 activation (Apo-ONE assay). In H4IIE the prenylated flavonoids showed no alteration of caspase-3/7 activation but in HepG2-cells Abyssinon IV and Abyssinon V exhibited an increased caspase-3/7 activity. As a further feature of apoptotic cell death nuclear fragmentation was observed in HepG2 cells. We conclude that the ground root bark of *Erythrina addisoniae* contains several pharmacologically active compounds. Prenylated flavonoids exhibited a prominent toxicity in both H4IIE and HepG2 cells inducing apoptotic cell death in HepG2 cells (caspase-3/7 activation). These substances may be active components responsible for anticarcinogenic actions of the plant extract.

[1]Institute of Toxicology, Heinrich-Heine-University, P.O. Box 101007, 40001 Düsseldorf, Germany
[2]Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitätsstraße 1, 40225 Düsseldorf, Germany

388

PROAPOPTOTIC COMPOUNDS ISOLATED FROM *ERYTHRINA ADDISONIAE*

Wätjen W.[1], Rohrig R.[1], Kulawik A.[1], Ruhl S.[1], Chovolou Y.[1], Kampkötter A.[1], Suckow-Schnitker AK.[2], Passreiter C.[2], Kahl, R.[1]

The genus *Erythrina* (Fabaceae) contains more than 100 different species, which are distributed in all tropical areas of the world. Diverse *Erythrina* species are used against cancer, e.g. stomach cancer in traditional medicine. In spite of these therapeutic effects of *Erythrina species*, little is known about pharmacologically active substances and the corresponding anticancer mechanisms. Therefore the ground root bark of the Ghanaian medicinal plant *Erythrina addisoniae* was extracted in a Soxhlet apparatus and different compounds were purified yielding different prenylated flavonoids (known substances like Abyssinon IV and Abyssinon V as well as the new flavonoids 3'-(2-hydroxy-3-methyl-but-3-enyl)-licoflavanone and its 4'-methylether), prenylated isoflavonoids (e.g. parvisoflavone A) and pterocarpanes (e.g. neorautenol, phaseolin). We analyzed the cytotoxic potential of these compounds in rat and human hepatoma cells (HepG2, H4IIE): Some of the prenylated flavonoids and also the pterocarpanes exerted a high toxicity in both cell lines (IC_{50} value in low micromolar range). We further investigated whether the cell death was mediated via apoptosis analyzing caspase-3/7 activation (Apo-ONE assay) and nuclear fragmentation: Some of the compounds (e.g. pterocarpane neorautenol) showed a high proapoptotic potency. We further investigated cellular mechanisms of this proapoptotic effect: For example, neorautenol (1 µM) strongly reduced phosphorylation of the ERK MAPK, first effects are found after 30 minutes (10 µM). We conclude that the ground bark of *Erythrina addisoniae* contains several pharmacologically active compounds. Distinct prenylated flavonoids as well as pterocarpanes exhibited a prominent toxicity in both H4IIE and HepG2 cells inducing apoptotic cell death. In case of neorautenol, apoptosis may be mediated via disruption of the ERK signalling pathway, this effect may be responsible for the anticarcinogenic actions of the plant extract. Further analysis of these substances may lead to new pharmacons to be used in cancer therapy.

[1]Institute of Toxicology, Heinrich-Heine-University, P.O. Box 101007, 40001 Düsseldorf, Germany
[2]Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitätsstraße 1, 40225 Düsseldorf, Germany

389

ACCUMULATION OF CURCUMIN IN MEMBRANE STRUCTURES OF HUMAN ISHIKAWA CELLS

J. S. Dempe, E. Pfeiffer and M. Metzler

Curcumin is the major yellow pigment of the rhizomes of the Asian plant *Curcuma longa*, which are commonly called turmeric. It is used as a food coloring agent and spice, e.g. in curry. Curcumin has been reported to exhibit anti-oxidative, anti-inflammatory and anti-carcinogenic properties. In previous metabolic studies, a high propensity of curcumin to bind to lipid membranes was noted. The aim of the present study was to characterize the accumulation of curcumin in membrane structures of mammalian cells in more detail. Human Ishikawa cells, derived from an endometrial carcinoma, were incubated with curcumin in culture media with and without fetal calf serum (FCS) for various periods of time up to 30 h. Subsequently, cells were harvested, homogenized and separated by ultracentrifugation into three fractions, i.e. cell debris, endoplasmic reticulum and cytosol. The cell fractions and incubation media were extracted with Folch reagent and ethyl acetate, respectively, and curcumin was determined in the extracts using HPLC. A rapid and time-dependent accumulation of curcumin in Ishikawa cells was observed in the absence of FCS. In culture medium containing FCS, the cellular uptake of curcumin was markedly lower, and a high degree of binding to FCS was noted. Within the cells, the major portion of curcumin was located in cell debris, whereas the endoplasmic reticulum contained less and the cytoplasm very little curcumin. The extract of the cytoplasm contained mostly the reductive curcumin metabolites hexahydro- and octahydro-curcumin. In cell culture media, parent curcumin decreased and the metabolites increased with time. Cells exposed to hexahydro-curcumin exhibited a reduced uptake of this metabolite as compared to curcumin. This study implies that curcumin rapidly penetrates the plasma membrane of mammalian cells and preferentially binds to the membranes of the endoplasmic reticulum, whereas its reductive metabolites exhibit a lower degree of membrane permeability and binding.

Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe , P. O. Box 6980, 76128 Karlsruhe

390

THE ISOTHIOCYANATE ERUCIN INDUCES APOPTOSIS BY MODULATION OF THE MITOCHONDRIAL MEMBRANE POTENTIAL AND ALTERATION OF THE p53-STATUS OF HUMAN HEPATOMA (HEPG2) CELLS

E. Lamy and V. Mersch-Sundermann

Experimental data provide strong evidence for the effective inhibition of tumorigenesis by isothiocyanates (ITC), enzymatic cleavage products of glucosinolates in *Brassica* vegetables. The chemopreventive effect of ITCs is due to various mechanisms including modulation of the xenobiotic metabolism, antiproliferative effects and the induction of apoptosis. In the present study we investigated the molecular mechanism underlying apoptosis induction induced by 4-Methylthiobutylisothiocyante (MTBITC, Erucin) in human hepatoma cells (HepG2). This ITC is a structure analogue of the well studied and promising chemopreventive agent sulforaphane and present in amounts of mg/kg d.w. in plants like e.g. rocket, cabbage turnip or cauliflower. Induction of apoptosis could be observed after treatment of HepG2 cells with ≥ 10 µM MTBITC. A concentration of 10 up to 30 µM MTBITC led to a time-dependent disruption of the mitochondrial membrane potential. Exposure of cells to 10 µM MTBITC resulted in a loss of the MMP of about 50 % compared to control cells. These concentrations also led to a significant reduction of the Glutathione (GSH) content. In accordance with these observations was a significant increase in the production of radical oxygen species (ROS) in these concentrations. Additionally, the expression of tumor suppressor protein p53 was modulated in a time- and concentration-dependent manner.In conclusion we demonstrated here that MTBITC concentration- and time-dependently inhibit cell proliferation and induce changes in the cell death pattern in the human hepatoma cell line HepG2. Furthermore, the exposure of HepG2 to MTBITC (> 10 µM) led to a depletion of cellular antioxidant defences i. e. GSH, allowing for the generation of significant quantities of ROS and the disruption of the MMP. Therefore, the mitochondrial damage-dependent pathway seems to play an important role in MTBITC-mediated apoptosis in HepG2 cells. The p53-dependent pathway seems to be another death inducing mechanism involved in MTBITC-dependent induction of programmed cell death.

Institute of Indoor and Environmental Toxicology, University of Giessen, Aulweg 123, 35384 Giessen

391

INDUCTION OF APOPTOSIS BY TWO NATURALLY OCCURRING ISOTHIOCYANATES IN PRIMARY HUMAN OVARIAN CARCINOMA CELLS

V. Mersch-Sundermann, E. Lamy, D. Oey, F. Eißmann

Among gynecologic cancers, epithelial ovarian cancer is of the highest mortality rate. Isothiocyanates (ITC) are plant-derived constituents known for their chemopreventive activity both *in vitro* and *in vivo*. Two of them, 4-Methylthiobutylisothiocyanate (MTBITC) and benzylisothiocyanate (BITC) are present in high amounts (mg/kg d.w.) in plants like cabbage turnip or garden cress. These two ITCs have been shown earlier to inhibit proliferation of different carcinoma cell lines but their effect on primary human cancer cells is relatively unknown. In the present study we isolated primary ovarian carcinoma cells from ascites of human volunteers by magnetic-bead technology and studied the effects of MTBITC and BITC on programmed cell death in these cells. Qualitative evidence was accomplished by the detection of fragmented chromosomal DNA (DNA laddering), a quantitative verification of the results by flow cytometric (FACS) analysis of the subG1 DNA content and by measurement of apoptotic single stranded DNA (ssDNA). After treatment of the primary cells with MTBITC or BITC in a concentration of 5-50 µM, a dose-dependent increase in the induction of apoptosis could be observed compared to control cultures. Exposure of the cells to a concentration of 10 µM BITC or 20 µM MTBITC for 24 hours resulted in a 2-fold increase in the subG1 DNA content compared to control cells. No influence of the two substances on cell cycle could be detected. Both BITC- and MTBITC-treatment of the cells led to a loss in the mitochondrial membrane potential after 24 h-treatment. To further investigate the underlying mechanism we exposed the cells to the thiol antioxidant *N*-acetylcysteine (NAC) 1 h prior to treatment with MTBITC or BITC which led to a complete reduction in apoptosis-induction. In conclusion, MTBITC and BITC could induce apoptosis in primary human ovarian cancer cells in a concentration dependent manner. First evidence is given to a mechanism based on the production of radical oxygen species resulting in the induction of programmed cell death.

Institute of Indoor and Environmental Toxicology, University of Giessen, Aulweg 123, 35384 Giessen

392

OXYDATION PRODUCTS OF THE PYTOSTEROL STIGMASTEROL INTERACT WITH ESTROGEN RECEPTOR-DEPENDENT PROCESSES IN CULTURED HUMAN BREAST AND ENDOMETRIUM CELL LINES

C. Müller[1], J. Wagner[1], C. Johannes[2], R.L. Lorenz[2], L. Lehmann[1]

Phytosterols are constituents of plant membranes and are thus found at low concentrations in vegetable oil as well as in high concentrations in functional food meant to reduce serum cholesterol levels. Endogenous hormones such as androgens and estrogens are formed by several subsequent enzymatic oxidation reactions starting from cholesterol. Likewise cholesterol, phytosterols are oxidized chemically in food and by biotransformation *in vivo*. Therefore, the estrogenic and antiestrogenic activity of oxystigmasterols was determined in a cultured human breast and endometrium cell line and compared with the activity of structural similar oxycholesterols at the following endpoints: (i) the affinity to isolated human estrogen receptors (ER), (ii) the basal and 17β-estradiol (E2)-induced expression of the alkaline phosphatase (ALP) in human endometrial adenocarcinoma (Ishikawa) cells, and (iii) the basal and E2-induced proliferation of human breast adenocarcinoma (MCF-7) cells. ALP mRNA levels were determined by reverse transcription/competitive PCR and the ALP activity was measured using a chromogenic substrate. Cell numbers were determined by electronic cell counting. A mixture of various oxyphytosterols were able to replace E2 from human ERalpha and beta and induced a weak estrogenic response in MCF-7 cells. In contrast, the E2-induced activity of the ALP in Ishikawa cells as well as the E2-induced proliferation of MCF-7 cells were decreased at non-cytotoxic concentrations (up to 10 µM), indicating an antiestrogenic potential of oxystigmasterols. Studies using specific oxidation products of stigmasterol revealed that the antiestrogenic potential of the mixture was mainly due to 7beta-hydroxystigmasterol. In contrast, cytotoxicity interfered with a putative antiestrogenic potential of 7alpha-hydroxystigmasterol and both 7-hydroxycholesterols. In conclusion, 7beta-hydroxystigmasterol differs in its biologic activity (i.e. its affinity for the ER as well as its cytotoxicity) from other oxidation products of stigmasterol and also from the corresponding oxidation product of cholesterol. Supported by DFG (Me574/24-2).

[1]University of Karlsruhe, Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, Kaiserstr. 12, 76131 Karlsruhe. [2]University of Munich, Institute for Prophylaxis of Cardiovascular Diseases, 80336 Munich.

393

STUDIES ON THE GENOTOXICITY OF OCHRATOXIN A: INDUCTION OF MICRONUCLEI AND OXIDATIVE DNA DAMAGE IN CULTURED CELLS

C. Behm[1], Y. Lektarau[1], Ü. Ündeger[2], G. H. Degen[1], W. Föllmann[1]

The mycotoxin ochratoxin A (OTA), a known nephrotoxin and rodent carcinogen, is a frequently found contaminant of grains and other foodstuffs. Thus, a careful risk assessment for OTA is required. In this context, it is important to take into account information on genotoxic properties, dose-effect relationships and its mode of action, and to distinguish between direct and indirect modes of genotoxicity. Effects of OTA were analyzed by means of the Comet assay and the micronucleus assay in order to better characterize the OTA-induced DNA damage and dose-effect relationships in cultured cells. Both assays were performed with urothelial cells and V79 cells under serum-free conditions. In addition, cytotoxicity of OTA was assessed by neutral red uptake assay. The Comet assay was performed with and without addition of FPG enzyme (fpg-glycosylase known to convert oxidative DNA damage into strand breaks) in cells treated between 1 and 6 h with OTA or solvent only or with H_2O_2 as positive control for induction of oxidative DNA damage. For the MN assay the cells were treated for about 1.5 cell cycles with graded concentrations of OTA and with DMSO as solvent control. Micronuclei (MN) increased dose-dependently between 0.03 and 1 µM OTA (up to 4-fold compared to controls in V79 cells) whilst the lowest tested concentration of 0.01 µM OTA did not induce a higher frequency of MN in either cell type. In the FPG-modified Comet assay evidence for oxidative DNA damage was observed in V79 and in urothelial cells treated with OTA concentrations of 0.03 µM and more, with no indication of this genotoxic effects at lower OTA levels. At higher OTA levels a plateau was reached indicative of an indirect mode of action. In summary, OTA induced micronuclei at concentrations below those which are overtly cytotoxic. The shape of the dose-response curve at very low concentrations in this and in the modified Comet assay supports the existence of a threshold for its genotoxicity, an observation in accord with an induction of oxidative DNA damage by OTA. In conclusion, our data point to an indirect genotoxic mode of action for this mycotoxin.

[1]Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystr. 67, D-44139 Dortmund, Germany, [2]Hacettepe University, Faculty of Pharmacy, Ankara, Turkey

394

CYP1A1 INDUCTION BY CIGARETTE SMOKE CONDENSATE IN UROTHELIAL CELLS – A RESULT OF COMPLEX COMBINATION INTERACTIONS

S. Plöttner, C. Behm, H. M. Bolt, W. Föllmann

Cigarette smoking is a known risk factor for urinary bladder cancer development. Aromatic amines are thought to play a major role, but polycyclic aromatic hydrocarbons (PAHs) appear also relevant. We have shown that benzo[a]pyrene (B[a]P) strongly induces CYP1A1 mRNA in cultured porcine urinary bladder epithelial cells (PUBEC) (WOLF et al. 2005, *Toxicology* 207: 255-269). Now we present CYP1A1 protein induction data for PUBEC incubated with different cigarette smoke constituents. After treatment with B[a]P (0.001-50 µM) a concentration-dependent increase of CYP1A1 was detected. Flow cytometry revealed a sensitive cell population of 10% already responding to 0.1 µM B[a]P. Western blot analysis displayed CYP1A1 induction beginning at 1 µM B[a]P. This effect was augmented by co-incubation of PUBEC with 1 µM B[a]P and either 4-aminobiphenyl (3-50 µM) or 2-naphthylamine (3-10 µM), whereas CYP1A1 was not induced by the arylamines alone. Cigarette smoke condensate (CSC) of 2R4F reference cigarettes induced CYP1A1 already at 1 µg/ml. Regarding the amount of B[a]P within

cigarette smoke condensate, however, CYP1A1 induction cannot be explained by B[a]P alone, as its concentration within 1 µg/ml was only 2.4 pM. Also the sum of all PAHs does not explain the inducing effect. PAHs and arylamines seem to operate synergistically, but more complex interactions may play a decisive role. Human urothelial cells also respond to cigarette smoke by CYP1A1 induction: Exfoliated urothelial cells of smokers contain higher numbers of CYP1A1 responsive cells than those of non-smokers (DÖRRENHAUS et al. 2006, *Arch Toxicol* DOI: 10.2007/s00204-006-0134-9). The complex combination interactions that lead to CYP1A1 induction in urothelial cells are a matter of further research.

Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystr. 67, 44139 Dortmund, Germany

395

COMBINATION EFFECT OF CO-EXPOSURE WITH POLYCYCLIC AROMATIC HYDROCARBONS AND AROMATIC AMINES IN UROTHELIAL CELLS

W. Föllmann[1], S. Plöttner[1], A. Borza[2], C. Behm[1], H. M. Bolt[1], J. Kuhlmann[2]

Cigarette smoking is one of the most predominant life-style factors in induction of human cancer. Beside lung cancer, smoking is considered as a most prominent risk factor for the development of urinary bladder cancer. Aromatic amines have been advocated as a main cause of urothelial neoplasias. Utilizing an experimental setup based on primary porcine urinary bladder epithelial cells (PUBEC), we have analyzed the combination effect of co-exposures with two tobacco smoke related substance classes, namely polycyclic aromatic hydrocarbons (PAH) and aromatic amines, on the expression of genes encoding for drug metabolizing enzymes. Aromatic amines alone only marginally enhanced gene expression of phase I and II enzymes. In contrast, polycyclic aromatic hydrocarbons raised the amounts of mRNA of metabolizing enzymes in treated urothelial cells. Especially CYP1A1 expression was strongly induced after incubation with benzo[a]pyrene (B[a]P). Cyclooxygenase-2 and UDP-glucuronosyl transferase were also induced, but to a lesser extent. A co-incubation of B[a]P with 4-aminobiphenyl resulted in a synergistic effect on the expression of CYP1A1, cyclooxygenase-2 and UDP-glucuronosyl transferase mRNA. We also analyzed gene expression of the cytochrome P450 isoforms CYP4B1 and CYP1B1 which are thought to be relevant in the metabolism of aromatic amines and PAH in the urinary bladder. But no significant changes in their expression after incubation with B[a]P, 4-ABP or a combination of both could be seen. Our findings support the view that co-exposures with different classes of smoke related chemicals synergistically enhance CYP1A1 induction, leading to a stimulated activation of toxicants like PAH in urothelial cells.

[1]Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystr. 67, 44139 Dortmund, Germany
[2]Max-Planck Institut für molekulare Physiologie, Otto-Hahn-Str. 11, 44227 Dortmund, Germany

396

WATER PIPE SMOKING AND HEALTH HAZARDS: MORE NAPHTHALENE METABOLITES IN THE URINE OF SMOKERS COMPARED TO NON-SMOKERS

T.G. Schulz, A. Seidel*, T. Skladnikiewicz, A. Luch

The water pipe (shisha, narghile) is a traditional aid for tobacco consumption in Asia and Northern Africa. In recent years, the number of people smoking water pipes in Germany and many other European countries has considerably grown. The information on health hazards related to this source, however, is rare. Only very few data have been published on the composition of the water pipe smoke. The Federal Institute for Risk Assessment recently published an evaluation of the risk connected with the habit of smoking water pipes (http://www.bfr.bund.de/cm/216/gesundheits_und_suchtgefahren_durch_wasserpfeifen.pdf).

Naphthalene, phenanthrene and pyrene are well known compounds in cigarette smoke. Accordingly, hydroxynaphthalene, 1-, 2-, 3-, 4-, and 9-hydroxyphenanthrene and 1-hydroxypyrene serve as well established biomarkers for cigarette consumption. To investigate the possible exposure to naphthalene, phenanthrene and pyrene through the smoke of water pipes, a study was initiated with 5 non-smoking controls and 10 water pipe smokers. The water pipe smokers were interviewed about their smoking habits. After consumption of one tobacco head, the smokers collected their urine for 24 hours. Non-smoking controls provided urine accordingly. Urine samples were analyzed for metabolites of the three compounds under consideration. The total quantity of hydroxynaphthalene, 1,2-dihydroxynaphthalene and 1,4-dihydroxynaphthalene was detected two-fold higher in the urine of water pipe smokers compared to the controls. Surprisingly, no differences were observed between smokers and non-smokers regarding the excretion rates of hydroxyphenanthrenes, 1-hydroxypyrene, and dihydroxypyrene. It will be investigated whether phenanthrene and pyrene are constituents of the water pipe smoke. However, no standardised smoking regimen for the water pipe is available at the moment. Further studies will also focus on the contribution of tobacco-specific nitrosamines possibly present in water pipe smoke.

Our results presented here may not suggest that the water pipe is a rather harmless alternative to cigarettes.

Federal Institute for Risk Assessment, Berlin, Germany
* Biochemical Institute for Environmental Carcinogens, Prof. Dr. Gernot Grimmer-Foundation, Grosshansdorf, Germany

397

CIGARETTE MAINSTREAM SMOKE AFFECTS ARTERIAL THROMBOSIS AND VESSEL REMODELING AFTER VASCULAR INJURY IN APOLIPOPROTEIN E-DEFICIENT MICE

T. Wallerath[1], M. Schroeter[2], K. Meurrens[3], S. Lebrun[1], S. Konstantinides[2], R. Schleef[4], and K. Schäfer[2]

Cardiovascular disease and its thrombotic complications, particularly myocardial infarction and stroke, are a main cause of death in industrialized countries. Currently, little is known about the underlying pathomechanisms of cigarette smoke on thrombosis and subsequent vessel wall remodeling, partly due to a lack of adequate *in vivo* models. We investigated the biological effects of sub-chronic inhalation exposure to cigarette mainstream smoke (MS) from the University of Kentucky Reference Cigarette 2R4F at doses of 600, 1200, 1800, and 2400 µg total particulate matter per liter per day on arterial thrombosis and intimal hyperplasia using the ferric chloride model of vascular injury in male apolipoprotein E-deficient (ApoE-/-) mice. Arterial thrombosis was analyzed in the left carotid artery by determining the time to thrombotic occlusion and patency rate after vascular injury. A dose-dependent decrease was seen in median time to occlusion (14.9 min for control, 9.7 min for MS-1800, and 8.0 min for MS-2400; $p < 0.001$ compared to control) and lower vascular patency rates after 25 minutes (40% for control, 10% for MS-1800, and 0% for MS-2400; $p < 0.001$ compared to control). Three weeks later, intimal hyperplasia was analyzed using immunohistochemistry and morphometry. Immunohistochemistry of neointimal lesions revealed an MS exposure-associated increase in the amount of α-actin-positive smooth muscle cells (range: 16% (control) to 25% (MS-2400); $p < 0.05$ for MS-2400 compared to control). No differences were seen in the number of apoptotic cells. Morphometry revealed an MS exposure-associated increase intima-media ratio ($p < 0.05$ for MS-1800 compared to control and $p < 0.01$ for MS-2400 compared to control). These data indicate that subchronic exposure of ApoE-/- mice to MS promotes arterial thrombosis and modulates the size and composition of vascular lesions.

[1]PHILIP MORRIS Research Laboratories GmbH, Cologne, Germany
[2]Department of Cardiology and Pulmonary Medicine, University of Göttingen, Germany
[3]PHILIP MORRIS Research Laboratories bvba, Leuven, Belgium
[4]Philip Morris USA, Richmond, VA, USA

398

CLOSTRIDIUM DIFFICILE TOXIN A-INDUCED APOPTOSIS

S. Nottrott, J. Schoentaube, H. Genth, I. Just, and R. Gerhard

Clostridium difficile, causative for antibiotic-associated diarrhoea, produces the homologous glucosyltransferases toxin A (TcdA) and toxin B. Both toxins glucosylate and thereby inactivate RhoA, Rac1, and Cdc42. The aim of this study is to elucidate the mechanism by which TcdA induces apoptosis in intestinal epithelial cells. TcdA-induced apoptosis was analysed in HT29 cells in terms of activation of caspases (3,8,9), the release of cytochrome *c* from mitochondria, and the fragmentation of DNA. Cleavage of procaspase-8 and -9 was responsive to inhibition by Z-VAD-fmk, a pan-caspase inhibitor. Interestingly, cleavage of procaspase-3 and truncation of Bid was not inhibited by z-VAD-fmk, indicating an activation by a non-caspase protease. The cathepsin inhibitor ALLM inhibited activation of all three caspases (3, 8, and 9) as well as truncation of Bid in TcdA-treated cells, suggesting a role of cathepsins in the activation of caspases and the truncation of Bid. The induction of apoptosis by TcdA was based on the glucosylation of Rho proteins. The apoptosis strongly correlated with the glucosyltransferase activity of mutant forms of TcdA. Rho glucosylation by TcdA induced actin re-organization and a subsequent G2/M arrest of the cells. Both effects, however, were also induced by latrunculin, an actin de-polymerizing agent, that did not induce apoptosis. Thus, TcdA-induced apoptosis was based on the glucosylation of Rho proteins independently of actin cytoskeleton. TcdA-induced apoptosis did not depend on p53, as p53 was neither activated nor up-regulated in TcdA-treated HT29 cells. In this line, the initiation of apoptosis by TcdA was comparable in p53 $^{-/-}$ and wild-type HCT 116 cells. In conclusion, TcdA induced apoptosis in a p53-independent manner, suggesting that glucosylation of Rho proteins by TcdA represents a cytotoxic rather than a genotoxic stimulus.

Institute of Toxicology, Hannover Medical School, Germany

399

CHARACTERISATION OF APOPTOSIS SIGNAL TRANSDUCTION INDUCED BY SPONGISTATIN 1 IN MCF-7 CELLS

U. M. Schneiders, L. Schyschka, N. Barth and A. M. Vollmar

Many chemotherapeutic agents mediate their effects by induction of apoptosis in cancer cells. The marine substance spongistatin 1, a highly cytotoxic macrocyclic lactone polyether, isolated from the sponges *Spirastrella spinispirulifera* and *Hyrtios erecta*, exhibits a strong cytotoxic potence in the human tumour cell line screen of the National Cancer Institute. But the apoptotic mechanisms involved remain unknown. In our study, we examine the apoptosis-inducing potential and the underlying apoptotic signaling pathway of spongistatin 1 in MCF-7 cells, an epithelial breast cancer cell line deficient in caspase-3. Our results show the induction of apoptosis by spongistatin 1 in a time and dose dependent manner in MCF-7 cells. Spongistatin 1 triggers the release of cytochtome c, Smac, AIF and Omi/HtrA2 from the intermembrane space of mitochondria to the cytosol, a key effect in the intrinsic pathway. Cytochrome c leads to the activation of procaspase-9 acting as a initiator caspase by activating the effector caspases-6 and –7 which are able to adopt the role of caspase-3. Interestingly, treatment with the pan-caspase-inhibitor zVAD.fmk reduced the apoptosis rates of spongistatin 1 about 10%, indicating for an additional involvement of caspase-independent apoptotic pathways. We found that spongistatin1 induces the translocation of the mitochondrial proteins AIF and Omi/HtrA2 to the nucleus, important factors acting independently of caspases during apoptosis. Consequently, our research focuses on the detection of upstream effectors of apoptosis. Since spongistatin 1 acts as a tubulin depolymerizing agent, microtubule interacting factors could play a important role in the induction of apoptosis by spongistatin 1. Therefore, the proapoptotic Bcl-2 family member Bim (Bcl-2 interacting mediator of cell death) is of particular interest to our ongoing work.

Department of Pharmacy, Center of Drug Research, University of Munich, Germany

400

IDENTIFICATION OF NOVEL XIAP INHIBITORS TARGETING BIR3 BY A PHARMACOPHORE-BASED VIRTUAL SCREENING

N. Barth[1], L. Schyschka[1#], C. Bliem[2], H. Stuppner[2] and A. M. Vollmar[1]

Targeting apoptosis control provides a novel therapeutic approach to the treatment of cancer. Several of the inhibitor of apoptosis protein (IAP) family members regulate apoptosis in response to various cellular assaults. Over-expression of these proteins leads to resistance to apoptotic triggers, but if they are inhibited, cells get sensitive for cell death again. The most intensively studied family member, X-linked IAP (XIAP), is a potent inhibitor of caspase activity, which selectively binds and inhibits caspases-3, -7 and -9. The focus of our work was to find new small molecule XIAP-BIR3 domain antagonists preventing the inhibitory effect of XIAP on caspase-9 and therefore blocking caspase-cascade at the initiator level. A NMR structure of a SMAC peptide complexed with the BIR3 domain of XIAP served as primary information for a pharmacophore model serving as a tool for the discovery of potent leading structures. The compound T8, a hit candidate from a screen of commercially available databases, was tested on its ability to enhance apoptosis in cancer cells which over-express XIAP. In Jurkat cells T8 does

not significantly induce apoptosis itself, however potentiates apoptosis induced by subtoxic concentrations of etoposide and also sensitizes for reduction of clonogenic survival of etoposide treated Jurkat cells. As expected from a XIAP inhibitor T8 has a strong impact on cleavage of caspase-9 and-3 proforms induced by etoposide and so the caspase activities induced by etoposide are increased. Moreover XIAP protein levels were reduced by T8 alone as well as in combination with etoposide. Summarizing the pharmacological data T8 revealed a distinct apoptosis enhancing effect and seems to be a promising chemosensitizing compound targeting XIAP.

supported by the Bayrischen Forschungsstiftung

1 Department of Pharmacy (http://www.chemie.uni-muenchen.de/pb/aks/vollmar/, University of Munich, Munich, Germany, e-mail: nicole.barth@cup.uni-muenchen.de
2 Institute of Pharmacy, Pharmacognosy, University of Innsbruck, Innsbruck, Austria

401

ROLE OF E2F-1/p73 APOPTOSIS SIGNALLING IN FOTEMUSTINE TREATED MALIGNANT MELANOMA CELLS

S.C. Naumann, W.P. Roos, M. Christmann and B. Kaina

The transcription factor and p53 homologue, p73 is able to induce apoptosis following DNA damage by transcription of p53-related pro-apoptotic genes. Two different promoters for the p73 gene have been identified. One leads to a transcriptionally active and pro-apoptotic form (TAp73), regulated by E2F-1. The second to a transcriptional inactive and anti-apoptotic form (deltaNp73), regulated by p53. Here, we examined the role of both p73 forms in apoptosis, induced by the DNA-interstrand cross-linker fotemustine (FM), using two malignant melanomas cell lines that differ in their p53 status. FM is used in the first line chemotherapy of malignant melanomas. FM induced apoptosis in D14 (p53-mutant) and D05 (p53 wild-type) melanoma cells, and p53-mt cells are more sensitive. In both, p53wt and mt cells, FM caused accumulation of cells in G2/M. In p53wt cells, this G2/M block was paralleled by a decrease in E2F-1. Also, the phosphorylated form of Rb (pRb) decreased. In p53mt cells, the accumulation of cells in G2/M following FM treatment was paralleled by an increase in E2F-1, while no change was observed for pRb. Furthermore, p53mt cells also showed increased TAp73 levels, along with increased p21, Puma and Noxa. This clearly points to a role of TAp73 in mediating the apoptotic response of melanoma cells to FM. In p53wt, however, E2F-1 decreased and, along with the increase in p53, leads to increased transcription of deltaNp73. As deltaNp73 inhibits the p53 pro-apoptotic transcription, it was no surprise that Puma and Noxa expression was unaffected by FM treatment in p53wt cells. DeltaNp73 had no effect on p21 expression in p53wt cells, as an increase was observed. Collectively, these data show that the alkylating agent FM induce apoptosis in malignant melanoma cells. We also show for the first time that the E2F-1/TAp73 pathway can been a substitute for the transcription of pro-apoptotic p53 related genes in p53mt malignant melanoma cells, while p53 protects against FM triggered apoptosis by deltaNp73 expression.

Work was supported by DFG KA724/13-1 and 13-2.

Department of Toxicology, Johannes-Gutenberg University of Mainz, Obere Zahlbacher Straße 67, 55131 Mainz, Germany

402

MAFOSFAMIDE INDUCED APOPTOSIS IN HUMAN LYMPHOBLASTOID CELLS: ROLE OF DNA REPLICATION, RNA TRANSCRIPTION, p53 AND Chk1/Chk2

M. Goldstein, W. P. Roos and B. Kaina

DNA interstrand crosslinks induced by mafosfamide are considered to be responsible for its cytotoxicity. How this cytotoxicity is brought forth, is still largely unknown. We show that mafosfamide induces apoptosis in TK6 (p53wt) and WTK1 (p53mt) lymphoblastoid cells dose and time dependently, with p53wt cells clearly more sensitive. We elucidated two upstream processes that could be triggered by mafosfamide-induced DNA lesions: DNA replication blockage and RNA transcriptional inhibition. In P493-6 lymphoblastoid cells, which can be manipulated to proliferate or not, we found that proliferation clearly increased the level of apoptosis induced by mafosfamide, suggesting that replication blockage leads to apoptosis. Using the transcriptional inhibitor α-amanitin, on its own or in combination with mafosfamide, we also show that RNA transcriptional inhibition also contributes to apoptosis initiation. Mafosfamide caused p53 stabilization and activation of ATM/ATR and Chk1/Chk2. Inhibition of PI3-kinase as well as Chk1/Chk2 attenuated the apoptotic response in p53wt cells. Mafosfamide-induced apoptosis was mediated via both the Fas and the mitochondrial pathway, which was dependent on caspase activation. For low dose-treated cells caspases were preferentially activated in the S-phase, which was not the case with high dose. We conclude that at low dose of mafosfamide, DNA replication blockage is the dominant apoptosis-inducing event, while with increasing dose RNA transcriptional inhibition comes into play. Therefore, replicating cancer cells should be targeted with lower mafosfamide doses, while at higher doses the normal non-proliferation cells will start dying.

Department of Toxicology, University Mainz, Obere Zahlbacher Str. 67, 55131 Mainz Germany

403

UV-INDUCED LESIONS TRIGGER APOPTOSIS IN P53-MUTATED GLIOMA CELLS

L.F.Z. Batista[1, 2], W.P. Roos[1], C.F.M. Menck[1,2] and B. Kaina[1]

We have recently shown that the O^6-methylating agent Temozolomide (TMZ) induces apoptosis in human glioma cells and that p53 wild-type cells are more sensitive to this treatment than p53 mutated cells (Roos et al, Oncogene, *in press*). We are now interested in finding treatments that render glioma p53-mutated cells sensitive to apoptosis. We decided to generate helix-distorting lesions on DNA, and verify if those lesions could induce apoptosis in p53-mutated cells. Since ultraviolet light (UV) is a known agent able to induce these kinds of lesions, namely CPDs and 6-4 PPs, we decided to verify the effect of this agent in our cells. To this end, we used human glioma cells with different p53 status treated with TMZ or UV-C. Apoptosis experiments showed that p53-mutated glioma cells are extremely sensitive to UV light. In p53 proficient cells, this protein accumulates within few hours after irradiation, and dot-blots against CPDs confirmed that this protein has a role in the removal of photolesions. Inhibition of p53 sensitized wild-type cells to UV. We show that Fas activation is not necessary for UV-induced apoptosis in p53-mutated cells, and that the pro-apoptotic protein Bak is up-regulated after irradiation, indicating that apoptosis occurs trough the intrinsic pathway. Caspase activation profiles confirmed these findings. Although transcription was impaired on both cell lines after UV, only p53 wild-type cells were able to recover from transcription inhibition. Although this indicates that transcription blockage was triggering apoptosis in p53-mutated cells, specific inhibition of transcription did not induce cell death. On the other hand, inhibition of DNA replication in UV-irradiated cells led to a reduction in apoptosis. Taken together our results show that photoproducts induce cell death preferentially in p53-mutated cells and that damaged-DNA replication is the main signal leading to apoptosis. The work provides new insight into the mechanisms of DNA damage triggered apoptosis. In addition, it provides a model system for screening chemotherapeutics drugs that could mimic UV-irradiation thereby being effective in killing p53-mutated glioma cells.

[1] Institute of Toxicology, University of Mainz, Germany
[2] Department of Microbiology, ICB, University of São Paulo, Brazil

404

MECHANISM OF METHYLATING AGENT INDUCED CELL DEATH: ROLES OF MGMT, MISMATCH REPAIR AND DNA DOUBLE-STRAND BREAK REPAIR

W. P. Roos, L.F.Z. Batista, S. C. Naumann, O. Kiedron, M. Christmann and B. Kaina

Methylating agents are commonly found in the environment, food and tobacco smoke. They are also used in the treatment of cancers like Hodgkin's disease, non-Hodgkin's lymphoma, malignant melanoma, pancreatic (islet cell) cancer, prostate cancer, astrocytoma, glioblastoma and brain metastasis from solid tumours. Although the mechanism for mutagenesis of these agents is well understood, the mechanism of cell killing still remains to be clarified. Using multiple experimental cell systems; including primary human lymphocytes, glioblastoma, melanoma and embryonic stem cells, we found the following: Upon methylation of DNA with clinical relevant doses, O^6-methylguanine (O^6MeG) was identified as the major cell killing lesion. In all cell systems tested, cells die by apoptosis due to O^6MeG. Apoptosis is executed in p53 wild-type cell via the death receptor (FAS/CD95) pathway, and in p53 mutant cells via the intrinsic mitochondrial pathway. The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) protects against O^6MeG-triggered apoptosis. Cellular proliferation, along with MutSα dependant mismatch repair, is absolutely required for the execution of O^6MeG-triggered apoptosis. Furthermore, in all cell systems tested, a wave of DNA double-strand breaks (DSBs) was observed following methylating agent treatment, which preceded apoptosis and only occurred in replicating cells. By comparing DNA DSB repair mutants for non-homologous end joining (NHEJ) and homologous recombination (HR) with their un-mutated counter parts, following methylating agent treatment, we have also identified that the HR proteins xrcc2 and BRCA2 are required for protecting cells from O^6MeG-triggered apoptosis.

Department of Toxicology, Johannes-Gutenberg University of Mainz, Obere Zahlbacher Straße 67, 55131 Mainz, Germany

405

CATALASE OVEREXPRESSION AND ITS ROLE IN DOXORUBICIN INDUCED APOPTOTIC SIGNALLING IN HUMAN CELLS

R. Lüpertz, Y. Chovolou, A. Kampkötter, W. Wätjen, R. Kahl

Doxorubicin is an anthracycline compound widely used as a chemotherapeutic agent. It acts as a topoisomerase II inhibitor and can induce apoptosis through a variety of mechanisms including DNA intercalation and production of reactive oxygen species (ROS). Besides inducing apoptosis one of the characteristic features of doxorubicin is the activation of the transcription factor nuclear factor-κB (NFκB). NFκB is known to control numerous gene products that play crucial roles in cell survival. One major point for the activation of NFκB is the amount of ROS inside the cell. Since doxorubicin is known to produce ROS we studied the effect of the antioxidant enzyme catalase on doxorubicin induced cytotoxicity and NFκB activation. Catalase is a peroxisomal enzyme catalyzing the breakdown of hydrogen peroxide to water and oxygen. Since H_2O_2 can be converted to the highly reactive hydroxyl radical it is generally assumed that catalase fulfils a cytoprotective role against oxidative stress. Catalase expression and activity were markedly reduced after incubation with doxorubicin at moderately cytotoxic concentrations for 24h in Hct-116 human colon carcinoma cells. To investigate whether the observed cytotoxicity is causally related to the loss of catalase activity we used transiently transfected Hct-116 cells overexpressing catalase. In contrast to expectations, these transfectants were not protected against doxorubicin cytotoxicity but were even more sensitive to doxorubicin than the wild-type cells. This raises the question whether the lack of sufficient amounts of H_2O_2 caused by catalase overexpression affects the ROS responsive NF-κB signalling pathway. The capability of catalase overexpression to affect the NFκB pathway after doxorubicin treatment is under investigation.

Institute of Toxicology, University of Düsseldorf P.O Box 10 10 07, D-40001 Düsseldorf, Germany

406

OVEREXPRESSION OF TUMOR NECROSIS FACTOR-ALPHA POTENTIATE ACETAMINOPHEN-INDUCED CYTOTOXICITY IN HUMAN CELLS

S. Sydor, Y. Chovolou, A. Kampkötter, W. Wätjen, R. Kahl

Acetaminophen is well known as a safe analgesic and antipyretic drug while overdoses of acetaminophen can cause serious liver damage. Toxicity requires conversion of acetaminophen to the highly reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). In addition to cellular damage inflicted by NAPQI a variety of other factors (e.g. cytokines) contribute to acetaminophen induced cytotoxicity. The present study investigates the role of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) in acetaminophen mediated cytotoxicity using as a model system a human hepatoma cell line (Huh7) and a human breast adenocarcinoma cell line (MCF-7). To determine whether overexpression of TNF-α increases the sensitivity of Huh7 and MCF7 cells to acetaminophen induced cytotoxicity, both cell lines were transiently transfected with a human TNF-α expression construct. The viability of the cells was determined by neutral red assay. TNF-α overexpressing cells were much more susceptible to the cytotoxic effects of acetaminophen than wild type cells. Further studies are needed to characterize the mode of cell death and the signalling pathways that contribute to the TNF-α mediated sensitization of the cells to acetaminophen.

Institute of Toxicology, University of Düsseldorf, P.O. Box 101007, D-40001 Düsseldorf, Gemany

407

TUMOR NECROSIS FACTOR-ALPHA INCREASES THE SUSCEPTIBILITY OF RAT HEPATOMA CELLS ACETAMINOPHEN-INDUCED CYTOTOXICITY

Y. Chovolou, A. Kampkötter, W. Wätjen, R. Kahl

Acetaminophen, also known as paracetamol, is widely used as an analgesic and antipyretic drug. While acetaminophen is a safe drug at therapeutic doses, an acute or cumulative overdose can lead to the generation of large amounts of the highly reactive and toxic metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI) by cytochrome 2E1 which leads to oxidative stress and can cause severe liver injury. Other factors contributing to the hepatotoxicity of acetaminophen include proinflammatory and cytotoxic mediators, such as tumor necrosis factor-alpha (TNF-α). However, the precise role of TNF-α in acetaminophen-induced hepatotoxicity is controversial. Whereas some studies have suggested that TNF- α contributes to acetaminophen cytotoxicity, others indicate that it has no effect.

The aim of this study was to elucidate the role of the proinflammatory cytokine TNF-α in acetaminophen-mediated cytotoxicity using rat H4IIE hepatoma cells overexpressing TNF-α as a model system. TNF-α overexpressing cells but not wtH4IIE cells exhibited increased toxicity after treatment with acetaminophen for 24h. The loss of cell viability after acetaminophen treatment was characterized by the appearance of cells displaying apoptotic morphology and DNA fragmentation in hepatoma cells overexpressing TNF-α. Furthermore, caspase-2,-3 and -8 activity was significantly increased only in TNF-α overexpressing H4IIE cells. Pretreatment of wtH4IIE cells with a non-toxic TNF-α concentration markedly increased acetaminophen-induced cell death as well as the caspase-2, -3 and -8 activity. Furthermore, intracellular ROS concentrations were increased by acetaminophen in TNF-α overexpressing cells compared to wtH4IIE cells. The NFκB signalling pathway is one of the key signalling pathways that is activated in response to oxidative stress. NF-κB nuclear binding activity as determined by EMSA remained unchanged throughout acetaminophen treatment in wtH4IIE cells. In contrast, pretreatment with TNF-α markedly induced NF-κB nuclear binding activity, suggesting a proapoptotic action of NF-κB in this model system. In conclusion, these data support the hypothesis that TNF-α aggravates acetaminophen-mediated cytotoxicity in rat hepatoma cells.

Institute of Toxicology, University of Düsseldorf, P.O. Box 101007, D-40001 Düsseldorf, Gemany

408

APOPTOTIC RELEASE AND POSTTRANSLATIONAL MODIFICATIONS OF THE CHROMATIN PROTEIN DEK

F. Kappes[1], M. Khodadoust[1], J. Fahrer[2], M. Moreno-Villanueva[2], A. Bürkle[2], D. Markovitz[1], and E. Ferrando-May[2]

DEK is a ubiquitous and abundant chromatin-associated protein lacking significant homologies to any known protein family. DEK was originally identified as part of the DEK-CAN fusion protein in some cases of acute myeloid leukaemia. Since its discovery, DEK was linked to numerous cellular functions, including carcinogenesis, cellular senescence and autoimmunity. In a recent proteomic screen of apoptotic nuclei, we identified DEK as a heavily posttranslationally modified target protein in early apoptotic events in T cells, suggesting a new role of DEK in cell death. Here we show that different apoptotic stimuli result in a release of a DEK subpopulation from the nucleus into the cytoplasmic compartment. This DEK population was found to be highly phosphorylated and more interestingly, was not associated with cytoplasmic apoptotic oligonucleosomes. In contrast, remaining nuclear DEK is dephosphorylated and immobilized on apoptotic chromatin. In addition, we show that apoptotically released DEK is also modified with poly(ADP-ribose) (PAR) *in vivo*, suggesting a link between DEK and members of the PARP protein family. In agreement with the *in vivo* findings, *in vitro* experiments showed that DEK is an efficient acceptor of PAR, which in turn negatively influences the DNA binding affinity of DEK and its ability to introduce topological changes in DNA templates. In a recent study, DEK was found to be secreted from activated macrophages via exosomes, which is one proposed mechanism by which DEK autoantibodies may be generated. In this study we found that apoptotically released DEK is also present in cell culture supernatants. Under conditions of limited phagocytic clearance, this might be a further mechanism contributing to the generation of DEK autoantibodies. Furthermore, we established stable DEK knock down cells, utilizing a lentiviral shRNA delivery system. DEK expression was significantly decreased in these cells. This appeared to interfere with the cells´ ability to repair DNA double strand breaks and sensitized them towards DNA damaging agents like etoposide, camptothecin and UV irradiation. Taken together these findings unveil a novel link between DEK and DNA repair, which may help understand the role of this protein in cancer pathogenesis and suggest an alternative way by which DEK autoantibodies might be generated.

[1] University of Michigan, Department of Internal Medicine, Division of Infectious Diseases, Ann Arbor, MI, USA
[2] University of Konstanz, Department of Biology / Molecular Toxicology, Konstanz, Germany

409

CORRELATION OF DNA ADDUCTS WITH OTHER ENDPOINTS OF GENOTOXICITY IN L5178Y MOUSE LYMPHOMA CELLS TREATED WITH METHYLATING AGENTS OF SN_1 OR SN_2 TYPE

A. Brink, B. Schulz, I. Richter, H. Stopper, W.K. Lutz

The biological significance of exposure-related increments of DNA adducts above background is under discussion. We addressed this question quantitatively by comparing increments of DNA adducts from an exogenous source with the increments measured for subsequent endpoints of genotoxicity. Two methylating agents with different profiles of DNA methylation, methyl methanesulfonate (MMS; SN_2-type) and methylnitrosourea (MNU; SN_1-type) were investigated for the formation of O^6-methyl-2'-deoxyguanosine (O^6-mdGuo) and 7(N)-methylguanine (7-mG). Large batches of L5178Y mouse lymphoma cells were treated with MMS or MNU at 0, 50, 100, 200, 300 µM concentration. Adducts were measured by LC-MS/MS. In parallel, aliquots of the cell suspensions were used to determine DNA breakage by the alkaline version of the comet assay, chromosomal damage by the micronucleus frequency assay, and gene mutations by the thymidine kinase $tk^{+/-}$ mutation assay. The dose response at low dose did not deviate from linearity, and slopes were estimated by regression. MMS produced 330 7-mG and 3.7 O^6-mdGuo per 10^9 nucleotides per µmol concentration, whereas MNU produced 113 and 16.1, respectively. This is in line with the SN_1-type reactivity of the methyl diazonium ion released from MNU, which results in a higher rate of oxygen methylations. DNA strand breaks and chromosomal damage increased with greater slopes after MMS treatment, whereas MNU was about three times more potent than MMS for the induction of mutations at the TK locus. This is in agreement with the high potency of O^6-mdGuo for mispairing with thymidine and induction of base-pair mutations. Expressed relative to background, slopes for adduct formation were greater by a factor of about 10 compared to the subsequent endpoints of genotoxicity. This means that the dose required to double the background of the DNA methylations was much smaller than the dose required to double DNA breakage or mutation. This should be considered when adduct measurements are interpreted in terms of their contribution to spontaneous mutagenicity and carcinogenicity.

Department of Toxicology, University of Würzburg, Germany

410

INFLUENCE OF PROBENECID ON DNA ADDUCT FORMATION BY 1-HYDROXYMETHYLPYRENE AND 1-HYDROXYMETHYL-8-METHYLPYRENE IN THE RAT

C.Donath, A.Seidel[1] and H.R.Glatt

Some alkylated polycyclic aromatic hydrocarbons (alk-PAH) are much more abundant in the environment than the reference PAH benzo[*a*]pyrene. Two of these compounds, 1-methylpyrene and 1,8-dimethylpyrene, are converted to the benzylic alcohols 1-hydroxymethylpyrene (1-HMP) and 1-hydroxymethyl-8-methylpyrene (1-HM-8-MP), respectively, via cytochrome P450-mediated benzylic hydroxylation. They are further bioactivated by sulfotransferases (SULT) to the corresponding reactive sulfuric acid esters, which are mutagenic and form DNA adducts *in vitro* and *in vivo*. In the rat, the liver shows the highest 1-HMP sulfonation activity, which exceeds that of kidney and lung 200- and 3200-fold, respectively. Nevertheless, high DNA adduct levels were found not only in liver but also in kidney and lung of rats after application of 1-HMP or 1-HM-8-MP. These findings point to hepatic export of reactive sulfuric acid esters and their transport by circulation, accompanied by their stabilisation by binding to plasma proteins. Moreover, we suspect that the excretion of sulfuric acid esters is carried out by active renal uptake in the proximal tubule cells. To study this hypothesis, male Wistar rats received a concurrent treatment with the benzylic alcohols and probenecid, a widely used inhibitor of the excretion of organic anions, whereas control animals received the benzylic alcohol and the solvent only. DNA isolated from liver, kidney and lung was then analysed for DNA adducts by ^{32}P-postlabelling. Probenecid significantly increased DNA adduct levels in liver of rats that had received 1-HMP or 1-HM-8-MP (up to 7-fold). DNA adduct levels in kidney were decreased up to 2-fold and DNA adduct levels in lung showed a 2-fold increase with both benzylic alcohols, but these findings were not statistically significant. We conclude that probenecid mainly inhibited hepatic export of reactive sulfuric acid esters and had only a weak inhibitory effect on the renal uptake of these metabolites. Moreover, we could show that the toxicological effects of some alk-PAH can be strongly affected by interaction with other chemicals e.g. pharmaceutical drugs such as probenecid.

Research described in this abstract was supported by Philip Morris Incorporated.

German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany
[1]Biochemical Institute for Environmental Carcinogens Grosshansdorf, Germany

411

DETECTION AND CHARACTERIZATION OF DNA ADDUCTS IN RAT AND HUMAN TISSUES AFTER CONSUMPTION OF *BRASSICA* VEGETABLE

C. Baasanjav, M. Schreiner[a], R. Iori[b], H. R. Glatt

Some damaged plants may release reactive molecules as a defense against microbial and animal enemies; some of these molecules may damage DNA. Here, we present results obtained with *Brassicaceae* plants. DNA from freshly prepared Arabidopsis homogenates showed no adducts. After incubating the homogenates for 2 h, five adduct spots (S1-S5) were detected using the ^{32}P-postlabelling technique. Similar patterns of adducts were found in broccoli and cabbage homogenates. Treatment of mammalian cells or bacteria (Ames's *Salmonella* strains) with such plant extracts led to the induction of mutations and the characteristic *Brassica* adducts in these cells, as observed in the plant DNA. Volunteers ate and extensive chewed *Brassica* vegetables. High levels of *Brassica*-typical adducts were formed in buccal mucosa after consuming raw broccoli or cabbage salad; adduct spot S3 persisted for many days in the mucosa, the others decreased faster. S3 was also detected in lymphocytes of volunteers, although at low levels. Much lower DNA adduct levels were found in buccal mucosa after consumption of cooked, compared to raw broccoli. We also fed rats with broccoli (*ad libitum* along with standard rat diet). DNA adducts (primarily S3) were detected in many tissues, including liver, kidney, small and large intestine. Whereas cooking of broccoli strongly decreased the buccal DNA adducts in humans, it did not affect, or even enhanced, the formation of adducts in internal tissues of the rat. Glucosinolates are typical constituents of *Brassicaceae.* Their hydrolysis by myrosinase, present in the plant itself as well as intestinal bacteria, leads the formation of reactive molecules. Thus, we treated various individual glucosinolates with purified myrosinase in the presence of DNA. When glucobrassicin was used, adducts S1, S2 and S4 were formed. These adducts were also generated when DNA was treated with indole-3-carbinol, a decomposition product of glucobrassicin. S5 was formed by various aliphatic glucosinolates treated with myrosinase and thus may be chemically heterogeneous. We are still working on the elucidation of the chemical nature of S3, the most prominent adduct(s) formed in animal and human tissues after consumption of *Brassica.*

This work was financially supported by BioProfil Nutrigenomik (project BIO/0313053A).

German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany; [a]Institute of Vegetable and Ornamental Crops, Grossbeeren, Germany; [b]Consiglio per la Ricerca e la Sperimentazione in Agricoltore, Bologna, Italy.

412

ASSESSMENT OF BIOMARKERS IN EARLY STAGES OF COLON CANCER IN FEMALE F344 RATS

E. Eder*, E. Biskup*, J. Müller**

4-Hydroxynonenal-2'-deoxyguanosine (HNE-dGuo) propano adducts are promutagenic DNA lesions originating from free radically-induced lipid peroxidation of polyunsaturated fatty acids. These adducts are not repaired by base excision repair, only by nucleotide excision repair. We have developed a 24 months animal testing to induce chronic colitis in female F344 rats by repeated application of sodium dextran sulphate (MW 36000-50000) (DSS) via drinking water. We are interested in the effect of a vitamin E and selenium deficient diet on the early stages of the development of colon cancer. The rats were divided into 5 groups distinguished from diet and treatment: group 1 (standard chow, drinking water), group 2 (standard chow, repeated DSS treatment), group 3 (deficient diet, drinking water), group 4 (deficient diet, repeated DSS treatment) and group 5 (deficient diet, repeated DSS treatment and long-term application of acetylsalicylic acid in drinking water). DSS is administered every 6 months at a 3 percent concentration (w/v) for 5 days followed by 3 days drinking water. The treatment is repeated twice to assure epithelial irritation. ASA is constantly applied at a concentration of 20 mg/l drinking water to find out whether the constant low dose intake of ASA has an effect on the development of colon cancer. To investigate the early stages of colon cancer we determine the levels of HNE-dG in liver and colonic epithelia by ^{32}P-postlabeling. This method allows detecting DNA alterations with a high sensitivity. Additionally, KI67 as a marker for cell proliferation, apoptotic rate, distribution of beta-catenin and E-cadherin in colonic sections are assessed by IHC staining. The progress in the histological alteration of the colon is evaluated by parameters as crypt architecture and length, number of goblet cells and eosinophilic/neutrophilic infiltrate. We assess levels of prostaglandin-$F_{2\alpha}$ as an inflammatory marker and 8-iso-prostaglandin-$F_{2\alpha}$ as a marker for radically-induced oxidative stress in liver and tissues of small and large intestine via a two-column-LC/MS-MS method.

*Department of Toxicology, University of Würzburg, Germany
**Institute of Pathology, University of Würzburg, Germany

413

INDUCTION OF DNA-STRANDBREAKS AND *HPRT*-MUTATIONS BY 3-*N*-NITROSO-OXAZOLIDINONES, *N*-(2-HYDROXYETHYL)-N-NITROSOUREA AND THE WEAK MUTAGEN GLYCIDAMIDE IN V79 CELLS

S. Thielen[1], M. Baum[1], R. N. Loeppky[2], G. Eisenbrand[1]

N-Nitroso-oxazolidinones (NOZ's) can be formed from nitrosated amino acids and dietary aldehydes. Their decay leads to highly reactive electrophiles, supposed to covalently react with nucleophilic centers in DNA. Induction and disappearance of DNA-strandbreaks (SSB) was studied with the putative DNA-hydroxyethylating *N*-Nitroso-oxazolidin-2-one (NOZ-2) and with the putative carboxymethylating/methylating *N*-Nitroso-oxazolidin-5-one (NOZ-5). The hydroxyethylating *N*-(2-hydroxyethyl)-N-nitrosourea (HENU) was used as reference for hydroxyethylation. Glycidamide (GA), representative for preferentially N^7-guanine alkylating agents was used as second reference. DNA-damage was measured by the Comet-assay (±FPG), mutagenicity by the *hPRT*-forward-gene-mutation-assay in V79 cells. The DNA-repair-enzyme FPG recognizes products of N^7-G-alkylation, including ring opened and abasic sites, rendering additional SSB from such lesions. NOZ-2 achieved maximum SSB-induction already after 15' incubation (≥3µM). HENU induced SSB less potently and more slowly (300µM, 4h). Additional FPG-treatment did not increase NOZ-2- and HENU-induced SSBs. GA and NOZ-5 were only marginally active without FPG-treatment (≥300µM, GA: 4h; NOZ-5: 15', for maximum efficacy); FPG-treatment increased SSB (≥10µM, GA: 4h; NOZ-5: 15', for maximum efficacy).

Within 8h, GA- and NOZ-5 induced SSB disappeared nearly completely, while those induced by NOZ-2 and HENU were more persistent. NOZ's (3-10µM) and HENU (≥30µM) potently induced *hPRT*-mutants, whereas GA (≥800µM) was much less potent.

Conclusions: NOZ-2 and HENU are expected to induce phosphodiester-hydroxyethylation at the DNA. These phosphotriesters rapidly decompose into SSB. Additionally N^7-G-adducts and promutagenic O^6-2-hydroxyethyl-G-adducts are expected to be formed. The latter are not repaired in MGMT-deficient V79 cells. Thus, the *hPRT*-response to NOZ-2 and HENU is thought to reflect overall DNA-hydroxyethylation, including SSBs induced by phosphotriester formation. In contrast to NOZ-2, carboxymethyl-/methyl-phosphotriesters formed by NOZ-5 are not expected to spontaneously decompose into SSBs. As a consequence, base-oxygen- and N^7-G-alkylation might become prominent. Since products of N^7-G-alkylation are FPG-substrates, as evidenced by the FPG-mediated enhancement of SSBs after NOZ-5- and GA-treatment, they are supposed to be largely repaired during the course of the 5d *hPRT* expression period. Since at the concentrations tested, GA is expected to primarily alkylate N^7-G, effective repair of these lesions contributes to ensure genomic integrity and provide for an about 80-fold higher threshold of *hPRT*-mutagenicity, as compared to NOZ's and HENU.

1. University of Kaiserslautern, Department of Chemistry, D-67663 Kaiserslautern, Germany
2. University of Missouri, Department of Chemistry, Columbia, Missouri 65211, USA.

414

LOW LEVELS OF XPA PROTEIN DO NOT ACCOUNT FOR THE SENSITIVITY OF TESTIS TUMOR CELLS TO CISPLATIN AND UV IRRADIATION

B. Köberle[1,2], H. Dürk[1], S. Schmitt[1], R.D. Wood[2], B. Kaina[1]

Most testicular germ cell tumors (TGCT) are curable using cisplatin-based chemotherapy, and cell lines from TGCT are unusually sensitive to cisplatin and other DNA-damaging agents. We investigated the induction of apoptosis after cisplatin treatment and found increased apoptosis in testis tumor cell lines. Testis tumor cell lines generally have lower levels of the DNA nucleotide excision repair proteins XPA and ERCC1-XPF in comparison to cell lines from other types of tumor which may be relevant for the unusual cellular sensitivity and increased apoptosis of TGCT cell lines. We asked whether over-expression of XPA protein would affect the cellular sensitivity and DNA repair capacity of a testis tumor cell line. Over-expressing XPA protein was not correlated with increased resistance of 833K testis tumor cells to cisplatin. Further, increasing XPA levels in 833K cells did not increase their resistance to UV irradiation or enhance their rate of removal of UV photoproducts from the genome. Thus, although the amount of XPA in testis tumor cells is low, it is sufficient for proficient repair of UV induced damage and cannot explain the sensitivity of testis tumor cells towards DNA damaging agents. Currently we are investigating the effect of over-expression of ERCC1-XPF on cellular sensitivity, induction of apoptosis and repair capacity in 833K cells.

[1]Institute of Toxicology, University of Mainz, Germany
[2]Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, USA

415

INVOLVEMENT OF DNA REPAIR FACTORS IN LATE ACTIVATION OF STRESS KINASES (SAPK/JNK) BY DNA CROSS-LINKING AGENTS

J. Damrot, B. Kaina and G. Fritz

Activation of c-Jun-N-terminal kinases/stress-activated protein kinases (SAPK/JNK) is part of the cellular response to genotoxic stress, finally regulating gene expression and cell survival. The significance of specific DNA lesions for genotoxin-triggered signaling to SAPK/JNK is fairly unknown. Here, we investigated the effect of DNA cross-linking agents (i.e. UV-C light, cisplatin and transplatin) on the activation of SAPK/JNK using rodent and human cell lines impaired in DNA damage processing. The data show that both UV-C, cisplatin and transplatin efficiently induce dual phosphorylation of SAPK/JNK (Thr183/Tyr185). If used at equitoxic doses, the SAPK/JNK activating potency of the cross-linkers is different. Analyzing the effectiveness of UV-C, cisplatin and transplatin on early and late activation of SAPK/JNK in cell lines deficient for either ATM, DNA-PKcs, PARP, CSB, XPC, XPA, ERCC1 or FANC-C, we observed large cell type and agent specific differences. For example, the UV-C-stimulated late (i.e. up to 24 hours after exposure) activation of SAPK/JNK was not affected in the absence of DNA-PKcs and CSB protein, whereas lack of PARP, ATM or XPA had impact on signaling to SAPK/JNK. Yet, late signaling to SAPK/JNK stimulated by cisplatin was not changed in ATM, PARP and CSB deficient cells as compared to the wild type. Regarding transplatin, SAPK/JNK activation was affected in CSB and ERCC1 compromised cells only. The results of these still ongoing experiments will be presented and discussed. Based on the data, we suggest that the SAPK/JNK activating potency of chemically different types of DNA cross-links is different. Furthermore, DNA repair factors are important modulators of DNA damage-triggered late activation of SAPK/JNK. The significance of particular DNA repair factors for modulating signaling to SAPK/JNK appears to be dependent on the chemical nature of the DNA adduct(s) formed by a given DNA cross-linking compound.

Department of Toxicology, Johannes Gutenberg University of Mainz, Obere Zahlbacher Straße 67, D-55131 Mainz, Germany.

416

A ROLE FOR C-FOS IN THE REGULATION OF DNA REPAIR AND PROTECTION AGAINST ULTRAVIOLET LIGHT-INDUCED CELL DEATH

M. Christmann, M.T. Tomicic-Christmann, J. Origer and B. Kaina

Cells deficient in c-Fos are hypersensitive to the cytotoxic and genotoxic effects of ultraviolet (UV-C) light[(1)]. The molecular mechanism behind was enigmatic for more than ten years. Here we provide evidence that mouse embryonic fibroblasts lacking c-Fos (*fos-/-*) are defective in the repair of UV-C induced DNA lesions. c-Fos deficient cells show an increased rate of DNA strand breaks, which are intermediates of nucleotide excision repair (NER), indicating an incomplete repair. This is also shown by the fact that fos-/- cells are unable to remove cyclobutane pyrimidine dimers (CPDs) from DNA. A search for genes responsible for the hypersensitivity and the DNA repair defect of *fos-/-* cells revealed a differential expression of the endonucleases XPF and XPG, which are responsible for the excision step during NER. UV-C exposure induces a transient block to transcription and thereby abrogates the expression of *xpf* and *xpg* mRNA in both cell lines. Only in the wild-type (wt) subsequent re-synthesis of *xpf* and *xpg* mRNA occurred. In consequence, the XPF protein remained undetectable 6-18 h after irradiation in *fos-/-* cells, explaining the defect in NER, whereas it recovered quickly in the wt[(2)]. In *fos-/-* cells the impaired removal of CPDs induced apoptosis via sustained activation of JNK and long lasting induction of the *fasL*. Inhibition of early JNK activation by the JNK inhibitor SP600125 increased UV-C induced apoptosis whereas inhibition of late JNK activation in *fos-/-* cells attenuated the apoptotic response. This indicates that the immediate-early induction of the JNK/*c-fos* pathway stimulates the CPD removal by resynthesis of XPF and XPG, which protects against apoptosis. If DNA repair does not occur, the persisting transcriptional inhibition leads to a reduced expression of MPK1, sustained JNK activation and *fasL* induction and finally to activation of the death receptor pathway[(3)].

[(1)]Haas *et al.*, (1995) Carcinogenesis [(2)]Christmann *et al.*, (2006) Nucleic Acids Res. [(3)]Christmann *et al.*, (2006) Carcinogenesis

Department of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

417

IFN INDUCES CELL KILL IN PANCREATIC CANCER CELL LINES ON ITS OWN AND SENSITIZES THESE CELLS IN COMBINATION WITH IONISING RADIATION

E. Jöst[1,2], W.P. Roos[1], H. Schmidberger[2], B. Kaina[1]

Today, pancreatic cancer is one of the most lethal human cancers as patients diagnosed with this cancer show a five-year survival of less than 1%. A possible method for improving postoperative therapy is a combination of radiation therapy with type-one interferons (IFNs). To this end, we determined whether pancreatic cancer cells respond to IFN treatment, and whether IFN could increase the efficacy of ionizing radiation (IR) in these tumor cells. The possible mechanism involved in these effects should be elucidated. We used a panel of pancreatic cancer cell lines that were derived from primary ductal pancreatic adenocarcinomas, or metastases of pancreatic adenocarcinomas. Cells were treated with 300 I.U./ml of either IFN-α or IFN-β. IFN alone caused a decrease in cellular survival, ranging between 20%-70%, in eight out of the ten cell lines used, as measured by colony survival. Generally, IFN-β was more effective than IFN-α as, on average, 15% more cell kill was observed with IFN-β. The radiosensitization effect for IR and IFN combined treatment was only observed in three out of the eight cell lines, and ranged between 5% and 20% of increased cell kill compared to IR alone. Once again, IFN-β was more effective. All eight IFN responders showed an increase in the expression of the CDK4 inhibitor p15 following IFN treatment alone along with a decreased progression through S-phase. The radiosensitization effect was paralleled with a decrease in expression of the homologous recombination (HR) repair genes Rad52 and Rad54l. Collectively, the data support a therapeutic

effect of using IFN alone or in combination with IR for the treatment of pancreatic cancer. Furthermore, it points to a possible mechanism for the radiosensitizing effect of IFN, where HR repair genes are down-regulated via p15 triggered cellular functions.
[1]Department of Toxicology, University Mainz, Obere Zahlbacher Str. 67, 55131 Mainz Germany
[2]Department of Radioonkology and Radiationtherapy, Langenbeckstraße 1, 55131 Mainz

418

INVOLVEMENT OF WRN HELICASE IN THE RESISTANCE TO TOPOISOMERASE I AND II INHIBITORS

C. Gestrich, M. Christmann and B. Kaina

The Werner syndrome helicase/3'-exonuclease (WRN) is a major component of the DNA-repair and replication machinery. To analyse if the WRN helicase is involved in the repair of topoisomerase-induced DNA damage, we utilized U2-OS cells expressing WRN-specific si-RNA (*wrn-kd*) and the corresponding non-transfected cells (*wrn-wt*). Here we show that WRN is involved in the repair of topoisomerase I but not topoisomerase II-induced DNA damage. *Wrn kd* cells are hypersensitive to treatment with the topoisomerase I inhibitor topotecan, as shown by WST assay, colony formation and induction of apoptosis. Interestingly there is no cross-sensitivity to topoisomerase II inhibitors, since *wrn kd* cells show the same sensitivity to etoposide than *wrn-wt* cells. Upon etoposide treatment, both cell lines show a clear accumulation in the G2 phase. Upon topotecan treatment, no cell cycle block was observed and both cell lines entered apoptosis directly from the G1-phase, which constantly declined. The strongest difference between the two drugs was observed by measuring single and double-strand DNA breaks. Whereas etoposide induces DNA single-strand breaks at a high level, which are persisting for at least 24 h in *wrn-kd* and *wrn-wt* cells, topotecan induces single-strand breaks only transiently 6h after treatment. Those breaks are repaired after 12 h to 24 h in *wrn–wt* cells, but in *wrn-kd* cells they are converted into DNA double-strand breaks. In addition, the *wrn-kd* cells show a strong and long lasting phosphorylation of H2AX, which is an indicator for non-repaired double-strand breaks. Our data suggests that WRN is not involved in the repair of double-strand breaks as they arise upon treatment with topoisomerase II inhibitors, but plays a role in the procession of double-strand breaks that arise from DNA repair-mediated resolution of stalled replication forks upon topoisomerase I inhibiton.

Department of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

419

TOPOTECAN-DRIVEN DEGRADATION OF TOPOISOMERASE I IS P53-DEPENDENT AFFECTING CELL SURVIVAL: ROLE OF GAMMA-H2AX-PHOSPHORYLATION, CASPASE ACTIVATION AND SURVIVING

Maja T. Tomicic, Markus Christmann, Bernd Kaina

The anticancer drug topotecan (TPT), widely used in the therapy of melanoma, ovarian, and colorectal carcinoma, and glioblastoma, belongs to the class of topoisomerase I (topo I) inhibitors. In the presence of TPT, topo I cleaves the DNA but is unable to religate the single-strand breaks, leading to stabilization of topo I-DNA bound complexes (irreversible cleavable complexes) and accumulation of topo I-linked DNA strand breaks that may interfere with DNA replication. The molecular mechanism of controlling the repair of topo I-DNA covalent complexes and its impact on sensitivity of cells to TPT is largely unknown. Here we used mouse embryonic fibroblasts (MEFs) wild-type for p53 (wt) and deficient in p53 (p53 -/-) in order to elucidate the role of p53 in TPT-induced cell death. We show that the p53 -/- cells are significantly more sensitive to TPT than the wt cells, displaying a higher frequency of TPT-induced DNA strand breaks and apoptosis. Treatment of wt cells with pifithrin-alpha, an inhibitor of the *trans*-activating activity of p53, caused reversal of the phenotype, making wt cells more sensitive to TPT. Upon TPT exposure, topo I was degraded in wt but not in p53 -/- cells. Topo I degradation was attenuated by the proteosomal inhibitor MG132. Similar data were obtained with human glioblastoma cell lines. U138 (p53 mutated) and U87sip53 (p53 knock-down) cells were significantly more sensitive to TPT than U87 (p53 wt) cells showing no topo I degradation. The data suggest that p53 causes resistance of cells to topo I inhibitors due to stimulation of TPT-triggered topo I degradation, which may impact TPT-based cancer therapy. A strong difference in TPT-induced apoptosis between p53 wt and p53 -/- cells can furthermore be explained by much higher induction of double-strand breaks (gamma-H2AX phosphorylation) in p53 -/- cells and, interestingly, by inhibition of the active caspase-3 by surviving in wt cells.

Department of Toxicology, University of Mainz, D-55131 Mainz, Germany

420

PROTECTION OF HAEMATOPOIETIC STEM CELLS (HSC) AGAINST HIGH-DOSE CHEMOTHERAPY BY VIRTUE OF THE O^6-METHYLGUANINE-DNA-METHYLTRANSFERASE (MGMT) REPAIR ENZYME

N. Cabezas-Wallscheid[1], L. Eshkind[1], C. Antunes[1], S. Schmitt[2], R. Heck[1], G. Nagel[1], S. Ohngemach[1], B. Kaina[1] & E. Bockamp[1]*

Strategies that confer chemoprotection to haematopoietic stem and progenitor cells have two important future therapeutic applications. First, clinical anti-cancer chemotherapy is often limited by severe and lethal haemato-toxicities, affecting HSC and early progenitors. In many clinical settings, this toxicity precludes the use of effective high-dose chemotherapeutic treatment against malignant tumour cells. Second, in the context of genetic diseases, overexpression of a chemoresistance gene like MGMT in stem and progenitor cells can be used for the enrichment of small numbers of genetically modified stem cells, which will subsequently produce sufficient numbers of differentiated therapeutic cells *in vivo*. To evaluate the protective potential of DNA-repair enzymes in stem cells and, in addition, to also provide a source of stem cells, protected against clinically relevant alkylating and chloralkylating anti-cancer drugs, we will make use of the MGMT DNA-repair mouse model. In this model a mutated O^6-methylguanine-DNA-methyltransferase (MGMT) repair gene will be conditionally expressed in HSC. In this respect, we have already generated a tet on/off SCL knock-in mouse (Bockamp et al. 2006) which can be used as a driver for conditionally "switching" the expression of therapeutic genes like the MGMT P140K in HSC.

* Bockamp, E., Antunes, C., Maringer, M., Heck, R., Presser, K., Beilke, S., Ohngemach, S., Alt, R., Cross, M., Sprengel, R., Hartwig, U., Kaina, B., Schmitt, S. and Eshkind L. (2006): Tetracycline-controlled transgenic targeting from the SCL locus directs conditional expression to erythrocytes, megakaryocytes, granulocytes and c-kit expressing lineage negative hematopoietic cells
Blood 108,1533-1541
[1] Institute of Toxicology, Johannes Gutenberg-Universität Mainz, Germany
[2] FACS and Array Core Facility, Johannes Gutenberg-Universität Mainz, Germany

421

COMPARING MGMT ACTIVITY AND PROMOTER METHYLATION IN GLIOBLASTOMAS AS PREDICTIVE TOOLS

G. Nagel[1], D. Wiewrodt[2], A. Perneczky[2], C. Sommer[3], M. Christmann[1] and B. Kaina[1]

The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) removes methyl groups from the O^6-position of guanine to itself and thereby gets inactivated. If not repaired, O^6-methylguanine leads to the induction of mutations, chromosomal breakage and cell death. Tumour therapy with alkylating agents such as temozolomide, ACNU or CCNU utilizes the cytotoxic properties of O^6-guanine alkyl adducts to kill cancer cells. The therapeutic effect of these alkylating drugs is critically dependent on the amount of active MGMT molecules present in the tumour cell. To determine, whether MGMT promoter methylation or MGMT activity is a predictor for the therapeutic outcome of glioblastoma tumour therapy with alkylating drugs, we determined the MGMT promoter methylation (n=36) and measured the MGMT activity in 37 primary glioblastoma tumours. The data revealed that there is no correlation between MGMT promoter methylation and MGMT activity in the tumour tissue. We found, however, a correlation between MGMT activity in the primary tumour and the patient's survival after therapy with alkylating agents: Patients with very low MGMT activity in primary tumour survived better after therapy with temozolomide/CCNU/ACNU than patients expressing high MGMT levels in the tumour. Determination of MGMT activity is therefore a predictive method for the therapeutic response of glioblastoma patients treated with alkylating agents.

[1]Department of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany; [2]Department of Neurosurgery, University of Mainz, Langenbeckstr. 1, D-55131 Mainz, Germany; [3]Department of Neuropathology, University of Mainz, Langenbeckstr. 1, D-55131 Mainz, Germany

422

DISSECTING THE ROLES OF PARP-1, PARP-2 AND PARG IN MODULATING THE CELL DEATH SIGNAL POLY(ADP-RIBOSE)

C. Blenn *, O. Cohausz *, M. Malanga, F. R. Althaus

Poly(ADP-ribose) (PAR) has been identified as a DNA damage-inducible cell death signal upstream of AIF (apoptosis inducing factor). PAR causes the translocation of AIF from mitochondria to the nucleus and triggers a caspase-indepent cell death (Andrabi *et al.*, PNAS, 2006, 103(48), 18308-13). In living cells PAR varies in size from short dimers to long branched polymers. Using RNA interference, we determined the roles of poly(ADP-ribose)polymerases-1 and -2 (PARP-1, PARP-2) and of poly(ADP-ribose)glycohydrolase (PARG), the key enzymes configuring PAR molecules following DNA damage. We found that PARP-1 is exclusively responsible for generating the PAR signal that causes AIF translocation and triggers apoptotic cell death. Moreover, abrogating PARP-1 did not have any effect on signals upstream of AIF.

Institute of Pharmacology and Toxicology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich
* These two authors contributed equally to this work.

423

ASSESSMENT OF CELLULAR POLY(ADP-RIBOSYL)ATION IN PERMEABILISED AND IN INTACT CELLS BY FLOW CYTOMETRY

A. Kunzmann [1], M.Schmitz [1], D. Liu [1], K. Annett [2], A. Bürkle [1]

Poly(ADP-ribosyl)ation is a reversible posttranslational modification of nuclear proteins and represents an early cellular response to DNA damage generated by endogenous and exogenous damaging agents. The synthesis of poly(ADP-ribose) chains covalently attached to target ("acceptor") proteins is catalysed by poly(ADP-ribose)polymerases (PARPs), using NAD^+ as substrate. Acceptor proteins included DNA repair enzymes, histones and mostly PARP itself. The best known member of this family of enzymes is PARP-1, which is responsible for over 90% of the poly(ADP-ribosyl)ation capacity in cells. Poly(ADP-ribosyl)ation is involved in several cellular processes including DNA repair and maintenance of genomic stability, and thus possess a cytoprotective, physiological function. But due to the fact that overactivation of PARP-1 can deplete the NAD^+- and ATP-pools, causing major energy deficiency and thus cell death, poly(ADP-ribosyl)ation takes also part in pathophysiological events. The measurement of cellular poly(ADP-ribosyl)ation capacity is therefore relevant for research addressing a large variety of scientific questions. We recently established a robust, FACS-based immuno assay for the detection of maximal poly(ADP-ribosyl)ation capacity in permeabilised cells. The applicability of this new method was confirmed by the determination of the IC_{50} value of the classical PARP inhibitor 3-aminobenzamide. We further developed an FACS-based immuno assay for the detection of poly(ADP-ribosyl)ation in intact cells. Using this assay we are able to closely monitor the dose and time-dependent cellular response of polymer formation after irradiation with high resolution. Both of these newly developed methods will be very useful for the comparison of PARP-1 activity of large sets of samples and will enable further studies on the role of poly(ADP-ribosyl)ation in the maintenance of genomic stability in cells.

[1] Molecular Toxicology Group, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany.
[2] Cancer and Ageing Research Group, School of Biomedical Sciences, University of Ulster, Coleraine, Northern Ireland, UK.

424

THE SPECIFIC INTERACTION OF POLY(ADP-RIBOSE) AND BINDING PROTEINS DEPENDS ON CHAIN LENGTH

J. Fahrer (1), M. Altmeyer (1), R. Kranaster (2), S. Beneke (1), A. Marx (2) and A. Bürkle (1)

Poly(ADP-ribosyl)ation is a DNA damage driven modification of proteins catalyzed by the family of poly(ADP-ribose) polymerases [PARPs]. PARP-1 is the founding member and its activity is triggered by DNA strand breaks resulting in the synthesis of linear and branched chains of poly(ADP-ribose) [PAR]. This biopolymer also interacts non-covalently with a plethora of DNA repair and cell cycle checkpoint proteins. Binding is mediated by a sequence motif comprising 20 amino acids. Aim of our project is to analyze the interplay of PAR and specific binding partners like the tumor suppressor protein p53 as a function of chain length. PARP-1, the xeroderma pigmentosum-A [XPA] protein and p53 were overexpressed using the baculovirus

system and purified to homogeneity. PAR was produced *in vitro* and end-labeled using the carbonyl-reactive linker biocytin hydrazide. ELISA experiments with neutravidin coated plates confirmed the successful covalent modification with biotin. Following separation of PAR by high resolution HPLC the collected fractions were characterized on modified sequencing gels revealing discrete ADP-ribose chains from 6-60 ADP-ribose units. Subsequently, selected fractions were purified by avidin affinity chromatography and used for PAR-protein interaction studies in solution. p53 promoted complex formation with long ADP-ribose chains (55-mer) in a concentration dependent manner ($K_{D\text{-}p53}$= $1.31x10^{-7}$M) as monitored by electrophoretic mobility shift assay [EMSA]. Interestingly, we observed that p53 induces the formation of at least three specific complexes with long PAR. p53 was also able to bind short PAR chains (15mer, $K_{D\text{-}p53}$= $2.46x10^{-7}$M), yet forming only one discrete complex. By contrast, XPA did not specifically interact with short polymer, but was shown to produce a complex with long ADP-ribose chains (55mer) in a concentration dependent manner ($K_{D\text{-}XPA}$= $3.21x10^{-7}$M). Additionally, we succeeded in immobilizing fractionated PAR on a microarray platform providing a useful tool to corroborate the data obtained from the EMSA experiments.

(1) Lehrstuhl für Molekulare Toxikologie, University of Konstanz, Germany
(2) Lehrstuhl für Organische Chemie/ Zelluläre Chemie, University of Konstanz, Germany

425

ESTABLISHMENT OF A KNOCK-IN MOUSE MODEL WITH EXPRESSION OF HUMAN POLY(ADP-RIBOSE) POLYMERASE-1 (HPARP-1)

A. Mangerich [1], O. Popp [1], J. Diefenbach [1], U. Kloz [2], F. van der Hoeven [2], and A. Bürkle [1]

As a caretaker of genomic integrity, poly(ADP-ribose) polymerase-1 (PARP-1) modifies various nuclear proteins with the biopolymer poly(ADP-ribose) upon DNA damage, leading to the activation of cellular DNA repair. We have shown previously that cellular poly(ADP-ribosyl)ation capacity correlates with genomic integrity and mammalian longevity. On the basis of the DNA damage theory of cancer and aging, we postulate a potential causal relationship between poly(ADP-ribosyl)ation capacity, DNA repair capacity, and longevity. Therefore, enhanced cellular poly(ADP-ribosyl)ation capacity may result in improved genomic integrity, and hence, in a lower cancer frequency, and a prolonged lifespan. Several in vitro studies supported this hypothesis, but a corresponding in vivo model remains to be established. Because the poly(ADP-ribosyl)ation capacity of human PARP-1 is significantly higher than that of the rodent enzyme, *hPARP-1* knock-in (ki) mice represent a suitable model to study the effects of increased PARP-1 activity without gene overexpression on an organismal level. For the generation of such mice, the endogenous murine *Parp-1* coding sequence was replaced with the human orthologous sequence by gene targeting in embryonic stem (ES) cells. The targeting construct comprised the entire *hPARP-1* sequence (46 kb) flanked by the murine regulatory sequences. Upon transfection of ES cells, we identified three ES cell clones with full-length sequence replacement and hPARP-1 protein expression. These *hPARP-1* ki ES cells were used for the generation of *hPARP-1* ki mice. Consistent with our working hypothesis heterozygous *hPARP-1* ki mice have so far (4 months of age) developed normally without any sign of pathology. Currently we are breeding the mice in order to obtain a fully homozygous *hPARP-1* ki mouse line on a C57BL/6 background. As a next step, it will be interesting to assess the biochemical and biological phenotype of cells derived from such mice under conditions of genotoxic stress.

[1] Molecular Toxicology, Department of Biology, University of Konstanz, Germany
[2] Transgenic Core Facility, German Cancer Research Center (DKFZ), Heidelberg, Germany

426

GENERATION OF TRANSGENIC MICE WITH T LYMPHOCYTE SPECIFIC EXPRESSION OF THE HUMAN PARP-1 PROTEIN

T. Eltze [1], A. Buerkle [1], U. Kloz [2]

Poly(ADP-ribosyl)ation is an immediate cellular response to DNA strand breaks mostly being catalysed by poly(ADP-ribose) polymerase (PARP-1). Poly(ADP-ribosy)lation is a key regulator of genomic stability under conditions of genotoxic stress where PARP-1 plays a major role in DNA repair, transcription regulation and the recovery of cells after DNA damage. A positive correlation of cellular poly(ADP-ribosyl)ation capacity and life span has been demonstrated in leukocytes of 13 mammalian species, whereby the longest-lived species studied (man) displayed 5-fold the level of maximal PARP activity of the shortest-lived (rat), while PARP-1 protein levels in the species were identical, providing support for the notion that longevity is linked with a high poly(ADP-ribosyl)ation capacity. We aim to generate transgenic mice with a T-cell specific over-expression of the human PARP-1 protein, which is motivated by the fact that the immune system interacts with every tissue and organ in the body and is profoundly affected by ageing. Generation of tg mice by DNA microinjection carrying an expression cassette for human PARP-1 under the control of a strong promoter, yet "locked" by a sequence that can be eliminated in vivo by expression of cre-recombinase as a result of crossing with appropriate tg mice expressing cre in a T-cell specific fashion. Analysis of huPARP-1 expression will be performed with species-specific anti-huPARP-1 antibodies. DNA microinjection into pronuclei of fertilized mouse oocytes was performed in collaboration with Deutsches Krebsforschungszentrum in Heidelberg. The expression of huPARP-1 protein, analysed with species-specific anti-huPARP-1 antibodies by immunofluorescence technique after co-transfection of a rodent cell line with a cre recombinase expression vector and the expression cassette for human PARP-1, was successful. We expect to observe a higher poly(ADP-ribosyl)ation capacity which should lead to a delayed accumulation of DNA damages and have positive impacts on the age-related decline of immunosenescence and host defence responses.

[1] Molecular Toxicology, University of Konstanz
[2] Deutsches Krebsforschungszentrum (DKFZ), Heidelberg

427

PARP-1 ACTIVITY AND EXPRESSION IN LONG TERM HUMAN LUNG CELL CULTURE

M. H. Ahmad, A. Torky, H. Foth

Poly(ADP-ribosyl)ation plays an important role in modulating the cellular response to stress. The extent of poly(ADP-ribosyl)ation, mainly via the activation of the Poly(ADP-ribose)polymerase-1 (PARP-1), correlated to the severity of genotoxic stress and this determines the cellular response. Therefore, this study was directed to examine the PARP-1 activity and expression in normal human bronchial epithelial cells (NHBEC) grown directly from normal human lung tissue after exposure to variable stress factors. We have examined the PARP activity in primary human lung cells under heavy metal stress and prolonged cultivation. Bronchial epithelial cells and peripheral lung cells from lung cancer patients were grown as explant cultures (3-4 weeks) and were seeded on glass coverslids. Induction of DNA damage by H2O2 (100µM), as a stimulating factor for PARP-1 activity, for 5 min in NHBEC led to formation of ADP-ribose polymers which were detected immunohistochemically by means of fluorescence detection. PARP-1 expression was proved by Western Blotting in normal human bronchial epithelial cells (NHBEC) and tumor lung cell A549. Copper sulfate (0.05mM- 24h) alone did not trigger PARP-1 activity in NHBEC but decreased PARP-1 activity induced by H_2O_2 (100µM). Similarly mercury(II)chloride (0.03 mM-24h) exerted no effect on PARP-1 activity and decreased the activity of PARP-1 upon co-incubation with H_2O_2 (0.1mM).

The inter-individual variation in PARP activity in the first explant (in 7 independent experiments) was up to 30 %. We have analyzed PARP-1 activity in progressive generations and passages of bronchial and peripheral lung cells cultivated for 10 weeks to examine the effect of prolonged cultivation of lung cells. In the higher passages and generations H_2O_2-induced PARP activity decreased, this reflects an adaptive response of cells to cultivation. The expression of PARP-1 was almost the same in the different generations of the same patient. This means that PARP-1 expression is not affected by prolonged cultivation and that it is a stable protein.

This study is supported by the DFG Graduate College GRK 416 (MH Ahmad)

Institute of Environmental Toxicology, Martin-Luther-University Halle, Germany

428

INFLUENCE OF OXIDATIVE STRESS CAUSED BY SOLAR RADIATION AND UVA ON THE REPAIR OF OXIDATIVE BASE MODIFICATIONS IN MELANOMA CELLS AND PRIMARY SKIN FIBROBLASTS

W. Eiberger[1] and B. Epe[1]

Irradiation of mammalian cells with solar light generates oxidative DNA modifications such as 7,8-dihydro-8-oxoguanine (8-oxoG) in addition to pyrimidine dimers. Under normal conditions, the oxidative modifications are rapidly repaired by base excision repair, which in the case of 8-oxoG is initiated by the repair glycosylase Ogg1. However, the cellular oxidative stress caused by the irradiation might well affect the DNA repair. To test for such an effect, we exposed various types of melanoma cells and normal fibroblasts of the same patients to radiation from a solar simulator or UVA and compared the repair rates of the induced oxidative base modifications with and without a preceding depletion of glutathione by BSO (DL-buthionine-[S,R]-sulfoximine). In all cells tested, the impaired antioxidant defence in the glutathione-depleted cells resulted in reduced repair rates, as determined in an alkaline elution assay in combination with Fpg protein as a probe for oxidative purine modifications. Furthermore, the hOGG1 glycosylase activity of the various melanoma cells after pre-treatment with BSO and irradiation with visible light was found to be significantly decreased. Glutathione depletion did not increase the extent of the oxidative DNA damage generated by solar radiation or UVA, neither in the various melanoma cells nor in the corresponding skin fibroblasts. Interestingly, the susceptibility of fibroblasts to DNA damage induction by UVA - but not by visible light - was significantly higher than that of melanoma cells. We conclude that oxidative stress is not only a source of oxidative DNA damage, but can also impair the processing of the DNA modifications.

[1] Institute of Pharmacy, University of Mainz, 55099 Mainz, Germany

429

BASAL LEVELS OF 8-HYDROXYGUANINE IN THE MITOCHONDRIAL DNA OF REPAIR-DEFICIENT (*OGG1*$^{-/-}$/*CSB*$^{-/-}$) MICE

C. Trapp[1], A.K. McCullough[2] and B. Epe[1]

The mitochondrial DNA (mtDNA) is assumed to be highly prone to damage by reactive oxygen species (ROS) because of its location in close proximity to the mitochondrial electron transport chain. Accordingly, mitochondrial oxidative DNA damage has been linked to various neurological diseases, ageing and cancer. Since 7,8-dihydro-8-oxoguanine (8-oxoG), one of the most frequent oxidative base modifications, is removed from the mitochondrial genome mostly or exclusively by the glycosylase OGG1, the basal levels of this lesion are expected to be highly elevated in *Ogg1*$^{-/-}$ mice. We have used a relaxation assay in combination with various repair enzymes (Fpg, MutY, endonuclease III, endonuclease IV) to determine the numbers of oxidative modifications in intact (supercoiled) mtDNA from the livers of mice deficient in OGG1 and/or the Cockayne syndrome B (CSB) protein and wild-type comparisons aged up to 23 months. The levels of all types of oxidative modifications were found to be lower than 12 per million base pairs, and the difference between wild-type and repair-deficient (*Ogg1*$^{-/-}$/*Csb*$^{-/-}$) mice remained below the limit of detection. Thus, the increase of 8-oxoG caused by the repair deficiency in intact mtDNA is not much higher than in the nuclear DNA, i.e. not more than a few modifications per million base pairs. We conclude that a selection against oxidatively damaged mtDNA during replication could prevent a higher accumulation of the modified bases even in the absence of repair.

[1] Institute of Pharmacy, University of Mainz, 55099 Mainz, Germany
[2] CROET/Mol. Med. Genetics, Oregon Health & Science University, Portland, OR97239, USA

430

NANOPARTICLE-INDUCED INFLAMMATION AND ITS EFFECT ON THE BASE EXCISION REPAIR PATHWAY IN RAT LUNGS

A. Wessels[1], C. Albrecht[1], A.M. Knaapen[2], R. Duffin[1], D. van Berlo[1], P. Voss[1], K. Unfried[1], R.P.F. Schins[1]

Exposure to combustion-derived nanoparticles is associated with an increased risk of lung cancer. These particles, as well as constituents adsorbed onto their large surface area such as transition metals and polycyclic hydrocarbons, have the properties to generate reactive oxygen species (ROS). Since particles can elicit inflammation, one has to discriminate between primary (particle-driven) and secondary (inflammation-driven) formation of ROS. These oxidants can induce DNA lesions such as 8-hydroxydeoxyguanosine (8-OHdG), which lead to G-to-T transversions. To prevent such mutagenic effects, cells are equipped with a specific DNA repair pathway, i.e. base excision repair (BER). To date, only few studies have described effects of particles on

DNA repair. To investigate the possible role of BER response after particle exposure we used bronchoalveolar lavage (BAL) and lung homogenates from rats, that were intra-tracheally instilled (4 weekly treatments) with toluene-extracted 14 nm carbon black (CB) and CB which had been reconstituted with benzo[a]pyrene (30 mg/g) with or without iron-sulphate (2.8 mM). We measured pulmonary inflammation by influx and activation of neutrophils in BAL fluid. Oxidative DNA damage was assessed by measuring 8-OHdG using HPLC/ECD and immunohistochemistry (IHC). The expression patterns of the BER-enzymes OGG1, APE1, XRCC1 and Pol β were measured by Western Blot analysis and IHC. All particle treatments caused a marked and persistent neutrophilic inflammation; however the observed inflammatory responses as induced by the different particle preparations did not enhance the level of 8-OHdG. Regarding these results, we found by Western Blot analysis, that the expression patterns of the BER-enzymes are significantly increased only in the particle treated animals. These findings were supported by IHC analysis. Currently, lung tissues are analysed by quantitative RT-PCR to determine whether mRNA expression of DNA repair enzymes is increased after nanoparticle treatment. Our data suggest that inhaled nanoparticles and/or their inflammatory effects influence the expression of BER-enzymes in the respiratory tract (Supported by DFG SFB-503).

[1]Institut für umweltmedizinische Forschung, Düsseldorf, Germany; [2]Maastricht University, The Netherlands

431

S-ADENOSYL-L-METHIONINE REDUCES ARSENITE-INDUCED GENOTOXICITY

U.Lakner[1], A.Brink[1] and H.Stopper[1]

Inorganic salts of arsenic are naturally occurring compounds considered to be human carcinogens. Chronic exposure of individuals to arsenic via drinking water has been associated with increased risk of skin, lung, liver, and prostate cancers. The mechanism of arsenic carcinogenesis is poorly understood. Arsenic is metabolized to methylated species by a methyl-transferase, using S-adenosyl-L-methionine (SAM) as a methyl donor. Because DNA methyltransferases also require SAM as a methyl donor, it has been proposed that carcinogenesis by arsenic could be mediated by interference with a common set of pathways affected by DNA methylation. These pathways encompass DNA damage/repair, cell cycle, and differentiation and have the potential to generate mutation and alter gene expression. We could show that the addition of SAM to sodium arsenite ($NaAsO_2$) reduced the DNA damage analysed by micronucleus test in three different cell lines (HL60, HeLa, and L5178Y) compared to treatment with $NaAsO_2$ alone. To understand the mechanism of arsenite-induced DNA damage and of the protective effect of SAM, we analysed kinetochore proteins, centrosomes and spindle pole formation by immunofluorescent staining. Results showed that the addition of SAM to arsenite-treated cells reduced the number of micronuclei containing whole chromosomes (kinetochore positive) and decreased the frequency of cells showing an abnormal number of three spindle poles. Flow cytometry and mass spectrometry analysis of methylated cytosine were used to investigate whether the observed effects may be related to the DNA methylation status. Arsenite-treated cells showed a significant DNA-hypomethylation which was prevented by addition of SAM. Furthermore, we could show that the frequency of mutant cell clones in the mouse lymphoma L5178Y $TK^{+/-}$ assay was elevated in cells treated with arsenite compared to cells treated with arsenite and SAM. Overall, our results support the hypothesis that arsenite-induced genotoxicity is mediated by methyl-group deprivation.

[1]Institute of Toxicology, University of Würzburg, Germany

432

GENOTOXICITY STUDIES WITH DISPERSE ORANGE 3 AND MIGRATES FROM COLORED TEXTILES

D. Fieblinger[1], T. Platzek[1], E. Heine[2]

In previous investigations it was shown that certain azo dyes for cosmetics and textiles are a possible source of exposure of consumers with aromatic amines. Some of these amines have an allergenic potential and some others are suspected to be mutagenic and / or carcinogenic. The azo dye disperse orange 3 (DO 3) is a well known contact allergen. In this study the migration of DO 3 from colored polyamide, polyester and acetate textiles and possible genotoxic effects of the migrates were investigated. This azo dye can be split into the amines p-phenylene diamine and 4-nitroaniline. These amines were described as genotoxic. The genotoxic potential of DO 3 was investigated in *S. typhimurium* TA 98, TA 100, TA 1537 and TA 1538 strains. In the plate incorporation test without an external metabolic activation system (S9) but also with Hamster-S9 a clear dose related mutagenic effect was observed. The data for two strains are compiled in the table.

Table 1: Mutagenic potential of DO 3 in S. typhimurium

Substance	Concentration		TA 98		TA 1538	
	[µg/plate]	[µmol/plate]	- S9-Mix	+ S9-Mix	- S9-Mix	+ S9-Mix
Solvent control (DMSO)			1.0	1.0	1,0	1.0
DO 3	2.5	0.0008	1.3	1.9	3.2	1.3
	6.4	0.004	1.6	2.8	6.4	2.9
	16	0.02	2.8	5.5	14.4	6.3
	40	0.10	5.1	11.5	36.4	12.7
	100	0.52	9.6	22.3	69.0	26.5
	250	2.60	21.6	31.1	131.2	48.9
Positive control *			14.8	32.3	65.6	17.2

* 3.16 µg/plate 2-Nitrofluorene (without S9-Mix) and 2 µg/plate 2-Aminoanthracen (with S9-Mix) were used as positive controls

Coloring of standard fabrics with DO 3 was performed in the German Wool Research Institute at the Aachen University of Technology. The colored fabrics were characterized by their color content and fastness. These textiles were subjected to different methods of dye release / migration. The released amounts of DO 3 depend on the extraction method, on the textile substrate and the color content with values from 0.9 to up to 210 µg/cm².

Some extracts of textiles, which were colored with DO 3, showed a clear mutagenic potential. There is concern regarding the use of DO 3 in consumer products considering its mutagenic and allergenic potential.

[1] Federal Institute for Risk Assessment, Berlin, Germany
[2] German Wool Research Institute at the Aachen University of Technology, Aachen, Germany

433

GENERATION OF REACTIVE OXYGEN SPECIES MAY PARTICIPATE IN THE CLASTOGENIC AND MUTAGENIC ACTIVITY OF TRANS-β-NITROSTYRENE

C. Ziemann[1], H. Rahmer[1], G. Leyhausen[2], H.J. Steinfelder[3], J. Volk[2]

β-Nitrostyrene (β-NS) is used as chain terminator in styrene type polymerization reactions for plastics, rubber, and resins production. Additionally, β-NS possesses antifungal, antibacterial, and insecticidal activities and displays a promising anti-tumour potential. Unfortunately, there exist only scarce, inconsistent data on genotoxicity. By using the comet assay, we already demonstrated strong clastogenic potential of β-NS (5-25µM) in different cell types, which requires the nitro group and the conjugated C=C bond. In the present study, β-NS, without metabolic activation (S9-mix), induced chromosomal aberrations (breaks and exchanges) in V79 cells and concentration-dependently enhanced mutant frequencies in the mouse lymphoma *tk* assay, with a significant increase at 7.5µM. Interestingly, in all genotoxicity assays, DNA-damage was significantly lower in the presence of S9-mix. Due to induction of DNA-double strand breaks, whose formation was prevented by aclarubicin (inhibitor of topoisomerase-II-binding to DNA), we have previously postulated that topoisomerase-II inhibition might be one mechanism of β-NS-mediated genotoxicity. This was supported by the fact that clastogenicity was strongly dependent on cell proliferation. As the antioxidant N-acetylcysteine was shown to decrease the pro-apoptotic effect of β-NS, we further speculated whether generation of reactive oxygen species (ROS) might also participate in β-NS-mediated DNA-damage. In the present study, β-NS indeed concentration-dependently induced ROS generation in L5178YTK$^{+/-}$ mouse-lymphoma cells (assessed by 2´,7´-dichlorodihydrofluorescein diacetate fluorescence) and mediated reduction in intracellular glutathione (measured by monobromobimane assay). Both effects were efficiently counteracted by *N*-acetylcysteine. Interestingly, *N*-acetylcysteine and reduced glutathione also inhibited DNA-damage, whereas pre-incubation of cells for 24h with L-buthionine-[S,R]-sulfoximine, which depletes intracellular glutathione, significantly increased the clastogenic potential of β-NS. Comet assays with human 8-hydroxyguanine DNA-glycosylase 1 are now necessary to further investigate hypothesised oxidative DNA-damage due to β-NS treatment. In conclusion, β-NS exhibits clastogenic and mutagenic potential *in vitro*, which might be in part due to ROS generation, a mechanism also known for nitroaromatic compounds.

[1]Fraunhofer ITEM and [2]Medical University of Hannover, Department of Conservative Dentistry and Periodontology Hannover Germany,[3]Dept. of Molecular Pharmacology, University of Göttingen, Göttingen, Germany.

434

MOLECULAR DESCRIPTORS FOR NON-SPECIFIC CHROMOSOMAL GENOTOXICITY BASED ON HYDROPHOBIC INTERACTIONS

S.B. Dorn*, G.H. Degen*, H.M. Bolt*, J.P.M. Lommerse♦

A concept relating compound lipophilicity and genotoxicity on a chromosomal level has been put forward by Schultz and Önfelt (Chem. Biol. Interact. 126:97-123, 2000). Aneuploidy in Chinese hamster V79 cells was elicited by lipophilic chemicals at concentrations related to their hydrophobicity (Log P), whereas toxicants with a specific mode of action acted at concentrations consistently lower than predicted based on their lipophilicity. We have now combined available data sets on aneuploidy/micronucleus formation with procedures used in QSAR modelling, in order to generate molecular descriptors for modelling non-specific chromosomal genotoxicity, and to optimise combinations thereof. Molecular structures of 26 chemicals were converted into single 3D models using Corina (version 3.20), and 11 descriptors of molecular properties were calculated. The data of 16 compounds, assigned to a non-specific mode of action, were imported into the QSAR module of the software package Cerius2 (version 4.10). Applying genetic function approximation (GFA), linear equations were set up relating molecular descriptors with experimental concentrations (LogC) at which doubling of micronuclei occurred in V79 cells. The number of variables (molecular descriptors) was limited to a maximum of three, and linear and quadratic terms were allowed. Based on the descriptions provided by the GFA procedure, logP was the most suitable *single* property to describe non-specific genotoxicity [r^2=0.88], confirming the original concept of Schultz and Önfelt. Using more descriptors (up to three in combination) resulted in improved correlations up to r^2=0.97. Such improved correlation coefficients were obtained by combinations (a) of the numbers of hydrogen bond acceptors, the polar surface and total surface areas of molecules on one hand, and by (b) the dipole moment, polar surface and total surface descriptors on the other hand. In essence, the relation of polar surface to the total molecular surface appears pivotal to determine the non-specific chromosomal genotoxicity of lipophilic compounds. Further validation will be carried out to support our findings.

*Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystr. 67, 44139 Dortmund, Germany
♦ Organon, Department of Molecular Design & Informatics, Oss, The Netherlands

435

ROLE OF CONNEXIN26 IN PHENOBARBITAL-MEDIATED TUMOUR PROMOTION OF MICE

P. Marx-Stoelting[1], S. Schreiber[1], J. Mahr[1], T. Ott[2], K. Willecke[3], A. Buchmann[1] and M. Schwarz[1]

Connexin (Cx) 26 and 32 are the major gap junction proteins in the liver. Like other connexins, they play an important role in tumour promotion and progression. In previous studies we could show that Cx32 is essential for phenobarbital (PB)-mediated tumour promotion in mouse liver. To investigate whether Cx26 plays a similar role, we injected Cx26-deficient mice and control mice with a single dose of N-nitrosodiethylamine (DEN 90µg/g) and subsequently kept one group on a diet containing 0,05% PB for 35 weeks, while a control group was kept on a PB-free diet. At the end of the experiment, the carcinogenic response in the liver of the animals was monitored. Mice from PB-treatment groups showed a strongly increased liver weight compared to mice treated with DEN alone, which was mostly due to a much higher tumour burden. Relative liver weights of PB-treated Cx26 knockout mice were slightly smaller than those of the respective wildtype mice. The tumour response in PB-treated mice of both strains was very similar, but the number of larger tumours was lower in PB-treated knockout mice. Cx26 knockout mice treated with DEN alone did not show an increase in tumour-prevalence when compared to Cx26 wildtype mice nor were there significant differences in terms of tumour-size between strains, which is in accordance with earlier findings. Our present data show that elimination of Cx26 has only minor effects on chemically induced mouse hepatocarcinogenesis,

in contrast to effects seen in Cx32 knockout mice, which demonstrate strongly increased susceptibility for DEN-induced hepatocarcinogenesis and a lack of response to PB-mediated tumor promotion. Since the major structural difference between Cx26 and Cx32 lies in a missing cytosolic C-terminal domain in Cx26, this may play an important role in PB-mediated signal transduction.

[1]Institute of Pharmacology and Toxicology, Department of Toxicology, [2]Institute of Human Genetics, University of Tübingen, [3]Institute of Genetics, University of Bonn

436

PROTEOME ANALYSIS OF CHEMICALLY-INDUCED MOUSE LIVER TUMORS WITH DIFFERENT GENOTYPE

J. Strathmann[1], K. Paal[2], C. Ittrich[3], E. Krause[4], K.E. Appel[2], H.P. Glauert[5], A. Buchmann[1], M. Schwarz[1]

Mouse liver tumors often show mutations in *Ha-ras*, *B-raf*, or *Ctnnb1* (encoding β-Catenin). A previous microarray analysis has demonstrated considerable differences in mRNA expression patterns between *Ha-ras* and *Ctnnb1* mutated tumors. We now conducted a proteome analysis with protein extracts from normal mouse liver and from liver tumors which were induced by a single injection of *N*-nitrosodiethylamine as initiator followed by multiple injections of 2 different polychlorinated biphenyls (PCBs) as tumor promoters, or corn oil as a control. Liver tumors were stratified into 2 classes: they were either mutated in *Ctnnb1*, and as a result positive for the marker glutamine synthetase (GS(+)), or they lacked *Ctnnb1* mutations and were therefore GS-negative (GS(-)). Proteome analysis by two-dimensional gel electrophoresis (2-DE) and mass spectrometry revealed 98 significantly deregulated proteins, 44 in GS(+) and 54 in GS(-) tumors. Only 12 of these proteins showed expression changes in both tumor types, and only 7 of them were deregulated in the same direction. Several of the identified enzymes could be assigned to fundamental metabolic or other cellular pathways with characteristically different alterations in GS(+) and GS(-) tumors, such as ammonia and amino acid turnover, cellular energy supply, calcium homeostasis and deregulation of cytoskeletal proteins. Our data suggest that GS(+) and GS(-) tumor cells show a completely different biology and use divergent evolutionary strategies to gain a selective advantage over normal hepatocytes.

[1]University of Tübingen, Department of Toxicology, Wilhelmstr. 56, 72074 Tübingen, Germany; [2]Federal Institute for Risk Assessment, Center for Experimental Toxicology, Thielallee 88-92, 14195 Berlin, Germany; [3]German Cancer Research Center, Central Unit of Biostatistics, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany; [4]Leibniz Institute for Molecular Pharmacology (FMP), Robert-Rössle-Strasse 10, 13125 Berlin, Germany; [5]Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY 40506, USA

437

THE NOVEL TUBULIN-ANTAGONIST SPONGISTATIN-1 IS AN EXTREMELY POTENT ANTI-ANGIOGENIC AGENT

A.S. Rothmeier, A.M. Vollmar, S. Zahler

In the last years, wide screenings of natural compounds were performed, to find new agents that may be set in battle against cancer. One of these agents is Spongistatin-1, a compound that is isolated from a marine sponge and binds to tubulin-heterodimers via a specific binding site, thereby destabilizing microtubules. We investigated the anti-angiogenic potency of Spongistatin-1. Beside the typical influence of tubulin-antagonists on mitosis, we examined the impact of Spongistatin-1 on non-mitotic functions of microtubules. First, we performed angiogenic assays (i.e. proliferation, migration, and tube formation) with Spongistatin-1 in comparison to well known tubulin-antagonists Vinblastin, Combretastatin-A4P, and Taxol. These assays revealed Spongistatin-1 to be the most potent of the tested drugs. Proliferation of the endothelial cells was markedly inhibited (IC50: 100 pM). Also in cell cycle analysis, Spongistatin-1 was the most capable agent (IC50: 1 nM). Using live cell imaging, we documented the disassembly of interphase microtubules and the dynamics of migration and tube formation inhibition of Spongistatin-1 treated endothelial cells. In addition, we could detect a rearrangement of phosphorylated focal adhesion kinase by confocal microscopy. We further analyzed the impact of Spongistatin-1 on small Rho-GTPases, which are in close dependency to the polymerizing status of microtubules. In experiments investigating the influence of Spongistatin-1 on cellular transport, we showed that endocytosis activity of Spongistatin-1 treated cells is inhibited. One striking point of our work is that especially proliferating endothelial cells are extremely sensitive towards Spongistatin-1. So we investigated signalling pathways that are essential for proliferation like the Akt/mTOR pathway. We detected an impact of Spongistatin-1 on mTOR and Akt phosphorylation. In ongoing studies we try to discover the mechanisms behind these effects. The fact that Spongistatin-1 acts at a very low dose, and that proliferating endothelial cells are very sensitive towards it, proposes Spongistatin-1 to be a potential anti-angiogenic drug. In further investigations we examine the impact of Spongistatin-1 on angiogenesis *in vivo* in a mouse model to reveal its aptitude as a therapeutic agent.

Department of Pharmacy, University of Munich, Germany

438

GENE EXPRESSION PROFILING IN HUMAN FIBROBLASTS EXPOSED TO A GENOTOXIC DENTAL RESIN MONOMER

H. Schweikl, K.-A. Hiller, C. Bolay, A. Eckhardt, G. Schmalz

Triethylene glycol dimethacrylate (TEGDMA) is a genotoxic and mutagenic dental resin monomer. It induced gene mutations and elevated numbers of micronuclei in V79 cells. The induction of DNA damage was also associated with the inhibition of the cell cycle in various human cell lines. Thus, we hypothesized that the monomer might modify the expression of genes related to the regulation of the cell cycle. First, normal human fibroblasts were exposed to 1.0 and 3.0 mmol/L TEGDMA, and the distribution of cells among the G1, S, and G2 phase was determined by FACS analysis. TEGDMA delayed the cell cycle in G1 after a 2h-exposure, because an increase of the numbers of cells from 53% in untreated controls to 66% and 71% in cultures exposed to 1.0 and 3.0 mmol/L TEGDMA was detected. A similar effect was determined after a 6h and a 24h exposure period. Then, gene expression profiles were analyzed in three independent untreated controls and in three independent cultures exposed to 3 mmol/L TEGDMA for 6h using Affymetrix DNA microarrays (U133A). In a total probe set of 22277 genes, positive signals were identified for 10727 genes (48.2%), no signals were present for 7562 genes (33.9%), and intermediate (present/absent) expression was detected for 3935 genes (17.7%). Since the signals detected with three treated cultures were related to the signals present in each of the three controls, 9 signal log ratios (SLR, $\log_2$) were calculated for each gene. Based on SLR $\geq$ 1.0, fifty genes were upregulated, and 113 genes were down-regulated according to SLR $\leq$ 1.0. Using the Ingenuity Pathway Analysis tools, the gene functions were related to the regulation of the cell cycle, DNA replication and repair as well as cellular growth and proliferation. The vast majority of these differentially expressed genes did not act as independent units but were identified as members of seven overlapping networks.

Dept. Oper. Dent. and Periodontology, Univ. of Regensburg, D-93042 Regensburg, Germany.

439

IDENTIFICATION OF THE JunD-INTERACTION PARTNER IN AhR-DEPENDENT REGULATION OF CYCLIN A EXPRESSION

D. Faust*, I. Schreck[#], F. Oesch*, C. Weiss[#], and C. Dietrich*

The aryl hydrocarbon receptor (AhR) is a transcription factor involved in physiological processes, but also mediates most, if not all, toxic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Activation of the AhR by TCDD leads to its dimerization with ARNT and transcriptional activation of several phase I and II metabolising enzymes. However, this classical signalling pathway so far failed to explain the pleiotrophic hazardous effects of TCDD such as developmental toxicity and tumour promotion. Thus, there is an urgent need to define genetic programmes orchestrated by AhR to unravel its role in physiology and toxicology. Treatment of rat liver cells with TCDD leads to a release from contact-inhibition. We have recently shown that TCDD-exposure leads to an elevation of JunD protein levels and to transcriptional activation of Cyclin A in an AhR-dependent, and probably ARNT-independent way. Ectopic expression of Cyclin A in confluent cultures overcomes G1-arrest indicating that increased Cyclin A levels are indeed sufficient to bypass contact-inhibition. Elevation of JunD precedes that of Cyclin A suggesting a role of JunD in Cyclin A induction. Indeed, down-regulating JunD by siRNA blocks TCDD-induced expression of Cyclin A. DNA affinity purification assays and reporter gene analysis indicate that JunD binds to an ATF / CRE consensus sequence in the rat Cyclin A promoter. In the present work we use *in vitro* DNA affinity purification assays and *in vivo* CHIP analysis to identify the hitherto unknown interaction partner of JunD.

(This work was partly supported by ECNIS, a network of excellence operating within the European Union 6th Framework Program, Contract No 513943.)

*Institute of Toxicology, Johannes Gutenberg-University, Obere Zahlbacherstr. 67, 55131 Mainz, Germany

[#] Institute of Toxicology and Genetics, Research Center Karlsruhe, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen; Germany

440

ANTI-LEUKEMIC EFFECTS OF FLAVONOIDS AND INDOLES CAN BE MEDIATED BY THE ARYLHYDROCARBON RECEPTOR

A. Goergens, C. Esser

Ligands of the Arylhydrocarbon receptor (AhR) include polycyclic aromatic hydrocarbons but also many natural substances, such as flavonoids and indoles. Some of these latter ligands are known for their anti-leukemic potential. Suggested mechanisms for the anti-tumorigenic potential of flavonoids are induction of CDK2 genes, promotion of caspase-3 dependent apoptosis, and interference with calcium signaling. Interestingly, all of these pathways are targets of the ligand-activated AHR as well. We analyzed AHR-dependent signaling in HL-60 cells, a human acute myeloic leukemia cell line for association with anti-leukemic effects. First, we verified expression of the AHR and its dimerisation partner ARNT in the cells, necessary for complete AHR-signalling, and the cells capacity for AhR-mediated induction of CYP4501A1. Four potential AHR agonists, the flavonoids quercetin (Qn) and curcumin (Cn), and the indoles indirubin-3´-oxim (In) and rutaecarpin (Rn) were used. Qn is a substance abundant in food, such as apples and onions, Cn, In, and Rn are secondary plant products from spices or medicinal herbs. Qn, Cn, In, but not Rn inhibited HL-60 proliferation by the induction of caspase-3 mediated apoptosis. Inhibition was dose-dependent, with IC50 values ranging from 1µM to 200µM, depending on the ligand. We transiently transfected HL-60 cells with a plasmid coding for an AhR-GFP fusion protein. We tested Qn and Cn and found that both ligands shuttled the AhR-GFP protein into the nucleus, indicating AhR-activation. Furthermore, proliferation inhibition and caspase-3 induction could be abrogated by AhR antagonist α-naphtoflavone in Cn treated HL-60 cells, whereas treatment of Qn and In showed increased apoptosis. This is the first evidence for AhR-mediated signalling as a contributing mechanism of action for anti-leukemic flavonoids. We currently investigate further the molecular AHR activation and subsequent nuclear signalling by flavonoids in HL-60 cells and primary leukemia cells.

This research is supported by the Deutsche José Carreras Leukämie-Stiftung, DJCLS R05/15

Institut für Umweltmedizinische Forschung (IUF) an der Heinrich Heine-Universität Düsseldorf, Auf˙m Hennekamp 50, 40225 Düsseldorf

441

PROPYLENE OXIDE INDUCED CELL PROLIFERATION IN RESPIRATORY MUCOSA OF RATS RESULTS FROM SEVERE PERTURBATION OF THE GLUTATHIONE STATUS

M.D.H. Khan, D. Klein, L. Quintanilla-Fend*, D. Oesterle, J.G. Filser

Propylene oxide (PO), a high-volume chemical intermediate, induces inflammatory lesions and cell proliferation in respiratory nasal mucosa (RNM) of rats. Upon long-term inhalation exposure to high concentrations (≥300 ppm) nasal tumours developed. The linear concentration-dependent formation of DNA adducts in RNM does not correlate with the exposure response for tumour formation, indicating that carcinogenic potential of PO does not result solely from its genotoxicity. In order to investigate whether cell proliferation in RNM is triggered by GSH depletion, male Fischer F344/N rats were exposed by inhalation (6 h/day, 3 days) to 50, 100, 200, or 300 ppm PO or treated i.p. for 3 days with diethylmaleate (DEM; 2x250 mg/kg/day and 1x500 plus 1x150 mg/kg/day, respectively) or with buthionine sulfoxime (BSO; 500 mg/kg/day). GSH was measured as non-protein thiol (NPSH), and cell proliferation was determined by BrdU immunostaining. Exposure to 50 ppm PO resulted in a partial NPSH depletion of approximately 48% of the control value; cell proliferation was not increased. At PO concentrations of 100-300 ppm, maximum NPSH depletion was reached (24% of the value in controls), and cell proliferation was significantly increased over controls (2.2-3.5-fold). Administration of 2x250

mg DEM/kg/day reduced the NPSH content in RNM to about 69% of that in controls. No effect on cell proliferation was observed. The dosage of 500+150 mg DEM/kg/day resulted in a NPSH depletion of 31% of the control value, thus reaching approximately the maximum depletion in PO exposed rats. Cell proliferation was significantly increased to 3-fold over controls. Likewise, after BSO treatment, NPSH depletion reached almost its maximum (37% of the value in controls), and cell proliferation was also significantly increased (about 2.5-fold over controls). These results strongly suggest that PO-induced cell proliferation in RNM of rats is caused by severe GSH depletion in this tissue.

GSF-Institute für Toxikologie und *Pathologie, Ingolstädter Landstr. 1, D-85764 Neuherberg, Germany

442

OCHRATOXIN A CARCINOGENICITY: TIME- ANF DOSE-DEPENDENT INCREASE IN RENAL CELL PROLIFERATION IN MALE F344 RATS

Rached, E.[1], Hard, G.C. [2], Blumbach, K. [3], Weber, K. [3], Draheim, R. [3], Steger, U. [4], Dekant, W.[1], and Mally, A.[1]

Ochratoxin A (OTA) is nephrotoxic and a potent renal carcinogen. In a 2-year carcinogenicity bioassay, tumor incidences in male F344 rats treated with 70 and 210 µg/kg b.w. were 20/51 and 36/50, respectively (*1*). In contrast, treatment with a low dose of 21 µg/kg b.w. did not result in increased tumor rates. Since the mechanism of OTA carcinogenicity is still largely unknown, this study was conducted to investigate the functional and pathological effects of OTA and to determine if sustained stimulation of renal cell proliferation might play a role in the mechanism of OTA carcinogenicity. Male F344 rats were treated with OTA for up to 13 weeks under conditions of the NTP bioassay. Cell proliferation was determined using bromodeoxyuridine (BrdU) and immunohistochemistry. Only minor changes in clinical chemistry parameters were evident throughout the study, indicating that OTA treatment did not markedly affect renal function. Histopathological examination showed renal alterations in mid and high dose animals involving single cell death and prominent nuclear enlargement within the straight proximal tubules. Treatment with OTA at doses of 70 and 210 µg/kg b.w. led to a marked dose- and time-dependent increase in renal cell proliferation, extending from the medullary rays into the OSOM. No significant effects were evident in the low dose group or in the liver, which is not a target for OTA carcinogenicity. A NOEL in this study was established at 21 µg/kg b.w., correlating with the dose in the NTP 2-year carcinogenicity that did not produce renal tumors. The apparent correlation between enhanced cell turnover and tumor formation induced by OTA indicates that stimulation of cell proliferation may play an important role in OTA carcinogenicity.

This work was supported by RCC Ltd.

(*1*) NTP, Natl Toxicol Program Tech Rep Ser 358, 1-142 (May, 1989).

[1]Department of Toxicology, University of Würzburg; [2]Private Consultant, Tairua, New Zealand; [3]RCC Ltd. CH-Itingen/Füllinsdorf; [4]Department of Surgery, University of Würzburg

443

FUNCTIONAL ANALYSES OF ARYL HYDROCARBON RECEPTOR RESPONSIVE GENES

M. Wormke, F. Dulich, M.Göttlicher

The AhR is a ligand-activated basic helix-loop-helix (bHLH) PAS transcription factor that heterodimerizes with the Arnt protein to activate or suppress a growing number of genes involved in xenobiotic metabolism and toxicity of dioxins, cell cycle and apoptosis. However, knowledge is incomplete for those genes that could mediate toxicity and carcinogenesis. Kolluri et al. (2001) identified several novel target genes of the AhR using the AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We are currently working to understand the cellular consequences resulting from AhR activation of three of these genes: epiregulin, *N*-myristoyltransferase 2 (NMT2), and the xCT cystine/glutamate transporter. Epiregulin is an epidermal growth factor (EGF) family member and a potent stimulator of hepatocyte proliferation. We have shown that epiregulin is upregulated in both rat 5L hepatoma cells and primary rat hepatocytes treated with TCDD. We are currently investigating whether TCDD-induced epiregulin expression effects proliferation of primary hepatocytes and hepatoma cells and constitutes a mechanism of TCDD-mediated promotion. The xCT cystine/glutamate transporter imports cystine into the cell where it is metabolized to cysteine and utilized for GSH synthesis. xCT has been shown to be induced by oxidative stress in glial cells. Our results show that TCDD induces xCT in rat 5L hepatoma cells indirectly by a cycloheximide-sensitive mechanism. Since cycloheximide also blocks TCDD- induced ROS production, we consider ROS as a potent mediator of xCT induction by TCDD. We have also identified a subset of cellular *N*-myristoylated proteins that undergo enhanced *N*-myristoylation as a result of TCDD-induced NMT2 enzyme levels. One of these proteins is the S4 subunit of the proteasome complex. We show that the non-myristoylated form of S4 localizes primarily to the nucleus, whereas the myristoylated form localizes primarily to cytosolic and nuclear membranes, suggesting that myristoylation status may play a critical role in localization and function of the S4 protein. In summary, the response of hepatocytes to TCDD involves multiple facets each of which could plausibly contribute to toxicity and carcinogenicity.

GSF National Research Center for Environment and Health, Institute of Toxicology, München-Neuherberg

444

BIO-INFORMATIC ANALYSIS REVEALS FLEXIBILITY AND CELL SPECIFICITY OF ARYLHYDROCARBON RECEPTOR SYSTEM

M. Frericks, M. Meissner, C. Esser

The arylhydrocarbon receptor is a sensor of a large number of small molecular weight chemicals, of either anthropogenic or biological origin. We had analysed the gene expression profiles of several immune cell types after (i) ligand activation of the AhR and (ii) in AhR deficient mice, and found a high number of genes whose expression was up- or down regulated, however, there was surprisingly little overlap between cell types[1]. In order to extend the analysis of AhR-dependent signalling to ligand independent situations, we data-mined published microarray experiments. We therefore performed linear global normalization on the data of 1968 published Affymetrix™ microarrays, i.e. the transcriptomes of >100 murine tissues or cell types. The mathematical transformation effectively nullified inter-experimental or inter-laboratory differences between microarrays, as shown by quantitative RT-PCR validation of selected transcripts. Using the data base we analysed components of the aryl hydrocarbon receptor (AHR) signalling pathway in various tissues. We identified lineage and differentiation specific variant expression of AHR, ARNT, and HIF1α in the T cell lineage, and high expression of CYP1A1 in immature B cells and dendritic cells. Performing co-expression analysis we found unorthodox expression of the AHR in the absence of ARNT, particularly in stem cell populations, and can reject the hypothesis, that ARNT2 takes over and is highly expressed when ARNT expression is low or absent. Analysis of differential gene expression in 308 different experimental settings ("conditions") revealed 53 conditions under which the AHR was regulated, numerous conditions under which an intrinsic AHR action was modified as well as conditions activating the AHR even in absence of known AHR ligands. Our data highlight the flexibility of AhR expression in response to intrinsic and extrinsic factors and support the growing evidence for its pleiotropic physiological role.

[1] *Frericks et al., Biol. Chem. 387:1219 (2006)*

Institut für Umweltmedizinische Forschung gGmbH an der Heinrich Heine-Universität Düsseldorf, Auf˙m Hennekamp 50, 40225 Düsseldorf

445

THE ARYHLYDROCARBON RECEPTOR IS INVOLVED IN THE CIRCADIAN RHYTHM OF HACAT KERATINOCYTES

J. Tigges, K. Ruwiedel, S. Wolff, U. Hübenthal, J. Abel, E. Fritsche

Circadian regulation of gene expression is a general phenomenon that has been observed in many organisms ranging from plants to animals. Circadian rhythmicity is thought to help the organism anticipate the cyclic changes in the ambient environment by regulating physiological and behavioral responses. Not only the brain regulates circadian rhythm of the organism, but also peripheral cells and organs possess rhythmicity that respond to environmental stimuli like ultraviolet light (UV). The basic molecular players of the circadian clock, the PAS (homologous to Per/ARNT/Sim) proteins Period, Clock and Bmal1 as well as the non PAS proteins Cryptochrome and Timeless exert rhythmic expression in skin cells. Chronobiological effects seem to be important for skin homeostasis in health and disease and may impact pharmacotherapy. However, the regulation of the underlying mechanisms of circadian rhythmicity in the skin is not well understood. We have shown now that HaCaT keratinocytes express gene products of the basic circadian machinery. This was shown earlier for primary skin cells. Moreover, the expression of Clock, Bmal1, Period and Cryptochrome in HaCaT cells shows rhythmicity over 24 hours. Deficiency of the arylhydrocarbon receptor (AhR) in these cells leads to a loss of Per and Clock circadian gene expression indicating that the AhR is involved in maintenance of circadian homeostasis in skin cells. Furthermore, we show that light (100 J/m^2 UVB) induces a shift in circadian gene expression in HaCaT cells. This was not observed when the cells were deficient for the AhR. In summary, our results demonstrate an involvement of the AhR in the basic circadian rhythm of HaCaT keratinocytes and indicate that the AhR participates in the mediation of adaptation of HaCaT cells to UV stimuli.

Institut für Umweltmedizinische Forschung (IUF) and der Heinrich-Heine-Universität, Auf˙m Hennekamp 50, 40225 Düsseldorf, Germany, email: ellen.ftitsche@uni-duesseldorf.de.

446

DIRECT CONTROL OF THE MAMMALIAN CIRCADIAN CLOCK GENES *Per1* AND *Per2* BY DIOXIN, AHR AND ARNT

B. Oesch-Bartlomowicz[*] H. Zatonski[*], E. Reszka[*], W. Wasowicz[#], G.T.J van der Horst[§], F. Oesch[*]

Circadian rhythmicity is a conserved physiological feature of almost all organisms. Through several photoreception systems, light is capable of synchronizing circadian oscillations to the environment. The core pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, whose neurons receive photic input signals from the retina via the retinohypothalamic tract. Circadian timekeeping is cell-autonomous, generated at least in part by a feedback loop involving clock proteins that inhibit the transcription of their own genes. Regulation of the transcriptional feedback loop by light is thought to mediate setting of circadian clocks to light-dark cycles. A negative feedback loop of mammalian *Period* genes 1 (*mPer1*) and 2 (*mPer2*) transcription is most probably central to the clock. A heterodimeric activator consisting of the basic helix-loop-helix (bHLH)-PAS proteins CLOCK and BMAL1 drives *mPer1* and *mPer2* transcription from E-boxes regulatory sequences, and the PER1 protein in turn acts to inhibit CLOCK-BMAL activity. Recently, mCRY1 and mCRY2 members of the light-harvesting cryptochrome/photolyase protein family were shown to be indispensable components of the molecular oscillators as mice with inactivated *mCry1* and *mCry2* genes completely lack a biological clock. It has been reported that that CRY1 and CRY2 inhibit CLOCK-BMAL1 transactivaton activity most probably by direct contacts between CRY1 and BMAL1, and between CRY2 and CLOCK. Dioxin signalling proteins aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) are both like CLOCK/BMAL bHLH-PAS proteins. Here we show that in mouse embryonic fibroblast (MEF) and in the NIH3T3 cells, *Per1* and *Per2* genes respond to dioxin in the reporter gene assay. Moreover, AHR and ARNT serve as dimerization partners for BMAL1 and CLOCK, respectively. Unexpectedly AHR-BMAL1 and ARNT-CLOCK were able to drive *Per1* and *Per2* transcription in the functional assay. As the CRY proteins are essential for maintenance of circadian rhythms we analysed the AHR-BMAL1 and ARNT-CLOCK E-box-dependent transactivation of the *Per1* and *Per2* genes in the *cry*-single-knockout mouse embryonic fibroblast cell lines *cry1* and *cry2* (derived from *cry1* and *cry2* mutant mice through gene targeting in embryonic stem cells) and *cry1* and *cry2* double knockout cells. Interestingly, ARNT-CLOCK dimer was transcriptionaly active in *cry2* but not in the *cry1* or *cry1*/*cry2* mutant cells. The AHR-BMAL1 was inactive in all these *cry* mutants. Surprisingly, dioxin has provided light-dependent and -independent regulation of *Per1* and *Per2* genes. Thus AHR and ARNT may act as significant components of the circadian clock and probably regulate *Per1* and *Per2* transcriptional cycling by contacting both the activator and its feedback inhibitors.

(This work was supported by ECNIS, a network of excellence operating within the European Union 6th Framework Program, Contract No 513943).

[*]Institut of Toxicology, University of Mainz, Obere Zahlbacherstr. 67, 55131 Mainz, Germany, [#],Institut of Occupational Medicine, Department of Toxicology and Carcinogenesis, Teresy 8 Str. Lodz, Poland, [§]Department of Cell Biology and Genetics, Erasmus University, 3000 DR Rotterdam, The Netherlands

447

REGULATION OF CONSTITUTIVE AND INDUCIBLE EXPRESSION OF THE HUMAN AHR REPRESSOR: ROLE OF SP-LIKE TRANSCRIPTION FACTORS

T. Haarmann-Stemmann, H. Bothe, E. Fritsche, J. Abel

The AhR is a ligand-activated transcription factor, which mediates the toxicity of environmental contaminants like dioxin. One major effect of AhR activation is the induction of a battery of target genes, especially of those encoding for drug-metabolizing enzymes. The recently discovered AhR Repressor (AhRR) is a new AhR target, whose gene product creates an inhibitory feedback loop of AhR signaling. Although some regulatory motifs within the murine and rat AhRR gene were identified, there are no data available concerning the molecular regulation of the human AhRR gene. Database-analysis revealed several putative binding sites for AhR/ARNT and sp1/sp3 within intron I of the AhRR gene. In order to study the functional importance of these putative binding sites, a 1kb DNA fragment of intron I was cloned, which harbours four putative xenobiotic-responsive elements (XRE) and two putative GC-boxes. Reporter gene assays in HepG2 cells showed a significant increase (7-fold) of luciferase activity after treatment with the AhR agonist 3-methylcholanthrene (3-MC). By generation of several deletion variants, one functional XRE within the cloned fragment was identified. Data of chromatin immunoprecipitation assays confirmed the binding of AhR and ARNT proteins to the identified XRE. Interestingly, 5bp upstream of this XRE a putative GC-box is present. To investigate the importance of this putative sp1/sp3 binding site, we treated transfected HepG2 cells with mithramycin A (MMA), a known inhibitor of sp1/sp3 transcription factors. MMA treatment leads to a significant decrease in the basal luciferase activity. Co-treatment with MMA and 3-MC resulted in a significant inhibition of AhRR induction. The obtained results were supported by AhRR mRNA analyses. Treatment of HepG2 and HeLa cells with MMA decreases the constitutive as well as the 3-MC-induced transcription of the AhRR gene. This effect was accompanied by an increase of CYP1A1 expression. The results indicate that AhR/ARNT and sp1/sp3 act co-operatively in regulation of constitutive and inducible expression of the AhRR gene in HepG2 and HeLa cells.

Experimentelle Toxikologie, Institut für umweltmedizinische Forschung an der Heinrich-Heine-Universität, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany

448

ß-CATENIN SIGNALING IN MOUSE HEPATOMA CELLS STABLY TRANSFECTED WITH HAIRLESS GENE

N. Armbruster, C. Rothmund, M. Schwarz and P.A. Münzel

Nuclear receptors and Wnt signaling are both important regulators of developmental and physiological processes. The hairless protein (HR) is a nuclear receptor corepressor and acts by regulating gene expression through cross talk with receptors like the thyroid-, vitamin D- and RAR orphan receptor (ROR). HR represses the transcription of genes involved in epidermal differentiation. A recent study revealed that HR represses expression of Wise, an inhibitor of Wnt signaling, leading to a model in which HR controls the timing of Wnt signaling required for hair cycling (Beaudoin III *et al.*, PNAS, 102, 14653, 2005). ß-Catenin, a mediator of the Wnt signaling pathway, is thereby involved in the periodic regeneration of hair follicles through a stem-cell-mediated process. To further elucidate the role of HR in the Wnt signaling pathway in hepatocytes, hairless gene was stably transfected into mouse hepatoma 53.2b cells, which have an intact Wnt signaling pathway. Positive clones were identified by Real Time PCR for the expression of hairless gene. In positive clones the expression of the ß-Catenin/TCF target genes axin 2 and GPR49 (G-protein coupled receptor 49) was measured. Expression of axin 2 was increased about 25 fold. GPR49 mRNA levels were elevated about 50 fold. CYP1A1 expression was also affected increasing up to 100 fold. HR seems to regulate the Wnt/ß-Catenin signaling and the ß-Catenin target genes axin 2 and GPR49 in a similar way, despite WISE is not expressed in the liver and therefore not the target for the corepressor HR. In summary, our data demonstrate that the stably transfected 53.2b hepatoma cells are a suitable model to study the mechanisms of HR function.

Institute of Pharmacology and Toxicology, Dept. of Toxicology, University of Tübingen, Wilhelmstr. 56, 72074 Tübingen, Germany

449

GPR49 IS A TARGET OF β-CATENIN AND NFκB SIGNALLING

Rignall, B., Marx-Stoelting, P., Loeppen, S., Braeuning, A., Koehle, C., Schwarz, M.

G-protein coupled receptors are among the most promising drug targets. GPR49, also called LGR 5 is a seven transmembrane domain G-Protein coupled receptor that was recently reported to be up-regulated in human hepatocellular and colon carcinoma bearing mutations in the β-catenin gene. Both tumours are among the most frequent tumours worldwide and a major cause of death. Here we do not only show that GPR49 is up-regulated in mouse liver tumours, which harbour a mutation in the *Ctnnb* gene but also demonstrate its up-regulation in primary hepatocytes after treatment with GSK3β inhibitors like LiCl or SB216763 and WNT3a conditioned cell culture medium. Furthermore we show that transgenic mice expressing an activated version of β-catenin (S33Y) in liver show a strong up-regulation of GPR49 on the mRNA level. These results give further proof for a regulation of GPR49 by the canonical Wnt signalling pathway. In addition, we could show that GPR49 is down- regulated after stimulation of NFκB-dependent signalling, an effect that could be specifically blocked by SC514, an inhibitor of this signalling pathway. These findings suggest that GPR49 is regulated by β-catenin and is also a possible target of NFκB signalling.

Institute of Pharmacology and Toxicology, Department of Toxicology, University of Tübingen

450

TRANSGENIC EXPRESSION OF S33Y MUTATED β–CATENIN IN MOUSE LIVER CONSTITUTIVELY ACTIVATES β–CATENIN SIGNALLING.

Schreiber, S.[1], Ott,T.[2] Koehle, C.[1], Marx-Stoelting, P.[1] Buchmann, A.[1], Schwarz, M. [1]

β–Catenin signalling plays an important role in a variety of processes such as cell differentiation and liver tumourigenesis in both mice and humans. Recent studies in our laboratory have shown that liver tumours of phenobarbital-treated mice bear β–catenin point mutations, e.g. S33Y in exon 2 of the *Ctnnb1*-gene. To investigate the role of the observed Ctnnb1 mutation in vivo, we created a conditional knock-in mouse model that expresses the S33Y mutated form of β–catenin in a liver-specific manner. By microinjection, we injected a *Ctnnb1*-S33Y expression vector containing a neo-polyA spacer in-between the CAG-promoter and the coding sequence into the pronuclei of B6D2F1-mice zygotes. To excise the flox-flanked spacer and activate mutated β-catenin, we crossed mice expressing a Cre recombinase under the control of the liver specific albumin promoter with mice carrying the S33Y construct. Expression of mutated β–catenin was verified by PCR and sequencing and its functionality was demonstrated by measuring the expression of β–catenin target genes. S33Y mutants showed increased expression of β–catenin targets such as glutamine synthetase (GS), various cytochromes P-450 (CYP) and GPR49 on both mRNA and protein level. Because β–catenin signalling is also known to be important for zonated expression of a number of liver proteins, we analysed zonation by immunhistochemistry. These experiments demonstrated abnormal zonation of CYPs and GS in S33Y mutant mice. Taken together, these results indicate that point-mutated β-catenin influences the expression of drug metabolising enzymes and other proteins in the transgenic animals. As mutated β–catenin seems to be responsible for a large percentage of mouse and human liver tumours, we hope that our model helps to further elucidate the role of β–catenin and its mutations in liver tumourigenesis.

[1]Institute of Pharmacology and Toxicology, Department of Toxicology, and [2]Institute of Human Genetics, University of Tübingen, Germany.

451

ROLE OF THE ESTROGEN RECEPTOR IN THE DIETHYLSTILBESTROL-INDUCED DISRUPTION OF THE EXPRESSION OF WNT5A AND WNT7A IN HUMAN ENDOMETRIAL (ISHIKAWA) CELLS

J. Wagner, A.M. Sauermann, P. Rittmann, L. Lehmann

Genes of the WNT family are involved in signal transduction cascades important for development and carcinogenesis. WNT5A is supposed to be an upstream regulator of WNT7A expression in a signaling pathway that transmits information among stromal and epithelial cells of the endometrium. Disruption of this pathway is associated with morphological abnormalities of the female reproductive tract, and mice treated *neonatally* or *in utero* with the trans-placental acting carcinogen diethylstilbestrol (DES) show the same abnormalities, whereas estrogen receptor (ER)alpha knockout mice are insensitive towards DES. The aim of the present study was to determine the influence of the synthetic estrogen DES on the mRNA expression of WNT5A and WNT7A in a human endometrial adenocarcinoma cell line (Ishikawa cells) by reverse transcription/competitive PCR. The role of the ER was examined by (i) the determination of the mRNA expression and the enzyme activity of the alkaline phosphatase (ALP), which is regulated by the ER in Ishikawa cells, (ii) the use of the ER antagonist ICI 182,780 (ICI), and (iii) silencing of the ERalpha by RNA interference using siRNA. After treatment of Ishikawa cells with DES at concentrations that stimulated the expression of the ALP for 6-48 h, a significant decrease in the mRNA levels of WNT7A was observed reaching maximum reduction after 24 h. DES-induced reduction of WNT7A mRNA levels was prevented by the ER antagonist ICI as well as by knock down of ERalpha by RNA interference. After treatment with DES for 24 h and longer, mRNA levels of WNT5A correlated with those of WNT7A; however, there were pronounced differences after treatment periods of 6 h and less. In conclusion, when challenged with DES, the Ishikawa cell line reacts similar to the endometrial epithelium of mice exposed to DES *in utero* or *neonatally*, thus providing a powerful tool for the investigation of endocrine disrupting of the female reproductive tract.

University of Karlsruhe, Section of Food Chemistry and Toxicology, Kaiserstr. 12, 76131 Karlsruhe

452

MECHANISMS OF BENZO[*A*]PYRENE-7,8-DIHYDRODIOL-9,10-EPOXIDE INDUCED ACTIVATION OF STRESS-ACTIVATED-PROTEIN-KINASES IN HUMAN CELLS

I. Schreck[1], S. Mayer[1], F. Oesch[2] and C. Weiss[1]

The polycyclic aromatic hydrocarbon and environmental pollutant benzo[*a*]pyrene (B[a]P), a constituent and contaminant of cigarette smoke, automobile exhaust, industrial waste and even food products, is carcinogenic to rodents and humans. B[a]P binds to the intracellular aryl hydrocarbon receptor thereby inducing its own metabolism by cytochrome P450s. Amongst several different metabolites the B[a]P-7,8-epoxide is considered as one of the most critical since, due to its metabolism by the microsomal epoxide hydrolase, it generates the precursor for the highly reactive electrophilic genotoxin and ultimal carcinogen B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE). During evolution, multiple surveillance and defense mechanisms have evolved to protect the cell from DNA damage. Specific signaling pathways operate to detect and repair different qualities of lesions. Prominent examples of such cell cycle and survival regulators are transcription factors such as p53 and stress-activated-protein-kinases (SAPKs). Previously, we analyzed BPDE effects on SAPKs in murine fibroblasts. Interestingly, within minutes of exposure we find rapid and strong phosphorylation of both SAPK family members JNKs and p38s. By the use of specific inhibitors of signaling mediators upstream of JNKs and p38-MAPKs we could demonstrate a role of c-src like kinases in SAPK-activation by BPDE. In the present study we extend our observations and establish a similar signal transduction cascade in the human cell lines HepG2 (hepatoma cells) and Hela (cervix carcinoma cells). As in rodent cells, BPDE induces rapid SAPK activation, which was reduced by pretreatment with the c-src kinase family inhibitor PP-1. To more rigorously proof an involvement of specific c-src like kinases, individual family members were depleted by the use of siRNAs. As a result, specific c-src like kinases could be identified contributing to SAPK activation by BPDE. Future experiments will address the mechanisms by which BPDE leads to activation of c-src like kinases and aim at the identification of the primary target(s) for BPDE induced signaling.

[1]Institute of Toxicology and Genetics, Forschungszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany
[2]Institute of Toxicology, University of Mainz, 55131 Mainz, Germany

453

GENOTOXIN-INDUCED DNA REPLICATION BLOCKAGE IS INVOLVED IN THE REGULATION OF STRESS KINASES (SAPK/JNK)

G. Fritz, T. Brachetti and B. Kaina

Activation of c-Jun-N-terminal kinases/stress-activated protein kinases (SAPK/JNK) is part of the cellular response to genotoxin exposure, eventually regulating gene expression, cell cycle progression and cell death. The physiological relevance of genotoxin-induced block in DNA replication for activation of SAPK/JNK is fairly unknown. To address this question, we compared the replication blocking efficiency of various genotoxins with their ability to stimulate dual phosphorylation of SAPK/JNK (Thr183/Tyr185) in mouse fibroblast (MEFs). The data obtained show that the potency of equitoxic doses of genotoxins to trigger SAPK/JNK phosphorylation is agent specific. There is no association between the intensity of genotoxin-induced DNA replication blockage and the extent of SAPK/JNK activation. On the other hand, stimulation of SAPK/JNK phosphorylation provoked by both methyl methanesulfonate (MMS) and UV-light was reduced by ~50 % in non-growing as compared to logarithmically growing cells. Repression of DNA replication by pharmacological inhibition of DNA polymerase using aphidicolin or hydroxyurea was sufficient to activate SAPK/JNK. Hence, apart from genotoxin-induced DNA damage-related effects on DNA replication, direct interference with the DNA replication machinery is also effective in triggering SAPK/JNK activation. Aphidicolin-stimulated signaling to SAPK/JNK was further enhanced by MMS treatment, but not by UV-C exposure. This finding indicates that the physiological significance of UV-C- versus MMS-induced replication block for the overall activation of SAPK/JNK is different. To further clarify the impact of DNA replication-dependent mechanisms on genotoxin-triggered signaling to SAPK/JNK, the potency of UV-light and MMS to activate SAPK/JNK in G1-, S- and G2-phase cells was analyzed. The data obtained will be presented and discussed. In summary, we suggest that both DNA replication-dependent and -independent mechanisms are relevant for activation of SAPK/JNK upon genotoxic injury. Furthermore, the significance of DNA damage-induced replication blockage for triggering signaling to SAPK/JNK appears to be agent specific.

Department of Toxicology, Johannes-Gutenberg University of Mainz, Obere Zahlbacher Straße 67, D-55131 Mainz, Germany.

454

THE INFLUENCE OF ß1-INTEGRINS AND EXTRACELLULAR MATRIX PROTEINS ON NANOPARTICLE INDUCED MAP-KINASE SIGNALLING

I. Gallitz, D. Lamers, U. Sydlik, K. Bierhals, J. Abel and K. Unfried.

Epidemiological studies convincingly demonstrate that combustion derived nanoparticles can cause adverse health effects in humans. Besides pro-inflammatory reactions, the induction of apoptosis and proliferation in lung epithelium are considered as pathogenic endpoints. Our earlier studies with cultured alveolar epithelial cells revealed that both, apoptosis and proliferation, are induced via different specific signalling pathways. Interestingly, the proliferative signalling via activation of protein kinase B and MAP-kinases ERK1/2 appeared to be strongly dependent on ß1-integrin transmembrane receptors. This kind of heterodimeric receptors are known to link extracellular matrix proteins (like fibronectin, laminin and collagen) to focal contacts, which represent intracellular signalling complexes responsible for outside-in as well as inside-out signalling. In order to elucidate the role of this signalling machinery in nanoparticle induced proliferative cascades, intervention studies on the level of ß1-integrins as well as ECM components were performed. Rat lung epithelial cells cultured on fibronectin, collagen, poly-D-lysin or on uncoated culture vessels, respectively, were treated with non-cytotoxic dosages of nanosize carbon particles (ufCB). In short-term experiments, culturing on fibronectin or collagen lead to an increase in ufCB-induced ERK1/2 and AKT activation without a change in total ß1-integrin protein amount. Long-term studies, however, revealed that cells cultured on collagen have a decrease in ß1-integrin protein amount, which is accompanied by an abolishment of ultrafine particle-induced ERK1/2 and AKT activation. Cells cultured on fibronectin maintained their ß1-integrin status as well as their specific nanoparticle induced cell responses. To further elucidate the specific role of ß1-integrins in these signalling events elicited by ultrafine carbon particles, ß1-integrin was blocked by specific inhibitors as well as by using specific shRNA. From these results we conclude that ß1-integrins are essential components of nanoparticle induced proliferative signalling which is dependent on the composition of the extracellular matrix.

(Project funding DFG UN110 3-1)

Institut für umweltmedizinische Forschung (IUF), Düsseldorf, Germany

455

THE ROLE OF ENVIRONMENTAL CONTAMINANTS IN ROS-MEDIATED SIGNAL TRANSDUCTION PATHWAYS TRIGGERED BY NANOPARTICLES IN LUNG EPITHELIAL CELLS

A. Weissenberg, U. Sydlik, R. Schins, J. Abel, K. Unfried

Ultrafine particulate matter (PM) has been shown to be involved in the occurrence of several lung diseases. Environmental PM is a heterogeneous mixture of variable composition, depending on its origin. The particle core, metals, and organic compounds associated to it have been recognized as the main pathogenically relevant fractions. Reactive oxygen species (ROS) generated by these components may lead to oxidative stress in the lung epithelium. This mechanism seems to play a key role in the regulation of apoptotic and proliferative signaling cascades. Ultrafine carbon particles, uncoated and coated with benzo[a]pyrene and/or $Fe_2(SO_4)_3$, were used as a model particle system for *in vitro* analyses of additive and synergistic effects on a molecular basis. Particle-treated rat lung epithelial cells (RLE 6-TN) were assessed for the induction of proliferative signaling involving phosphorylation of the MAP-kinase ERK1/2 as well as AKT. Specific inhibitors were used for studying the involvement of the EGF receptor and integrins in these signaling pathways. ROS generation was detected with specific fluorogenic markers, and the role of oxidative stress was analysed by treatment with the ROS scavenger N-acetylcysteine (NAC). Particle treatment induces phosphorylation of ERK1/2 and AKT. This effect is additively enhanced in the prescence of iron sulfate, whereas benzo[a]pyren shows no significant influence. Particularly at earlier time points, iron sulfate alone is also able to activate both signaling pathways. Particle-induced activation can be significantly reduced by inhibiting the EGF-receptor. Inhibition of ß1-integrins indicates their involvement in particle-induced signaling, but these receptors seem not to play a role in iron sulfate mediated activation. Particles are able to generate ROS, and the resulting oxidative stress is likewise involved in the activation of signaling cascades. Taken together, the particle-induced activation of proliferative and anti-apoptotic signaling can be significantly modulated by coating of the particle core, depending on the nature of the bound substances.

(Project grants: DFG SFB 503, Graduiertenkolleg 1427)

Institut für umweltmedizinische Forschung, HHU Düsseldorf.

456

NANOPARTICLE-GENERATED REACTIVE OXYGEN SPECIES AS INDUCERS OF APOPTOTIC AND PROLIFERATIVE SIGNALING PATHWAYS

U. Sydlik, A. Weissenberg, J. Abel and K. Unfried

Different kinds of nanoparticles are able to induce the pathogenic endpoints apoptosis and proliferation in rat lung epithelial cells, in a size and dose dependent manner. The specific signaling pathways deciding which endpoint is induced have been identified on the level of membrane receptors and protein-kinases. The initial events, however, triggering these membrane receptor-coupled signaling steps are not fully understood. Reactive oxygen species (ROS) are supposed to play an important role in eliciting these intracellular cascades. The generation of ROS was investigated in rat lung epithelial cells (RLE-6TN) treated with non-cytotoxic dosages of nanosized carbon particles (14 nm). The intracellular induction of ROS was first demonstrated by significant changes in the ratio of oxidized vs. reduced glutathione. Moreover, intracellular ROS generation was monitored using flouorogenic dyes. The relevance of these ROS on the endpoints apoptosis and proliferation was investigated using specific scavenging substances. Both nanoparticle specific endpoints, apoptosis and proliferation, as well as the activation of the respective signal pathways were significantly reduced in cells pre-treated with *N*-acetylcysteine (NAC). Moreover, diphenyleneiodonium (DPI) as inhibitor of NADPH-oxidases, exhibited comparable effects on apoptosis and proliferation indicating a specific role of mitochondrial ROS generation. In this context, the role of mitochondria in nanoparticle induced apoptosis was investigated on the level of cytochrome C release and the activation of specific caspases. Both, increased levels of cytochrome C and the subsequent activation of caspases 9 and 3 indicate a key role of mitochondria in nanoparticle induced apoptosis. Inhibitor assays for caspase 8 revealed no evidence for an involvement of the death receptor pathway .after nanoparticle treatment. Taken together, these data confirm the hypothesis that ROS induced by nanosized pure carbon particles are triggering pathogenic endpoints via specific signaling pathways. With regard to ROS generation as well as to the induction of apoptosis, mitochondria appear to play a key role in this scenario.

Institut für umweltmedizinische Forschung an der Heinrich-Heine-Universität Düsseldorf

457

STUDIES ON THE DELAYED CELLULAR EFFECTS CAUSED BY THE ACTIN-ADP-RIBOSYLTRANSFERASES *CLOSTRIDIUM BOTULINUM* C2 TOXIN AND *SALMONELLA ENTERICA* SPVB

S. Pust and H. Barth

The bacterial virulence factors *Clostridium botulinum* C2 toxin and *Salmonella enterica* SpvB mono-ADP-ribosylate actin, leading to a complete depolymerization of actin filaments. The C2 toxin is a binary exotoxin, consisting of the transport component C2IIa, which delivers the enzyme component C2I into the cytosol. In contrast to the C2 toxin, SpvB is supposed to be transported from the intracellularly located *Salmonella* into the host cell cytosol via a type-III-secretion system. To characterize the effects of SpvB in mammalian cells, we used a recombinant fusion toxin (C2IN-C/SpvB) to deliver the catalytic domain of SpvB (C/SpvB) into the cytosol via the C2 toxin specific uptake mechanism. Both ADP-ribosyltransferases modify monomeric G-actin at position arginine-177, resulting in depolymerization of actin filaments and cell rounding. Here, we focused on the delayed cellular responses, which determinate the fate of toxin-treated cells. We demonstrated that, in contrast to amoebae, for the tested mammalian cell types (Vero, HeLa) ADP-ribosylation was no trigger for an enhanced degradation of actin. Thus, ADP-ribosylated actin accumulated in toxin-treated cells. Cells, treated with C2 toxin, became round and remained in this morphology until they died. Compared with this, the cytopathic effect of C2IN-C/SpvB was transient, as rounded cells regained a flat but extremely enlarged morphology. Such cells stayed viable for several days but became poly-nuclear. The cellular recovery was accompanied by the *de novo* synthesis of actin and reconstitution of actin structures in the cellular periphery. However, C2IN-C/SpvB-treated cells showed an altered cytoskeletal organization. Moreover, the impulsive step for the recovery process was the degradation of C2IN-C/SpvB in the host cell cytosol. Accordingly, inhibition of proteasomal degradation by MG 132 led to no cellular recovery. Although, both toxins act by the same molecular mechanism and induce comparable early cytopathic effects, diverse delayed cellular responses occurred.

Institut für Pharmakologie und Toxikologie, Universität Ulm, Albert-Einstein-Allee 11, 89081 Ulm

458

ADP-RIBOSYLATION OF ACTIN BY BACTERIAL TOXINS RESULTS IN A DELAYED TYPE OF CELL DEATH IN MAMMALIAN CELLS

K. Heine and H. Barth

Various bacteria secrete protein toxins targeting cutting sites of eukaryotic cells, such as the cytoskeleton. On the one hand, there are the *Clostridium botulinum* C2 toxin and the *Salmonella enterica* SpvB, which mono-ADP-ribosylate actin leading to a depolymerisation of the actinfilaments. On the other hand, there is the large clostridial toxin TcdB of *Clostridium difficile,* which mono-glucosylates the Rho-GTPases, thereby resulting in a destroyed cytoskeleton, too. As for the C2 toxin the molecular mode of action is well characterized, we report here for the first time that the finale fate of toxin-treated cells is apoptotic cell death. We could show that in Hela cells the ADP-ribosylation of the total amount of cellular actin itself does not correlate simultaneously with the occurrence of apoptosis. However, within a lag of ~ 20 hours, apoptosis was observed in ~ 20 - 50% of the cells. Despite the fact that both, C2 toxin and SpvB, directly damage the actin cytoskeleton of eukaryotic cells, the cellular fate seems to process quite in a different way. Whereas the morphological changes, induced by C2 toxin, are irreversible some of the SpvB treated cells recover a flat-shaped morphology. Nevertheless, they are disturbed in their cytokinesis and mitosis thus display a bi- or multi-nucleic state. The "delayed" form of cell death is in contrast to the "fast" apoptotic effects observed with TcdB (3 –

5 hours after toxin-treatment). Here we state that in the same time-frame, as the GTPases are entirely glucosylated, apoptotic cells first show up. This striking distinct behaviour raises several questions. In both cases the entire actin is ADP-ribosylated thus affecting essential cellular processes. Why are the C2 toxin-treated cells still viable for a certain period of time? Does the modification of the Rho-GTPases mediate a "fast point-of-no-return" signal? Further experiments are under construction to elucidate the mechanism underlying the connection between destruction of the actin cytoskeleton and apoptotic cell death.

Institut für Parmakologie und Toxikologie, Universität Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

459

PREDICTIVE TOXICOLOGY: BIOMARKER IDENTIFICATION IN RAT PLASMA BY 2DE*

J. Pieh[1], M. Glückmann[2], B. von Eiff[1], M. Kröger[3], J. Hellmann[1], P.-J. Kramer[1], S. O. Müller[1]

In a former in vivo study with a genotoxic carcinogen, we have shown that proteomic analyses can help in identifying predictive safety markers. Here, we extended this study to identify predictive protein markers in plasma of rats treated with prototypical toxicants. For that purpose, we employed the subacute toxicity bioassay in rats (28 days/OECD TG 407) for the prediction of genotoxic and hepatotoxic compounds. Wistar rats were exposed to nine different chemical substances of three categories (genotoxic carcinogens, non-genotoxic and hepatotoxic, non-genotoxic carcinogens) and samples were taken at three different time points (day 3, 7 and 28). In this BMBF-funded project, genomic and proteomic analyses were done on liver tissue and plasma samples. In addition, effects on the gene and protein expression level were correlated with histopathological findings. In order to identify predictive biomarkers in plasma, we analysed these samples by two-dimensional-gel-electrophoresis (2-DE) followed by protein identification using mass spectrometry (MS). Candidate markers in plasma are generally difficult to detect since more than 99% of plasma proteins are high abundant ones. Therefore, we applied several depletion methods for high abundant proteins to increase the sensitivity. By using 2-DE, several differentially expressed proteins could be detected after 3 days as well as 7 or 28 days of exposure. By subsequent MS analysis, we identified some candidate biomarkers such as vitamin D-binding protein and betaine-homocysteine methyltransferase in the genotoxic carcinogen group. Besides that several high abundant proteins, e.g., apolipoprotein A1 and complement C3, molecules involved in lipid metabolism and immune response were affected. We are currently investigating the role of these putative markers in toxicity and will correlate our results with markers detected in liver tissue as well as by gene expression analysis.

[1]Merck KGaA, Institute of Toxicology, Darmstadt, Germany; [2]Applied Biosystems, Mass Spectrometry and Proteomics, Darmstadt, Germany; [3]Merck KGaA, Darmstadt, Germany;

* We thank the BMBF (grant 0313137) for funding.

460

MULTIPLEXED PROBING OF CYTOCHROMES P450 USING INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

A. Venkatachalam[1], C. Köhler[2], I. Feldmann[1], J. Messerschmidt[1], A. Manz[1], N. Jakubowski[1] and P.H. Roos[2]

Cytochromes P450 (CYP) are among the most important enzymes involved in the metabolism of xenobiotics. CYP profiles as co-determinants of adverse effects vary substantially and are subject to xenobiotic-dependent modulations. Hence, profiling methods for CYPs, associated receptors and involved signaling compounds are an important tool for toxicological research. ICP-MS has often been used in recent times for the element specific detection of proteins. On one hand, it can be utilized directly to detect the phosphorus in proteins through which phosphorylation status of proteins could be monitored. On the other hand, proteins of interest could be labeled and indirectly observed concurrently (Müller et. al, 2005, *J Anal Atom Spectr* 9, 907). Considering the different hyphenation techniques for ICP-MS, laser ablation is found to be the most suitable one for SDS-PAGE separated proteins. Laser ablation of blot membranes after PAGE separation gives the required quantitative information of proteins with high accuracy as it has lesser volumes, minimal contamination and is easy to handle compared to gels. Based on these aspects a new laser ablation cell has been designed and adopted (Feldmann *et al.*, 2006, *J Anal Atom Spectr* 21, 1006). For concurrent determination of proteins in a sample, downstream applications such as immuno-blotting could be useful provided specific antibodies are available. We make use of this technique and of differentially labeled monoclonal antibodies for CYP1A1 and CYP2E1. The CYP1A1 antibody was labeled with Europium via a covalently coupled chelator (DOTA = 1,4,7,10-tetraazacyclo-dodecane-tetraacetic acid) and the CYP2E1 antibody was iodinated. Suitability of the labeled antibodies for detection of blotted microsomal CYPs by ICP-MS and the detection sensitivity will be demonstrated. Concomitant application of the two differentially labeled antibodies to determine several CYPs in one step was successful.

[1]Institute for Analytical Sciences, P.O. Box 10 13 52, D-44139 Dortmund, Germany
[2]Institute for Occupational Physiology at the University of Dortmund, Ardeystr. 67, D-44139 Dortmund, Germany

461

COMPARATIVE ANALYSIS OF N-NITROSOMORPHOLINE- INDUCED DIFFERENTIAL GENE AND PROTEIN EXPRESSION IN RAT LIVER

A. Oberemm[1], H. Ellinger-Ziegelbauer[2], H.-J. Ahr[2], M. Kröger[3], M. Weimer[4], C. Ittrich[4], A. Kopp-Schneider[4], H.-B. Richter-Reichhelm[1], U. Gundert-Remy[1]

Transcriptomics and proteomics were applied simultaneously to analyse global gene and protein expression changes in rat liver following application of *N*-nitrosomorpholine at a concentration of 120 mg/L in drinking water for 7 weeks. 1 day, 1 week, 3, 8, 12, 20, 30 and 50 weeks after the start of the study, 5 animals of each treatment group (vehicle-control + NNM) were killed and left liver lobes were sampled for biochemical and histopathological processing. Gene expression was analysed using the Affymetrix rat genome chip U34A. Analysis of protein expression was performed on the basis of 2D electrophoresis (IPG method) and complementary use of MALDI and ESI-MS/MS mass spectrometry. Time courses of deregulation were distinctly different between gene and protein expression: trancriptional changes were most pronounced after 3 weeks of exposure and sharply dropped down after termination of exposure. Most deregulated protein spots were observed after 8 weeks and maintained a higher level compared to genomics during the post-exposure phase. Qualitative changes were compared after editing expression profiles of genes, which were deregulated as proteins. Overall, 7 genes were shown to be deregulated on both the transcriptional and translational level. Limited overlap between genes and proteins might partly be explained by technical limitations of each approach: on the gene level, only protein identities can be detected, which are present as annotated probe sets on the chip. On the protein level, only molecules showing a pH > 4 < 8 and a molecular weight > 10 < 100 kDa can be resolved. The detection of membrane proteins is difficult and low abundance proteins like transcription factors cannot be analysed at all. For this study, 132 proteins were calculated which potentially could be identified as well on the gene as on the protein level.

[1]Federal Institute for Risk Assessment (BfR), D-14195 Berlin; [2]Molecular and Genetic Toxicology, D-42113 Wuppertal, [3]Merck KGaA, Institute of Toxicology, D-64293 Darmstadt, [4]German Cancer Research Centre, Biostatistics, D-69120 Heidelberg

462

A NOVEL GLUCOSYLTRANSFERASE FROM *LEGIONELLA PNEUMOPHILA* TARGETS EUKARYOTIC ELONGATION FACTOR 1A

Y. Belyi§ and K. Aktories#

Legionella pneumophila, the causal pathogen of Legionnaires' disease, is an intracellular parasite, which proliferates within the eukaryotic target cells. After several hundredfold multiplication within host cells, the pathogens are released for new invasion by induction of apoptosis or necrosis. *L. pneumophila* produces a glucosyltransferase (Lgt1), which selectively modifies a ~50 kDa mammalian protein by using UDP-glucose as a co-substrate.

Here we analyzed the eukaryotic target of the *L. pneumophila* glucosyltransferase and studied the functional consequences of the modification by glucosylation. For this purpose Caco-2 cell lysates were treated with Lgt1 in the presence UDP-glucose. Labelled proteins were isolated by gel electrophoresis and analyzed by mass spectrometry (Ricarda Niggeweg and Matthias Wilm, EMBL, Heidelberg). This identified the protein substrate of Lgt1 as the mammalian elongation factor 1A. Modification of elongation factor 1A by Lgt1 occurs at serine-53, which is located in the GTPase domain of the elongation factor. Introduction of Lgt1 into eukaryotic target cells (e.g., embryonic bovine lung cells) by electroporation caused morphological changes and death of target cells after 1 to 2 days. Glucosylation of elongation factor 1A by Lgt1 results in inhibition of eukaryotic protein synthesis in vitro and also in inhibition of incorporation of labelled methionine into proteins of target cells. The data show a novel type of inhibition of protein synthesis caused by *L. pneumophila* glucosyltransferase Lgt1 and have major impact for understanding of the host-pathogen interaction of *L. pneumophila.*

§ Gamaleya Research Institute, ulitsa Gamalei 18, Moscow 123098, Russia
#Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, Albertstr. 25, 79104 Freiburg

463

PHYTOCHEMICALS INCREASE THE BIOCHEMICAL BARRIER OF THE INTESTINE AGAINST BENZO[*A*]PYRENE

Bettina Ebert[1], Albrecht Seidel[2], Alfonso Lampen[1]

Benzo[*a*]pyrene (B[*a*]P), a prominent member of the group of polycyclic aromatic hydrocarbons (PAH), represents a model compound of food contaminants which has been well characterized. Due to its ubiquitous occurrence and its high carcinogenic potential, B[*a*]P is a food-contaminant of high toxicological relevance. Many PAH are known to induce their own metabolism by upregulating Phase I and Phase II xenobiotic metabolizing enzymes via aryl hydrocarbon receptor (AhR)-dependent pathways and thereby reduce their bioavailability. The present study aimed at the identification of the unknown transport-protein which mediates the transport of hydrophilic conjugates of B[*a*]P across the cell membrane of the human intestinal cell line Caco-2. Furthermore, we questioned whether this unknown transport-protein would be inducible by its substrate (B[*a*]P). Since many phytochemicals (e.g. flavonoids) have been reported to be weak AhR-agonists, it was of great interest whether these naturally occurring compounds have an impact on the expression of this transport-protein. The results of the present study show that in Caco-2 cells the ABC half-transporter BCRP (breast cancer resistance protein) is responsible for the apically transport of both Phase II metabolites of B[*a*]P, B[*a*]P-3-sulfate and B[*a*]P-3-glucuronide. The results further show that the expression of BCRP is most likely regulated via AhR-dependent pathways. In Caco-2 cells, BCRP-protein was inducible by some xenobiotics (established AhR-agonists, e. g. TCDD, benzo[*k*]fluoranthene) and some food-derived compounds (e. g. quercetin, dibenzoylmethane and the synthetic antioxidant TBHQ). Western Blot analysis of AhR-deficient MCF-7 AHR_{200} cells show a very low basal expression of BCRP protein and it is poorly inducible in these cells upon treatment with AhR-agonists. It can be concluded that BCRP has a protective role against dietary pro-carcinogens such as B[*a*]P. BCRP can be induced by naturally occurring compounds from plant foods which stresses the important role of non-nutritive compounds in the prevention of food-related cancer diseases.

[1]Abteilung Lebensmittelsicherheit, Bundesinstitut für Risikobewertung, 14195 Berlin, Germany; [2]Biochemical Institute for Environmental Carcinogens, Prof. Dr. Gernot Grimmer Foundation, 22927 Grosshansdorf, Germany

464

URINARY AND FAECAL EXCRETION OF MERCAPTURIC ACIDS OF ALKYLATED POLYCYCLIC AROMATIC HYDROCARBONS: DIRECTING ROLE OF ORGANIC ANION TRANSPORTERS (OATs)

N. Bakhiya, M. Batke, J. Laake, A. Seidel[1], W. Engst, H. R. Glatt

Alkylated polycyclic aromatic hydrocarbons are common environmental contaminants. Some congeners are metabolically activated via benzylic hydroxylation and sulfonation into electrophilic, mutagenic and carcinogenic sulfuric acid esters. To a certain extent, they may be detoxified by conjugation with glutathione, often followed by further processing to mercapturic acids. Thus, excreted mercapturic acids may be used as non-invasive biomarkers indicating the formation of electrophilic intermediates, such as benzylic esters. After treating rats with 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene, we analyzed urinary and faecal metabolites using LC-MS/MS. Benzylic mercapturic acids were detected after both treatments. Interestingly, 1-methylpyrenyl mercapturic acid (MPMA) was primarily excreted in urine (80 %

of the total urinary and faecal level), whereas 1,8-dimethylpyrenyl mercapturic acid (DMPMA) clearly preferred the faecal route (90 %). We then studied whether these mercapturic acids are substrates of renal basolateral OATs. Uptake was studied in control human embryonic kidney HEK293 cells and in derivative cell lines engineered for stable expression of human OAT1 and OAT3. The uptake rate of MPMA by OAT1- and OAT3-expressing cells was 3.0±0.2 fold and 1.7±0.1 fold higher than that by control cells, indicating that OAT1 and, at lesser degree, OAT3 are able to transport MPMA. In contrast, there was no significant difference in the accumulation of DMPMA between OAT-expressing and control cells. In conclusion, we show that a minute structural difference (the presence or absence of an additional methyl-group in an alkylated four-ring polycyclic hydrocarbon) can strongly direct the excretion route of mercapturic acids. Differential interactions with specific anion transporters appear to be involved in this modulating effect of the methyl group. – Research described in this abstract was supported by Philip Morris Incorporated.

German Institute of Human Nutrition Potsdam-Rehbrücke, Department of Toxicology; and [1]Biochemical Institute for Environmental Carcinogens, Großhansdorf

465

PROINFLAMMATORY CYTOKINES REGULATE THE TRANSEPHITELIAL BARRIER IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS FROM EXPLANT-OUTGROWTH CO-CULTURED WITH HUMAN LUNG FIBROBLASTS

C. Pohl[1], I. Hermanns[1], M. Bock[1], K. Kehe[2], C. J. Kirkpatrick[1]

Airway epithelial cells provide a barrier to the migration of toxic materials. Tight junctions (TJ) play a key role in maintaining barrier functions and are responsible for the selective regulation of various substances through the paracellular space. Primary isolated normal human bronchial epithelial cells from small bronchi (NHBE) were cultured with lung fibroblasts as a bilayer on a 24-well HTS-Transwell filter plate. The cells were grown on collagen type I and maintained at an air-liquid interface (ALI) by feeding basolaterally with medium. Barrier properties and morphological phenotype were compared over 28 days. The integrity of the tight junctional complexes within the cells was assessed by staining for the tight junctional structural protein ZO-1 and occludin and the intercellular adhesion molecule (AJ) (ICAM-1) following immunofluorescence microscopy. NHBE in bilayer co-culture form a tight epithelial barrier with average TER-values of 583 ± 148 Ω*cm^2, correlating with a continous expression of TJ. To determine if the treatment with the combination of the cytokines TNF-α (10ng/ml) and IFN-γ (100ng/ml) modifies TER and the permeation of sodium fluorescein, cells were treated apically and basolaterally for 24 and 48h. After 24h there were no significant effects. After 48h exposure TER decreased by up to 60% to 50% of control cultures. Furthermore, increases in P_{app} after 48h were detected. With both apically and basolaterally stimulated cells the P_{app} was 4-fold greater than in untreated cells. The cytokines induce changes in the integrity of the TJ, leading to a decreased barrier function. Increased AJ levels may lead to accelerated sodium absorption. These data indicate that the exposure of the co-culture of NHBE and human lung fibroblasts to TNF-α and IFN-γ induced significant changes in the barrier functions and lead to alterations in ion transport.

[1] *Institute of Pathology, Repair-Lab, Johannes Gutenberg University Mainz*
[2] *Institute of Pharmacology and Toxicology, Bundeswehr Munich*

466

INFLUENCE OF SUBSTRATE INTERACTIONS AT IM- AND EXPORT CARRIERS ON HEPATIC TOXICITY IN VITRO

C. Kneuer, K. Sommer, F. Demiraj, F.R. Ungemach, W. Honscha

For various drugs and toxins, transmembrane transport activity is one major determinant of intracellular concentration. The simultaneous expression of import and export carriers in one cell type and their overlapping specificity complicates predictions about the contribution of a single transporter to the intracellular toxicokinetics of a certain molecule. We therefore evaluated the interaction between carrier substrates in HPCT-1E3 cells as in vitro model of the hepatocyte. This cell line was previously found to possess a liver-like carrier expression pattern. HPCT-1E3 cells were seeded in 96well-plates at 15.000/well. The day later, two different substrates of either MDR, MRP, OAT or OATP family members were added in combination: substance A was applied at increasing concentrations to determine its toxicity (IC_{50}); substance B was added at sub-toxic concentrations known to inhibit the carrier under investigation. Cell viability was assessed after 48h using the MTT assay. Toxicity of carrier substrates was also compared between HPCT and HepG2 cells. Inhibition of toxin export by interference at a carrier of the MRP or MDR family, both known to extrude substances from cells, did frequently enhance its toxicity. For example, 15 or 50 µg/ml verapamil decreased the IC_{50} of terfenadine from 13.6 (12.3-15.0) to 11.0 (9.5-12.9) and 6.9 (6.0-7.8) µg/ml, respectively. Hill Slopes of the sigmoidal dose-response curves also decreased from 2.8 to 1.4 and 0.4. The majority of substrates for import carriers, however, did not result in interactions that were measurable as increase in toxicity. Nevertheless, the overall activity of import carriers may influence toxicity in liver cells, as HPCT cells were more sensitive to pravastatin (OATP), ketoconazol and methotrexate (OAT, Rfc1) than HepG2 cells. We conclude that liver cell toxicity is modulated by interference with export protein activity, while inhibition of one of many import carriers with overlapping substrate spectra is more likely to be compensated.

Institute of Pharmacology, Pharmacy und Toxicology, University of Leipzig, An den Tierkliniken 15, D-04103 Leipzig, honscha@vetmed.uni-leipzig.de

467

INVOLVEMENT OF CYSTEINE RESIDUES IN PROCESSING OF *CLOSTRIDIUM DIFFICILE* TOXINS A AND B

M. Egerer, T. Jank, T. Giesemann and K. Aktories

Clostridium difficile toxins A and B are the cause of antibiotics-associated diarrhoea and pseudomembranous colitis, occurring during treatment with antibiotics. Whereas the toxins' mode of action (glucosylation of Rho GTPases) is well characterized, their entry into target cells is still largely unknown. The toxins bind to not well characterized receptors and are then endocytosed. At the low pH of endosomes the toxins insert into membranes and form pores. It is suggested that the toxins translocate through the pores into the cytosol. Recently, it was shown that only the catalytic domain of toxin B, covering the N-terminal 543 amino acid residues, enters the cytosol, whereas the middle part and C-terminus of this large toxin is not translocated.

Here we studied the processing of *Clostridium difficile* toxin A and B. To get information about the proteases involved in toxin processing, we employed protease inhibitor studies. In order to describe the region necessary for recognition by the protease, we deleted the C-terminus and various parts of the middle portion of the toxins. In addition we changed the region of the cleavage site by site-directed mutagenesis to get information about the recognition sequence of the cleaving protease. Cleavage of toxin A and B was inhibited by addition of *N*-ethylmaleimide (NEM) indicating involvement of cysteine residues in toxin cleavage. To clarify the role of cysteine residues in toxin processing several mutants were made in which cysteine residues of toxin A and B were changed to alanine. These mutant toxins exhibited reduced susceptibility for proteolytic cleavage. Changes in specific cysteine residues also reduced the potency of the toxins to cause cytopathic effects in cultured cells. The data indicate that cysteine residues are important for proteolytic cleavage and biological activity of the toxins.

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Freiburg, Albertstr. 25, 79104 Freiburg

468

FUNCTIONAL ROLE OF A POLY-PROLINE STRETCH FOR THE UPTAKE OF THE ADP-RIBOSYLTRANSFERASE SPVB FROM *SALMONELLA ENTERICA* IN MAMMALIAN CELLS

E. Kaiser and H. Barth

Salmonella enterica, a facultative intracellular pathogen, causes human diseases ranging from gastroenteritis to severe systemic infections. *Salmonella* produces a variety of virulence factors to promote the invasion of the intestine, and to mediate systemic disease. The SpvB protein acts as an ADP-ribosyltransferase modifying actin and thereby destructing the host cell cytoskeleton. Biochemical studies on intact cells are limited, as the SpvB protein is not taken up when applied to cultured cells. To deliver the catalytic domain of SpvB (C/SpvB) into cells, a fusion toxin was constructed (C2IN-C/SpvB), which uses the binary *C. botulinum* C2 toxin as a cellular delivery system. The fusion toxin consists of C/SpvB and the N-terminal domain of C2I, responsible for cell entry. As the wild type SpvB protein contains 7 proline residues connecting its N-terminal and C-terminal domain, this proline stretch was also introduced in the C2IN-C/SpvB fusion toxin. To determine the functional role of this proline stretch, different fusion toxins were constructed containing either no, 5, 7 or 9 proline residues. In a cell free system, the four fusion toxins ADP-ribosylated actin independently of the length of the proline stretch. We demonstrated that the cytopathic effect of the fusion toxins on intact cells directly correlated with the length of the proline stretch: the more prolines were present, the more cells were rounded up due to the destruction of the cytoskeleton. However, the fusion toxin containing no proline stretch did not induce a cyctopathic effect. We have experimental evidence that the presence and length of the proline stretch determines the translocation efficiency of the toxins across the cell membrane. The proline stretch, which connects the two domains, may enhance the flexibility of the fusion toxin to facilitate its unfolding and/or refolding during translocation across membranes.

Institut für Pharmakologie und Toxikologie, Universität Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

469

ESTABLISHMENT AND VALIDATION OF A PULMONARY *IN VITRO* MODEL Calu-3 AND INVESTIGATION OF ITS TRANSPORT ACTIVITIES

B. Baumstümmler, U. Bock, A. Kraft, E. Haltner-Ukomadu

The objective of the study was to establish and validate pulmonary an *in vitro* cell model for transport and toxicity studies. For that purpose Calu-3 cell line was established under liquid covered conditions (LCC) and characterized in transport experiments. Calu-3 is derived from a human bronchial adenocarcinoma and features epithelial morphology, adherent monolayer growth, tight junctions and Multi-drug Resistance (MDR1, MRP1, OCT, OAT) as well as lung specific proteins (LRP, CFTR, Caveolae). During the validation steps culture conditions and thresholds for TEER (Trans Epithelial Electrical Resistance) representing the cell integrity and tight growth as well as P_{app} (Apparent Permeation Coefficient) values representing permeation qualities were determined.

With a relevant subset of substances characteristical aspects were analyzed like cell integrity and functionality of tight junctions (^{14}C-Mannitol, paracellular marker), lipophilic transcellular transport (^{3}H-Propranolol), presence and activity of transporter proteins. ^{3}H-Digoxin is a substrate for MDR1 (Multi-drug Resistance1), Rhodamine123 is transported by MDR1, MRP1 (Multi-drug Resistance related Protein) and OCT (Organic Cation Transporter), Fluorescein is supposed to be actively transported by OAT (Organic Anion Transporter) but is often used as low permeable marker. Cyclosporine A and Verapamil were applied as competitive MDR1 inhibitors. Finally, known pulmonary drugs (Budesonide and Salbutamol) were tested.

(i) Stable TEER values during transport and non-directional low permeation of Mannitol demonstrate dense cell growth and functional tight junctions. (ii) High permeation of 3H-Propranolol, Budesonide (transcellular) and low permeation of Salbutamol (paracellular) approve non-directional diffusion. (iii) Directional carriage of transporter protein substrates and successful competitive inhibition point to the presence and activity of transporter proteins. Even Fluorescein showed directional transport as a prove for OAT activity.

All this facts lead to the conclusion that Calu-3 cell line with epithelial monolayer growth, functional tight junctions and transporter proteins can be used for *in vitro* transport and toxicity studies for respiratory uptake.

Across Barriers GmbH, D-66123 Saarbrücken, Germany

470

INTELLIGENT IVIV TESTING – RATIONALE SCHEME FOR FAST TRACK DEVELOPMENT

U. Bock[1], M. Maringer[2], B. Lecher[2], E. Haltner-Ukomadu[1]

The fast track development of an oral dosage form for a toxic active compound (API) in a cascade of *in vitro* and *in vivo* investigations will be presented. As a consequence of the individual testings an intelligent in vitro – in vivo (IVIV) testing strategy will be deducted. The active drug (API, acidic structure) has been stopped during the development of the oral dosage form as a result on inadequate pharmacokinetic and toxic properties. Therefore as an alternative

for the highly potent API a prodrug concept (ester of the API) was developed. The modification of esterification of the API include the variation of the lipophilic chain of the API for solubility and stability. The presented investigations concern on the physicochemical, ADMET and compatibility parameters of the prodrug and the corresponding API. A detailed focus will be driven on the drug interaction of the prodrug/API according to the FDA draft guidance for drug interaction studies (FDA 2006). In accordance to the recommendations of GLP (OECD 2004), GCCP (Coecke 2005) and FELASA (Working Group on Health Monitoring of Rodent and Rabbit Colonies, 2001) quality controls of the individual in vitro and in vivo assays will be presented. The intelligent IVIV profiling for oral dosage forms indicate a useful tool to i) proof a drug delivery strategy, ii) select modifications of the API and iii) develop an adequate formulation of the API. The individually tailored, staged combinations of in vitro and in vivo assays noticeably shorten the development times and costs. Taken into account the present scheme for other dosage forms like topical or inhalative formulations we will adopt the scheme for individual demands of the applications and the project requests (Pharma, Biotech, Cosmetics, Drug Discovery, Drug Delivery) to the present IVIV profiling. Furthermore the IVIV profiling will be a useful tool in the safety evaluation of APIs, excipients (or formulations) for pharmaceutics, cosmetics and chemicals in the context of REACH.

[1] Across Barriers GmbH, 66123 Saarbrücken, Germany
[2] MFD Diagnostics GmbH, 55234 Wendelsheim, Germany

471

AT_1 RECEPTOR-MEDIATED GENOTOXICITY OF ANGIOTENSIN II

U. Schmid[1], N. Schupp[1], L. Krens[1], A. Heidland[2] and H. Stopper[1]

Increased activity of the renin angiotensin system with increased levels of Angiotensin II (AngII) leads to hypertension, oxidative stress with endothelial dysfunction and atherosclerosis. On top, epidemiological studies revealed higher cancer mortality and an increased kidney cancer incidence in hypertensive patients. The increased AngII level might contribute to carcinogenesis. Treatment of rat, porcine and human kidney cells with AngII showed different effects on the expression of the components of the renin angiotensin system. Renin, angiotensin converting enzyme (ACE) and angiotensinogen were up-regulated in all three cell lines. The AngII type 1 receptor (AT_1) shows an up-regulation in the rat cells, which indicates a positive feedback mechanism, and a down-regulation in the porcine and human cells, a fact that could suggest a protective mechanism. AngII in concentrations, which can be expected in primary urine of hypertensive persons, induced oxidative stress and subsequently DNA damage in several kidney cell lines and human blood cells. The AngII-induced oxidative stress could be decreased by co-incubation with the AT_1-antagonist candesartan as shown by flow cytometry. The DNA damage which was detected by comet assay and micronucleus frequency test could be reduced by co-incubation with candesartan whereas an AT_2-antagonist had no effect. This implies a role only for the AT_1 receptor and its downstream signalling in AngII-induced DNA damage. A time course of AngII-mediated oxidative stress that was measured by laser scan microscopy as well as flow cytometer showed that reactive oxygen species are released at multiple time points. This suggests the activation of different signalling pathways: phospholipase C, protein kinase C and Ca-release. Inhibitors of components of these pathways will indicate which one is involved in AngII-induced DNA damage.

[1]Institute of Toxicology, University of Würzburg, Germany, [2]Department of Internal Medicine, University of Würzburg, Germany

472

CAMPHORQUINONE INDUCES CYTOTOXIC AND GENOTOXIC EFFECTS IN PRIMARY HUMAN GINGIVAL FIBROBLASTS

J. Volk[1], W. Geurtsen[3], G. Leyhausen[1], H. Günay[1] , A. Beckedorf[1], H. Rahmer[2], and C. Ziemann[2]

Leaching of substances from resin-based dental materials has a potential impact on the biocompatibility as well as safety of these materials. One of the main identified leachables is camphorquinone (CQ). CQ is the most important photoinitiator used in dental adhesives and resin composites. Aim of this study was to evaluate the cytotoxic and genotoxic potential of CQ (0.05 to 2.5mM) on adherent primary human gingival fibroblasts (HGF) in the absence of visible-light (VL)-irradiation. Cell viability, levels of intracellular reduced glutathione (GSH), and intracellular generation of reactive oxygen species (ROS) were assessed by fluorescence spectroscopic assays, pro-apoptotic effects were evaluated on the level of DNA-fragmentation, and genotoxicity was investigated using the comet assay. During 3h of incubation in the dark CQ induced rapid concentration-dependent formation of ROS in HGF, without reducing cellular viability, even at higher concentrations. The maximum amount of ROS was found at a concentration of 2.5mM CQ, which was equivalent to a ~50-fold increase, as compared to untreated controls. After 24h of incubation, however, CQ concentrations of $\geq$ 1mM induced a significant reduction in cell number and cell viability with a $TC_{50\%}$ of about 2.5mM and 2.3mM CQ, respectively. After an incubation period of 16h with 2.5mM CQ, there was evidence of apoptotic DNA-fragmentation in HGF. Increase of ROS production, observed after 1.5 and 3h of 'CQ incubation', was followed in both cases by a marked GSH depletion. The comet assay revealed a significant induction of DNA-damage due to CQ, which was very likely caused by ROS. Therefore, the generation of oxidative DNA-damage and the effects of antioxidants will be further investigated. In conclusion, CQ, even without VL-irradiation, exhibits cytotoxic, genotoxic, and pro-apoptotic potential in primary HGF, which is most likely correlated to ROS generation and a reduced antioxidant capacity of the cells due to GSH depletion.

[1]Medical University of Hannover, Department of Conservative Dentistry and Periodontology, Hannover, Germany; [2]Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany; [3]University of Washington, Department of Restorative Dentistry/Division of Operative Dentistry, Seattle, USA

473

RELEVANCE OF ARYLHYDROCARBON RECEPTOR (AhR) REGULATED PHASE I ENZYMES FOR THE CARCINOGENICITY OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD)

S. Knerr [1] A. Mally [2], W. Dekant [2] and D. Schrenk [1]

The carcinogenicity of dioxins is mostly investigated based on the "prototype" compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In 1997 TCDD was classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) though the the mechanisms of TCDD´s carcinogenic effects are to a great extent still unknown. It was hypothetised that TCDD causes increased levels of reactive oxygen species (ROS) via an arylhydrocarbon receptor (AhR) mediated induction of phase I enzymes, especially of CYP1A1. This may lead to DNA damage, mutations and cancer. Microarrays representing genes involved in stress and toxicity were performed with hepatic RNA isolated from TCDD-treated wild type C57/BL6, and from transgenic mice, expressing a constitutively active AhR (CA-AhR). They were compared to the respective vehicle-treated controls. Microarray data indicated, among others, differential expression of hepatic genes involved in apoptotic processes for both the TCDD treated and the transgenic mice. Additionally it was shown that a (permanent) activation of the AhR in CA-AhR mice causes increased hepatic oxidative DNA damage, measured as 8-oxo-desoxyguanosine (8-oxo dG).

Using Supersomes™ (microsomes prepared from insect cells, expressing various single recombinant human or rat CYP enzymes) the relative ROS formation potencies of single CYP enzymes as well as the influence of selected test substances on ROS formation was investigated using the H_2DCFDA assay. Addition of various inhibitors and substrates of CYP1A enzymes (benzo[*a*]pyrene, 8-methoxypsoralene, 17ß-estradiol) did not change or decreased ROS formation. These findings do not support the hypothesis that estradiol-dependent redox-cycling plays a role in TCDD mediated liver tumor formation in female rats. In general the hypothesis was confirmed that AhR regulated CYPs are a potential source for ROS formation.

[1] University of Kaiserslautern, Food Chemistry and Environmental Toxicology, Erwin-Schroedinger-Str. 52, 67663 Kaiserslautern, Germany
[2] University of Würzburg, Institute of Toxicology, Versbacher Str. 9, 97078 Würzburg, Germany

474

THE CARCINOGEN AZOXYMETHANE CAUSES NECROSIS AND OXIDATIVE STRESS IN RAT LIVER WITHIN 48 HOURS AFTER INJECTION, A TOXIC EFFECT THAT IS HOWEVER STRONGLY MITIGATED BY PRETREATMENT WITH THE COFFEE COMPONENTS KAHWEOL AND CAFESTOL

W.W. Huber[a], E. Haslinger[a], D. Schachner[a], W. Parzefall[a], B. Grasl-Kraupp[a], A. Hochreiter[a], H. Lang[b], S.V. Torti[c], F.M. Torti[c], R. Schulte-Hermann[a]

Coffee consumption reduces the rate of colon and liver cancers and possibly of further cancers. Out of the >1000 coffee components, an important role in this chemoprevention appears to be played by kahweol and cafestol (K/C), two diterpenes contained in the coffee bean and in unfiltered drinking preparations. In several rodent studies, partly involving liver and colon, pretreatment with K/C reduced mutagenicity/tumorigenicity of several compounds relevant to humans. These observations could be largely explained by the wide pattern of effects of K/C on xenobiotic metabolism, resulting in diminished activation and enhanced detoxification of carcinogens. Moreover, K/C increased the hepatic repair capacity towards alkylated DNA which is e.g. caused by azoxymethane (AOM), a model carcinogen in colon and liver. In the present study, possible acute hepatotoxic effects of AOM in the male F344 rat were investigated at 12, 24, 36 and 48 hours after exposure (30 mg / kg body weight, i.p.). Furthermore, we monitored possible protection by K/C pretreatment (0.2%, 10 days, feed) against such early AOM-related damage. Indeed, AOM caused acute hepatotoxicty, i.e. increased serum ALT and liver necrosis, as well as hepatic oxidative stress, shown by higher levels of lipid hydroperoxides in the ferrous oxidation in xylenol orange [FOX] assay (however not in TBARS and MDA assays) and by enhanced liver ferritin. Methyl nitrosurea, another alkylating carcinogen, did not lead to any significant changes in ALT and oxidative stress. Increases in ALT (and marginally ferritin) by AOM displayed a biphasic course with a first peak at 12 hrs whereas increases in the FOX assay occurred in a single peak on day 2 only, suggesting the involvement of more than one AOM-related damaging mechanism. Notably, K/C mitigated all described indicators of AOM-related toxicity, e.g. only 2-fold vs. >3-fold maximum increase in the FOX assay and no change vs. 5-fold increase in liver ferritin. This antioxidant capacity of K/C may be partly explained by increases in hepatic glutathione and NAD(P)H:quinone oxidoreductase 1 activity (3-fold and 5-fold respectively). Importantly, AOM alone did not change both of these antioxidant mechanisms over the 48 hour observation period.

[a]Div. Institute of Cancer Research, Dept. of Medicine I, Medical University of Vienna, Austria, [b]Allg. Öffentl Krankenhaus Wr. Neustadt, Austria, [c]Dept. of Biochemistry, Wake Forest University, Winston-Salem, N.C., USA. Supported in part by Herzfelder´sche Familienstiftung.

475

ROSUVASTATIN PROTECTS AGAINST OXIDATIVE STRESS AND DNA DAMAGE BY UPREGULATION OF THE CELLULAR ANTIOXIDANT DEFENSE

N. Schupp[1], U. Schmid[1], A. Heidland[2] and H. Stopper[1]

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, may exert a number of beneficial effects independently of cholesterol reduction, including an antioxidative action. Human promyelocytic cells (HL-60) were used to examine the effect of rosuvastatin (RSV) on reactive oxygen species (ROS)-induced DNA damage, formation of ROS and expression enzymes of the cellular antioxidant defense system. DNA damage caused by phorbol 12-myristate 13-acetate (PMA) or by hydrogen peroxide (H_2O_2) was assessed by the comet assay. Oxidative stress induced by PMA was measured by flow cytometry and by quantification of the glutathione levels in the cells. The effect of RSV on the expression of enzymes of the glutathione metabolism and on heme oxygenase-1 was analysed by RT-PCR. DNA damage induced by PMA or by H_2O_2 was reduced to control values by RSV concentrations starting from 10 nM. PMA-provoked formation of ROS was also prevented by RSV. Intermediates of the cholesterol synthesis pathway did not reverse the reduction of DNA damage by RSV or the prevention of ROS formation. RSV restored the glutathione levels in cells treated with PMA and even after specific glutathione depletion by buthionine-sulfoximine. RT-PCR showed that the expression of enzymes of the glutathione metabolism were increased by RSV compared to PMA treatment. In addition, the expression of heme oxygenase-1 was significantly higher than in PMA-treated and control cells. RSV, in a concentration comparable to plasma levels in therapy, is capable of preventing oxidative stress and subsequent DNA damage. This effect seems to be mediated by strengthening of the antioxidant defense of the cells independently of HMG-CoA reductase inhibition.

[1]Institute of Pharmacology and Toxicology, University of Würzburg, Germany, [2]Department of Internal Medicine, University of Würzburg, Germany

476

ANTHOCYANIN/POLYPHENOLIC RICH FRUIT JUICE REDUCES OXIDATIVE CELL DAMAGE IN AN INTERVENTION STUDY WITH PATIENTS ON HEMODIALYSIS

Spormann, T.[1], Albert, F.W.[2], Rath, T.[2], Dietrich, H.[3], Will, F.[3], Eisenbrand, G.[1] and Janzowski, C.[1]

Patients with chronic renal failure undergoing hemodialysis (HD) are considered to face an elevated risk for cancer, artheriosclerosis and other diseases. This has been attributed in part to increased oxidative stress, resulting from bioincompatibility of the extracorporal blood circuit, malnutrition and other factors. Uptake of fruit juice with especially high flavonoid/polyphenol content has been shown to reduce oxidative cell damage in healthy probands (Weisel *et al.* Biotechnol. J., 1, 388, 2006) and might also be utilized as preventive measure in HD-patients. An intervention study was performed with mixed red fruit juice (total phenols: 3478 mg/L; trolox equivalent antioxidant capacity, TEAC: 31.3 mmol/L trolox). After a three week run-in phase, 20 HD-patients (non-smokers, stable HD) consumed for 4 weeks 200ml/d of the juice, followed by a three week wash-out phase. Blood sampling was performed weekly. DNA damage (COMET assay with/without formamidopyrimidine-DNA-glycosylase) and glutathione (kinetic photometric assay) in whole blood were monitored. Additionally, lipid peroxidation (HPLC/fluorescence of malondialdehyde), protein oxidation (carbonyls, photometric assay) and TEAC were determined in plasma; DNA binding capacity of the transcription factor NfκB (ELISA) was monitored in primary blood mononuclear cells. First results (from 13 patients) show a strong decrease of total DNA damage (with FPG) during juice uptake (mean TI%: run-in: 5.3/juice uptake: 3, $p<0.0005$; juice uptake: 3/wash-out: 3.9, $p<0.0005$), basic DNA damage (without FPG) was also reduced (mean TI%: run-in: 0.61/juice uptake: 0.51, $p<0.05$). Correspondingly, a decrease of protein oxidation ($p<0.05$) and lipid peroxidation ($p<0.01$) and an increase of total glutathione ($p<0.0005$) and of glutathione status ($p<0.0005$) were observed during the intervention, whereas the TEAC was not modulated. In conclusion, the red fruit juice clearly reduces oxidative cell damage in HD-patients. This effect can be attributed to its high content of flavonoids/polyphenols (Supported by Karin Nolte Foundation).

1: Technical University, Division of Food Chemistry&Environmental Toxicology, Kaiserslautern, Germany; 2: Westpfalz-Klinikum, Kaiserslautern, Germany; 3: Research Institute Geisenheim, Dept. of Wine Chemistry and Beverage Technology, Geisenheim, Germany

477

ORGANIZATION OF MITOCHONDRIAL DNA

J. Kienhöfer[1], M. Bachschmid[2], M. Moreno-Villanueva[1], V. Ullrich, A. Bürkle[1]

In our previous work we isolated intact nucleoids from rat and cattle (N.-S. Arch. Pharmacol., Volume 372, Supplement 1). We found that the Mn-SOD (SOD 2) as well as glutathione peroxidase is part of the nucleoid complex. In our current work we isolated intact human nucleoids and we found also in these nucleoids an association of the mitochondrial DNA (mtDNA) with the Mn-SOD. The isolation of the nucleoids was done by a density-gradient centrifugation and verified by the presence of the mitochondrial Transcription factor A (mtTFA) and the Polymerase gamma (Pol. γ), shown by several other groups that they are part of the nucleoids in mitochondria. We confirmed our results by a co-immunoprecipitation with several antibodies. Thereby it was possible to precipitate the nucleoids and to detect Mn-SOD by western blot. This finding could be an attractive basis for the explanation of fundamental processes during aging and the pathophysiology of some chronic diseases. It has been reported for the aging process, that 8-oxoguanine in the mtDNA is present in a much higher amount in old animals. This is supporting the assumption of increased oxidative damage in mitochondria, which could be due to an increased inactivation of Mn-SOD as previously shown by Tyr-nitration of this enzyme in old rats (van der Loo B. *et al.*, J. Exp. Med. 2000; 192: 1731-1744). In order to establish a highly sensitive assay to detect 8-oxoguanine we modified the automated version of the fluorescence-detected alkaline DNA-unwinding (FADU) assay. By the use of an 8-oxoguanine DNA glycosylase (Fpg) each 8-oxoguanine indirectly generates one single-strand break and it was possible to measure this generated single-strand break by the FADU assay. Preliminary data indicate that increased levels of 8-oxoguanine can be conveniently detected by this method in aging rats and mice.

[1] Molecular Toxicology Group, Department of Biology, University of Konstanz, Germany
[2] Vascular Biology Unit, Boston University School of Medicine, USA

478

COMPARISON OF HOMOVANILLIC ACID AND AMPLEX RED IN HORSERADISH PEROXIDASE-CATALYZED H_2O_2 DETECTION

K. Staniek, H. Nohl

Reactive oxygen species, including H_2O_2, are produced in various biological systems and were assumed to be involved in several physiological and pathophysiological processes. A prerequisite for the quantification of low concentrations of H_2O_2 formed in biological systems are sensitive and reliable detection assays. Exploring suitable detection systems for mitochondrial H_2O_2 production different enzyme-catalyzed redox reactions were tested. The enzymatic determination of H_2O_2 by horseradish peroxidase (HRP) is based on the oxidation of different H-donating substrates accompanied by changes of their spectrophotometric or fluorimetric properties. Homovanillic acid (HVA) and Amplex Red, both being nonfluorescent compounds, are oxidized by HRP to the fluorescent HVA dimer (λ_{ex} = 312 nm; λ_{em} = 420 nm) and resorufin (λ_{ex} = 563 nm; λ_{em} = 587 nm), respectively. The specificity of the assays was confirmed by catalase which dose-dependently inhibitted the H_2O_2-induced fluorescence increase. Both H-donors for HRP, namely Amplex Red and HVA, were applied to the H_2O_2-generating glucose / glucose oxidase system and compared for their sensitivity. The addition of bovine serum albumin, which is frequently used in mitochondrial or cell incubation media, significantly decreased the fluorescence intensity of the Amplex Red oxidation product while the fluorescence intensity of the HVA dimer was significantly increased in 0.15 M KCl but not in 0.3 M sucrose. The mitochondrial H_2O_2 formation was studied in antimycin A-inhibited succinate-respiring rat heart mitochondria in the presence and absence of superoxide dismutase which catalyzes the dismutation of primarily produced superoxide radicals to H_2O_2. Amplex Red turned out to be a more sensitive analytic tool for H_2O_2 detection than HVA.

Research Institute of Biochemical Pharmacology and Molecular Toxicology, University of Veterinary Medicine, Vienna, Austria

479

FORMATION OF FREE RADICAL ADDUCTS FROM DIFFERENT ETHYL-SUBSTITUTED EMPO-DERIVATIVES

K. Stolze[1], N. Rohr-Udilova[2], T. Rosenau[3], A. Hofinger[3], and H. Nohl[1]

Oxygen-centered radicals are playing a major role in the onset of many diseases. We therefore developed a series of novel spin traps in order to identify and localize free radicals in biological systems. Namely, the spin trapping behaviour of four ethyl-substituted EMPO derivatives, 5-ethoxycarbonyl-3-ethyl-5-methyl-pyrroline *N*-oxide (3,5-EEMPO), 5-ethoxycarbonyl-4-ethyl-5-methyl-pyrroline *N*-oxide (4,5-EEMPO), 5-ethoxycarbonyl-5-ethyl-3-methyl-pyrroline *N*-oxide (5,3-EEMPO), and 5-ethoxycarbonyl-5-ethyl-4-methyl-pyrroline *N*-oxide (5,4-EEMPO) towards a series of different oxygen- and carbon-centered radicals is described. Considerably different stabilities of the superoxide adducts (ranging from about 12 to 35 min), a considerable improvement compared to the commercially available spin trap DMPO ($t_{1/2}$ ca. 45 sec). The structure of all compounds was confirmed by ^{1}H and ^{13}C-NMR. Furthermore, spin adducts obtained from different carbon-centered radicals derived from methanol, ethanol, formic acid and linoleic acid hydroperoxide have also been characterized.

[1]Research Institute of Pharmacology and Molecular Toxicology, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria
[2]Institute of Cancer Research, Medical University of Vienna, A-1090 Vienna, Austria
[3]Department of Chemistry, University of Natural Resources and Applied Life Sciences (BOKU), Muthgasse 18, A-1190 Vienna, Austria

480

A HUMAN HEPATOCYTE LONG-TERM CULTURE SYSTEM SERVES AS A DRUG TESTING ALTERNATIVE TO ANIMAL MODELS: RESULTS OF METABOLIC ASSAYS AND ELECTRON MICROSCOPY

A. Ullrich[1], D. Beer Stolz[2], E.C. Ellis[3], S.C. Strom[3], G.K. Michalopoulos[3], C. Berg[1], J.G. Hengstler[4], and D. Runge[1]

Human hepatocytes are the *in-vitro* system of choice to study drug-induced processes in man. We have developed and validated HEPAC[2]: a serum-free long-term culture system for human hepatocytes. Cellular viability and liver functions (urea and albumin production) were monitored daily. These functions remained relatively constant for up to 3 weeks. We used HEPAC[2] to study the effects of repetitive drug treatments on hepatocellular functions and morphology. Acetaminophen (APAP) was used as a model substance. Hepatocytes were exposed to 18.6 mM APAP for 24 h. Subsequently, culture medium was replaced by medium without APAP and the same exposure scenario was repeated every 4 days. During APAP treatment urea and albumin secretion were reversible reduced by 15-30% and 70-80%. Cytochrome P450 2E1 and 1A2 were active for at least 3 weeks, since cellular response to APAP did not change during the first 4-5 cycles of exposure to APAP. Electron microscopy revealed that APAP led to a complete replacement of rough ER by smooth ER and degradation of glycogen. After removal of APAP, hepatocytes refilled their glycogen stores within 1 day, while it took about 2-3 days for complete regeneration of rough ER. Following the exposure protocol, the same ultrastructural changes were also found after the 3rd and 5th treatment of human hepatocytes with 18.6 mM APAP. Metabolism of APAP via glucuronidation and sulfation was analyzed by HPLC. Our data demonstrate the suitability of HEPAC[2] to serve as a tool for repetitive screening of drug-mediated changes on hepatocyte functions. Furthermore, it may help to overcome the sparse availability of human hepatocytes for testing drug-mediated responses in man.

[1]PRIMACYT GmbH, Schwerin, FRG, Dept. of [2]Cell Biology and [3]Pathology, University of Pittsburgh, [4]Center for Toxicology, University of Leipzig

481

FUNCTIONAL CHARACTERISATION OF LONG-TERM PRIMARY RAT HEPATOCYTE CULTURES AS A MODEL FOR REPEAT-DOSE TOXICITY

G. Tuschl*, J. Hrach*, P. G. Hewitt and S. O. Mueller

Assessment of repeat-dose toxicity *in vitro* is a challenging and important goal in predictive toxicology. For that, the preservation of *in vivo*-like liver functions in primary hepatocyte cultures is essential. We compared primary rat hepatocytes cultured with either serum-containing or serum-free medium for two weeks on a dried collagen film (monolayer) or between two layers of gelled collagen (sandwich). General gene expression analysis in differentially cultured hepatocytes revealed distinct patterns over time. Hierarchical clustering of gene expression data showed a destabilisation in both monolayer and the serum-containing sandwich culture. In contrast, after an initial adaptation period, the serum-free sandwich culture displayed stable expression over time. In addition, we analyzed the drug metabolizing cytochrome P450 enzymes (CYPs) as functional endpoints. While CYP1A2 gene expression was reduced under each of the investigated culture conditions, CYP2C and CYP3A1 were stably expressed in serum-free cultures. CYP1A induction diminished completely in monolayer cultures but was preserved in sandwich cultures. The three-dimensional extracellular matrix environment along with serum-free medium was also necessary for the responsiveness to Phenobarbital-mediated CYP2B induction. Interestingly, although transcription of CYP2C and CYP3A genes was strongly reduced under serum-containing conditions, induction occurred in sandwich cultures with or without serum equally. In the liver, conjugated metabolites are excreted into the bile. Sandwich cultured primary rat hepatocytes reorganise into cellular aggregates displaying the polar differentiation status of hepatocytes by the formation of bile canaliculi-like structures. The stability of these structures is especially maintained under serum-free conditions. Although there is no explicit difference in gene expression levels of apical membrane transporters, we showed by fluorescence microscopy that transporter-dependent excretion of a fluorescein derivative and accumulation in bile canaliculi was conserved over time particularly in serum-free sandwich cultures. Taken together, these results show that the serum-free sandwich culture of primary rat hepatocytes seems most suitable for the assessment of liver cell functions *in vitro*.

*both authors contributed equally to this work

Molecular Toxicology, Institute of Toxicology, Merck KGaA, 64271 Darmstadt, Germany

482

LPS-INDUCED GENE EXPRESSION CHANGES IN PRECISION-CUT RAT LIVER SLICES

N. Zidek[1], D. Müller[2], P.G. Hewitt[1]

Precision-cut liver slices (PCLS) represent the multicellular, structural and functional features of an *in vivo* tissue, making the use of this *in vitro* system very attractive. It has been shown that cultured rat PCLS are a suitable model to study metabolic pathways and xenobiotic metabolism. The aim of the current study is to examine whether an inflammatory process affects liver metabolism based on mRNA expression profiles and to compare to the effects of lipopolysaccharide (LPS)-induced gene expression *in vivo*. Briefly, PCLS were prepared from rats on three occasions using the Krumdieck Tissue Slicer and incubated in a shaken flask system. Following 2h pre-incubation, medium was replaced with medium containing 10 and 50µg/ml LPS for 6h, and then further incubated with medium without LPS. Samples were taken after 6h, 24h and 48h for gene expression analysis on Affymetrix RAE 230A Microarrays in accordance with the *in vivo* rat short-term study previously reported (Zidek, N. *et al.* 2005. *Archives in Pharmacology*, Vol. 371). PCLS showed effects on gene expression after LPS incubation and revealed multiple genes associated with the inflammatory process and their known secondary responses. Although higher variance in biological replicates was detected compared to the *in vivo* situation. We were able to identify genes commonly deregulated by both test systems with very good concordance seen after 6h of high dose treatment (190 significantly deregulated genes indicating a strong induction of chemokine-chemokine interactions and inflammatory mediators, e.g. *chemokine ligands Ccl2*, *Cxcl2* and *interleukin 1b* and *6*, a decrease in xenobiotic and lipid metabolism as well as liver specific transporter activity). Human liver slices are currently being tested to aid in the comparison between rat and human responses. In conclusion, PCLS showed pronounced gene expression changes after LPS treatment. Commonly deregulated genes reflected LPS mediated effects, and were comparable to the liver inflammation and injury previously observed *in vivo*, showing the validity of the liver slice model for toxicity studies.

[1]Institute of Toxicology, Merck KGaA, Darmstadt, Germany
[2]Institute of Pharmacology and Toxicology, Friedrich-Schiller University, Jena, Germany

483

GENOTOXICITY-TESTING WITH THE PHENION® FULL THICKNESS SKIN MODEL

K. Reisinger [1], J. Scheel [2], K. Schröder [1] and D. Eschrich [1]

The currently applied *in vitro* genotoxicity tests demonstrate a high sensitivity but very low specifity. Therefore, *in vivo* assays must often be performed in order to clarifying the biological relevance of the *in vitro* experiments. In order to reduce and finally replace *in vivo* genotoxicity testing we established a genotoxicity assay using the Phenion® Full Thickness Skin Model featuring tissue- and species-specific metabolism. This model represents the primary target of cosmetic ingredients and product testing and is often the tissue of maximum exposure. The model consists of a dermal and an epidermal layer facilitating interaction between both major skin compartments which are essential for normal skin physiology. In addition, major Phase I and II enzymes of the xenobiotic metabolism are expressed and regulated in the skin model (Wiegand *et al.*, Poster DGPT). First, the Micronucleus Assay for detecting the genotoxic impact of substances after topical application was established. This assay accounts for persisting damages of chromosomal structure or deficient segregation. A protocol was established for isolating keratinocytes from the *in vitro* skin model. First results are presented showing the suitability of this test system.

[1] Phenion GmbH & Co KG, Düsseldorf; [2] Henkel KGaA, Corporate SHE and Product Safety/Human Safety Assessment, Düsseldorf, Germany
kerstin.reisinger@henkel.com

484

QUANTIFICATION OF DNA STRAND BREAKS BY AN AUTOMATED VERSION OF THE FLUORESCENCE-DETECTED ALKALINE DNA-UNWINDING (FADU) ASSAY

M. Moreno, E. Ferrando-May and A. Bürkle

Quantification of DNA strand breaks and repair in living cells is an important endpoint in the assessment of genotoxicity. We established and automated a version of the Fluorescence alkaline DNA unwinding (FADU) assay based on the use of a commercial laboratory robot. The very high precision and reproducibility of this new assay makes it possible to measure the effect of very low concentrations of genotoxic agents after 5 minutes incubation and to observe the repair capacity of the cells in 5 minute intervals. After preparation of cells the automated steps take only 2 hours and thus the method can be used not only in experimental research but also to screen and characterise drugs that induce DNA strand breaks. Furthermore, it is possible to detect endogenous DNA strand breaks. Thus the assay is useful to investigate differences between groups of individuals, e.g. smokers/non smokers, young/old, before chemotherapy/after chemo-therapy.

Lehrstuhl Molekulare Toxikologie, Universität Konstanz, Konstanz, Germany

485

NEW HIGH-THROUGHPUT METHOD FOR ASSESSING DNA INTERSTRAND CROSSLINKS

M. Debiak, E. Müßig and A. Bürkle

DNA-crosslinking agents are a diverse group of agents used as drugs in cancer chemotherapy. Nitrogen mustards derivates, platinum compounds and mitomycins are among the first-choice drugs in treatment of lymphomas, leukemias, and solid tumours. The critical DNA lesions induced by these compounds are DNA interstrand crosslinks (ICL). Although such lesions are highly mutagenic and pro-apoptotic, they are induced only to a minor extent, rarely exceeding 10 % of total DNA damage. Thus, assessment of ICL formation and of the cellular repair capacity should be useful for optimization of the chemotherapy administered to patients. Monitoring has so far only been possible by using time-consuming specialized methods such as the comet assay. We have adapted a robust, automated high-throughput method, recently developed in our lab for the detection of DNA strand breaks, for the assessment of ICL. The assay principle is based on the FADU assay as originally described as a manually-operated version by Birnboim & Jevcak (Cancer Res 1981; 41:1889-92). We have been able to reduce the sample size to 100 µl of whole blood and to detect ICL induced in human PBCM by treatment with 1 µM mitomcycin C, within 2 h assay time.

Molecular Toxicology Group, University of Konstanz, D- 78457 Konstanz, Germany

486

CULTURES OF HUMAN LUNG CELLS AS MODEL SYSTEM FOR EFFECTS OF AS(III), CU(II) AND HG(II)

F. Glahn, A. Torky, M. Ströfer, E. Stehfest, S. Hofmann* , H. Foth

Human lung faces chronic exposure to low doses of metals, which might lead to DNA-damage or adaptation, e.g. by increased expression of Multidrug resistance associated proteins (MRP). The model system used in our experiments with As(III), Cu(III) and Hg(II) were human lung tumor cell lines A549 and H322 and primary cultures of normal human bronchial epithelial cells (NHBEC). We applied the MTT-assay to find sub-toxic concentrations for incubation. Moreover we could show increased resistance of NHBEC to As(III) after six weeks of exposure to 1µM arsenite. Transition metals like Cu might lead to DNA-damage, therefore we analysed Cu(II) treated cells in the COMET-assay. In NHBEC 72h incubation with Cu(II) caused significant DNA-damage starting at 150µM, in A549 already 75µM Cu(II) led to significant DNA-damage, co-incubation with vitamin C or tocopherols significantly reduced this effect. As MRP-transporters export a variety of substrates they might protect cells by extrusion of metal complexes. We found significantly increased sensitivity of H322 to As(III) when pre-incubated with MK571, an inhibitor of MRP1-4. Real-time RT-PCR analysis of MRP-transporters in NHBEC revealed clear differences between cultures derived from different donors: One week of treatment with As(III) up-regulated MRP3, MRP4 and MRP5 (1.3 to 2.3 fold) in cultures derived from B182/1, while As(III) down-regulated MRP1, MRP3 and MRP4 (0.5 to 0.6 fold) in B201/1. Five additional days of treatment decreased MRP1, MRP3, MRP4 and MRP5 (0.4 to 0.5 fold) in B201/1. In B182/1 MRP1 was decreased (0,75 fold). One week Cu(II) slightly increased MRP3 in B182/1. The second week of treatment down-regulated MRP1 (0.6 fold) in B201/1, while it increased MRP4 in B182/1 (1.9 fold). Incubation (5 d) with Hg(II) increased MRP3 (2.1 fold) in B182/1 while in B222/1 it increased MRP5 (1.7 fold). The second week decreased MRP1, MRP3, MRP4 and MRP5 (0.3 to 0.7 fold), in B201/1while it increased MRP3 (2 fold) in B182/1.

Institute of Environmental Toxicology, *Cardio-Thoracic Surgery, Martin Luther University, D-06112 Halle / Saale, Germany

487

DEVELOPMENT OF AN *IN OVO* MODEL FOR INVESTIGATION OF MITOCHONDRIAL TOXICITY OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

I. Garcia Moreno, M. Wiertz, D. Höschele

Nucleoside reverse transcriptase inhibitors (NRTIs), drugs used to treat human immunodeficiency virus (HIV) infection, inhibit cellular DNA polymerase γ and, as a result, cause impairment of mitochondrial (mt) DNA synthesis. Some NRTIs affect mitochondrial function also directly. Mitochondrial toxicity produced by these agents can lead to a wide range of severe adverse effects, of which lactic acidosis and hepatic steatosis are increasingly recognized in NRTI treated patients. At present no clinically relevant *in vitro* model exists for the investigation of mitochondrial toxicity, and specialized animal models are mostly used to confirm preliminary *in vitro* data. The aim of this study was to establish an *in ovo* model for the investigation of mitochondrial toxicity. The NRTIs zalcitabine (ddC), didanosine (ddI), stavudine (d4T), zidovudine (AZT), and lamivudine (3TC) were administered at days 5, 7 and 9 into the air cell of incubated hens' eggs at concentrations of 0.05 to 40 mg/egg. Ethidium bromide (EtBr) was used as a positive control, as it is known to deplete mtDNA in cell cultures. Mitochondrial function was determined at day 11, when the embryo´s nervous system is not yet completely established, by measuring blood lactate levels and ATP content in erythrocytes. Additionally, mtDNA content of the embryo's liver was determined by measuring the ratio of mtDNA to nDNA using a novel dual colour real-time PCR based assay. Treatment with AZT, ddI, ddC and EtBr resulted in significant concentration dependent increase in blood lactate levels. A significant decrease of ATP content was observed after treatment with AZT and EtBr. In liver cells a significant depletion of mtDNA was found for ddI, ddC and EtBr but not for d4T, AZT or 3TC, which are less potent inhibitors of the DNA polymerase γ. The *in ovo* model is a simple, sensitive and rapidly responding non-animal model, which is applicable for studying mitochondrial toxic effects on mitochondrial function and mtDNA.

Federal Institute for Drugs and Medical Devices, Kurt-Georg-Kiesinger Allee 3, D-53175 Bonn, Germany

488

INDUCTION OF ALKALINE PHOSPATASE BY 17BETA-ESTRADIOL IS DEPENDENT ON THE PRESENCE OF ESTROGEN RECEPTOR ALPHA IN THE ENDOMETRIAL ISHIKAWA CELL LINE

A.M. Sauermann, J. Wagner, L. Lehmann

The Ishikawa cell line which is derived from a human endometrial adenocarcinoma represents an acknowledged test system for the detection of estrogen active compounds. Its widespread use is based on the observation that both the mRNA levels as well as the activity of the alkaline phosphatase (ALP) are stimulated by estrogens and inhibited by estrogen receptor (ER) antagonists. However the molecular mechanism underlying the induction of ALP expression by estrogens has not been demonstrated unequivocally so far. The aim of the present study was to clarify the role of ERα in the induction of ALP by 17β-estradiol (E2) in Ishikawa cells. Therefore, we determined the basal and E2-induced ALP activity in Ishikawa (ER+) and transient Ishikawa (ER_{tr}-) cells. Ishikawa (ER_{tr}-) cells were generated by degradation of ERα mRNA using siRNA complementary to specific sequences of ERα ($siRNA_{ER\alpha}$). As negative control, cells were treated with control siRNA ($siRNA_K$). ERα and ALP mRNA levels were quantified by reverse transcription/competitive PCR. ALP activity was determined by the conversion of a chromogenic substrate. Ishikawa (ER+) cells expressed about $8x10^6$ copies of ERα mRNA per µg total RNA, whereas no ERα mRNA was detected in Ishikawa (ER_{tr}-) cells. Treatment of Ishikawa cells with transfection reagent with and without 10 nM $siRNA_K$ did not affect mRNA levels of ERα. Likewise, neither the basal (275±12 pmol/min/10^6 cells) nor the E2-induced (1331±124 pmol/min/10^6 cells) activity of ALP was affected. In contrast, both the basal and the E2-induced ALP activity were reduced by 30±6% and 84±1%, respectively, after treatment of Ishikawa cells

with 10 nM and more $siRNA_{ER\alpha}$ compared to that of cells treated with 10 nM $siRNA_K$. Thus, the induction of ALP expression in Ishikawa cells by E2 depends on the presence of ERα which verifies the importance of the Ishikawa cell line as a test system for the detection of estrogen active compounds.
Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe, D-76131 Karlsruhe.

489

ISHIKAWA CELL LINES WITH DISTINCT ESTROGEN RECEPTOR EXPRESSION: A NEW MODEL SYSTEM FOR DISCRIMINATION OF ESTROGEN RECEPTOR (ER)-DEPENDENT AND ER-INDEPENDENT ESTROGENIC SIGNALS

S. Simon, S. Schmidt and S. O. Mueller*

Estrogens regulate diverse physiological processes in various tissues including mammary and reproductive tissues. Their effects are mediated by the estrogen receptors (ER) belonging to the nuclear receptor superfamily of ligand-inducible transcription factors. Here we present, for the first time, a comparative characterization and quantitative global gene expression analysis of human endometrial Ishikawa cell lines expressing endogenous ERα (Ishikawa-plus) or lack ER expression (Ishikawa-minus). In an approach to analyze cellular responses to estrogens at the gene expression level in a time- and dose-dependent manner, we used the Illumina® Sentrix® BeadArray technology. Prior to global gene expression analysis, real time PCR-based TaqMan® Low Density Arrays (TLDA) were performed to determine appropriate time points and doses. Samples treated with a low dose estradiol (E2) and a combination of E2 and the anti-estrogen ICI 182,780 (ICI) for 2, 24 and 72h were selected and subjected to Illumina® whole genome human BeadArray analysis. Principle component analysis (PCA) showed that the two cell lines accumulate in two well-defined, separate clusters, confirming their different properties in gene expression. We were able to identify more than 1300 significantly deregulated genes in response to E2 in Ishikawa-plus with a clear time-dependent pattern. The expression of the majority of these genes was inhibited by cotreatment with ICI, indicating their E2-induced deregulation and confirming their ER-dependent modulation. In contrast, only 374 genes were deregulated in Ishikawa-minus. Interestingly, 17 genes were deregulated in both Ishikawa-plus and –minus, eight of them show diametric deregulation. These eight genes are therefore putative marker genes for ER-dependent effects. The nine genes showing unidirectional deregulations in both cell lines indicate ER-independent effects and therefore may facilitate the identification of estrogenic but ER-independent signals. Overall, these results show that Ishikawa cell lines with distinct ER expression provide a suitable model system to screen for and to analyze estrogenic effects with respect to ER-dependent or ER-independent signaling pathways.
[1]Institute of Toxicology, Molecular Toxicology, Merck KGaA, 64293 Darmstadt, Germany

490

BIOTRANSFORMATION OF RETINOL AND CYCLOPHOSPHAMIDE IN THE EMBRYONIC STEM CELL TEST USING A CO-CULTURE WITH HEPATIC CELLS

M. Binder*, J. Volland*, F. Meyer*, S. Lunkenbein**, H. Nau**, U. Hübel*, P.-G. Germann*

The embryonic stem cell test (EST) can detect direct acting teratogens, but not proteratogens, i.e. substances which need biotransformation before they exert embryotoxic action. In the present study, the possibility of co-culturing metabolically active hepatic cells with murine embryonic and adult cells to form a metabolically competent EST (mEST) was investigated. A co-culture was implemented by adding cell culture inserts with hepatic cells at each step of the EST experimental process. Retinol (RO) and cyclophosphamide (CPP), two well-known proteratogens, were used as model substances. A range of hepatic cells were tested: primary hepatocytes from rat and mouse, and the hepatoma cell lines H4IIE (rat), Hepa 1-6 (mouse), and HepG2 (human). They were selected with respect to their tolerance of co-culture conditions, and their metabolic activity. Their ability to metabolize RO to *all-trans*-retinoic acid (RA), the teratogenic metabolite of RO, was determined by HPLC-UV analysis. Only H4IIE and HepG2 cells were found to tolerate the co-culture conditions. Of these two, only HepG2 produced RA in relevant concentrations. Therefore, RO was tested in the mEST with HepG2 cells, whereas CPP was tested in the mEST with HepG2 and H4IIE cells. RO, RA, and CPP were also examined in the conventional EST. By comparing EST and mEST results, the efficiency of metabolic activation in the mEST can be determined. For RO as well as for CPP, a significant increase of their toxicity in the mEST compared to the EST could not be observed. The feasibility of the selected hepatoma cells, the model substances applied, and the mEST test conditions will be discussed.
*ALTANA Pharma AG, Institut für präklinische Arzneimittelsicherheit, Barsbüttel
**Stiftung Tierärztliche Hochschule Hannover, Institut für Lebensmitteltoxikologie und Chemische Analytik, Hannover

491

COMPARISON OF NOVEL IN VITRO ENDPOINTS FOR THE EVALUATION OF DEVELOPMENTAL OSTEOTOXICITY USING THE EMBRYONIC STEM CELL DIFFERENTIATION MODEL

L.A. Davis[1], D.E. Rancourt[1], N.I. zur Nieden[1, 2]

The teratogenic potential of a compound can devastate its chances as a drug candidate. Currently, embryotoxicity tests are performed in living animals. The drug industry has been in need for a practical *in vitro* embryotoxicity model for the last two to three decades. Ten years ago, the currently most promising *in vitro* embryotoxicity test, the embryonic stem cell Test (EST) has been called to life. In contrast to other *in vitro* embryotoxicity tests, the EST uses two permanent cell lines, murine embryonic stem cells (ESCs) and differentiated 3T3 fibroblasts and thus does not require the use of pregnant animals. The EST may be useful to identify developmental inhibition particularly in cardiac tissue. However, the analysis of cardiomyocyte differentiation alone as an endpoint does not suffice for the determination of embryotoxic effects. Recently, we improved the EST to include other tissue endpoints, such as bone, cartilage and neurons. ESC-derived osteoblasts reach a state of full maturity after 30 days of differentiation *in vitro* as assessed by the expression of osteoblast-specific genes (i.e. osteocalcin). This long test-duration is not desirable for the use in an *in vitro* assay. In the present study, we have thus begun to explore new means of evaluating the degree of osteogenesis. First, we have commenced to culture the differentiating ESCs in a monolayer instead of plating intact embryoid bodies. Recently, we further began to utilize IMAGE analysis to quantify osteogenesis, a process in which cultures are photographed and the percentage of black pixels is calculated. This is only possible because osteocalcin-expressing mineralized osteoblasts appear black in phase contrast microscopy. Furthermore, we analyzed osteoblast yield by measuring the amount of mineralized calcium incorporated into the matrix and ALP enzyme activity and compared this with quantitative real-time PCR for osteocalcin. Ultimately, we were able to show that by using monolayer cultures and IMAGE analysis we were able to predict the osteotoxic potential of retinoic acid and reduce the length of the embryotoxicity assay to 14 days.
[1] Institute of Maternal & Child Health, University of Calgary, Calgary, AB, Canada, T2N 4N1; [2] Fraunhofer Institute for Cell Therapy & Immunology, 04103 Leipzig, Germany

492

***IN VITRO* ESTIMATION OF THE EMBRYOTOXIC POTENCY OF VALPROIC ACID DERIVATIVES EMPLOYING THE EMBRYONIC STEM CELL TEST (EST)**

R. Buesen[1], A. Visan[1], E. Genschow[1], B. Slawik[1], K. Schlechter[1], M. Fernandes[2], H. Nau[2], H. Spielmann[1], A. Seiler[1]

In the field of reproductive toxicity mandatory test guidelines require *in vivo* experiments for the detection of the embryotoxic potential of chemicals and drugs. A promising alternative method for these purposes has been established by the use of embryonic stem cells (ES cells), the Embryonic Stem Cell Test (EST). This assay takes into account the inhibitory effects of a test compound on the development of ES cells into contracting myocardial cells and its cytotoxic effects on the proliferation of ES cells and 3T3 fibroblasts. Through a number of prevalidation and validation studies the EST has demonstrated to be a reliable alternative method for classifying test compounds into strongly, weakly and non-embryotoxic. The aim of the study presented here was to investigate whether the EST is sensitive enough to distinguish among structurally related compounds with gradually different embryotoxic potential. Valproic acid (VPA) is a potent antiepileptic drug with known teratogenicity in humans and rodents. VPA derivatives, which were synthesised and highly purified at the University of Veterinary Medicine Hannover, exhibit different and stereoselective embryotoxic potentials *in vivo* covering the range from highly to non-teratogenic. The results presented demonstrate that the EST is able to reflect structure-activity relationships of the investigated compound class *in vitro* and point out a high selectivity. Based on these results the *in vitro* assay may be a useful tool in the process of drug development and, in addition, saves experimental animals.
[1] Federal Institute for Risk Assessment (BfR), Centre for Alternative Methods to Animal Experiments (ZEBET), 12277 Berlin, Germany
[2] University of Veterinary Medicine Hannover, Centre of Food Science, Department of Food Toxicology and Chemical Analysis, 30173 Hannover, Germany

493

MOLECULAR IMPACTS OF FLAVONOIDS AND THE *GINKGO BILOBA* EXTRACT EGb761 IN *C. ELEGANS*

A. Kampkötter, R. Zurawski, C. Büchter, C. Timpel, C. Gombitang, R. Rohrig, T. Pielarski, Y. Chovolou, W. Wätjen and R. Kahl

Flavonoids are present in many herbal foods and are assumed to contribute to the health benefits from a diet rich of vegetables and fruits. They possess a broad spectrum of biochemical and pharmacological actions but precise mechanisms of their action have not been finally elucidated yet. It is discussed whether their impact is predominantly attributed to the antioxidative and free-radical scavenging properties of numerous flavonoids or to modulation of the activity of enzymes involved in signalling processes. There is a remarkable strong conservation in molecular and cellular pathways between *Caenorhabditis elegans* and mammals and many key discoveries in basic biology and medically relevant areas were first made in the worm. For these reasons we considered *C. elegans* as a suited model to examine the molecular mechanisms by which herbal flavonoids mediate their effects in a multicellular organism. The flavonoids quercetin and kaempferol as well as the flavonoid containing *Ginkgo biloba* extract EGb761 were demonstrated to be protective against thermal and oxidative stress in *C. elegans*. For the investigation of the mechanisms by which the protective effects are mediated in living worms we used transgenic C. elegans strains carrying GFP coupled reporter genes for the C. elegans FoxO transcription factor DAF-16, the antioxidant enzyme SOD-3 (MnSOD) and the phase II metabolism enzyme GST-4. The translocation of the transcription factor DAF-16 into the nucleus in response to the treatment indicates that signalling processes are affected by all three substances. The expression of SOD-3 – a target gene of DAF-16 – was induced by EGb761, repressed by quercetin and not affected by kaempferol whereas all substances caused a downregulation of stress-responsible GST-4. Since these results indicate that the investigated flavonoids and EGb761 exert their effects by different mechanisms involving modulation of signalling processes as well as antioxidative activity we perform western blot analysis to examine the mode of action in more detail.
Institute of Toxicology, Heinrich-Heine University, D-40225 Düsseldorf

494

INFLUENCE OF FLAVONOIDS AND EGb761 ON STRESS RESISTANCE OF C. ELEGANS

R. Zurawski, C. Büchter, T. Pielarski, C. Gombitang, Y. Chovolou, W. Wätjen, A. Kampkötter and R. Kahl

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin and may contribute to the health beneficial effect of a diet rich of vegetables and fruits. Oxidative stress caused by an excess of reactive oxygen species (ROS) is associated with diverse diseases and with ageing so that the antioxidative activity demonstrated for many flavonoids is regarded as a important aspect for their protective effects. In our study we used the nematode *Caenorhabditis elegans* as a model to investigate the impact of the flavonoids quercetin, kaempferol, rutin and fisetin as well as the *Ginkgo biloba* extract EGb761 on resistance against oxidative stress in a multicellular organism. There is a strong conservation in many basic biological processes between the worm and mammals so that *C. elegans* is commonly used in various areas of biological and medical research and the findings often reveal relevance to mammals. All substances with exception of rutin increased the survival of the worms under conditions of a lethal

thermal stress and thus enhanced the thermotolerance of *C. elegans*. The resistance against oxidative stress was also elevated by the treatment with all substances, except for fisetin. To investigate whether this effect was exerted by the antioxidative potential of the protective substances we measured the ROS accumulation in living worms at acute thermal stress. Besides fisetin, all substances reduced the ROS accumulation indicating the antioxidative activity of these substances. Since oxidative stress is regarded as a main factor in the process of ageing we analysed the impact of the substances to the established ageing marker lipofuscin and we found a reduction of the lipofuscin accumulation by all substances, except for rutin. This result suggests that these substances affect the process of ageing in *C. elegans*. To verify this assumption we currently determine the influence of the substances on the life span of *C. elegans*.
Institute of Toxicology, Heinrich-Heine University, D-40225 Düsseldorf

495

RESULTS OF THE ECVAM SKIN IRRITATION VALIDATION STUDY (SIVS)
H. Spielmann[1], M. Liebsch[1], H. Kandarova[1], P. Botham[2], J. Fentem[3], V. Zuang[4], T. Hartung[4], C. Eskes[4], R. Rouguet[5], T. Cole[6], S. Hoffmann[1], J. Cotovio[5], P. Jones[3], C. Robles[7], A. Gamer[8], R. Curren[9]
To replace the Draize skin irritation test (OECD TG 404), ECVAM has sponsored a formal validation study of three *in vitro* test systems, two employing reconstituted human epidermis models (EPISKIN, EpiDerm) and the skin integrity function test (SIFT) employing *ex vivo* mouse skin. The objectives were to conduct a validation study to assess the relevance (predictive ability) and reliability (reproducibility within and between laboratories) of these test systems with a set of 58 coded test chemicals for which high quality *in vivo* data were available. It was the goal of the study to assess if the *in vitro* tests would predict *in vivo* classification according to the two classes of the EU classification "R 38" and "non-irritant".). The SIFT test did not meet the acceptance criteria for the formal validation study. For testing with the human skin models the following tiered testing strategy was used: Cytotoxicity (MTT) was determined in tier 1 and 1L-1α in tear 2 in samples from chemicals providing MTT results below a threshold of 50% viability. The EpiDerm test was conducted in the following laboratories ZEBET (lead lab) Germany, Institute for *In vitro* Sciences (IIVS) USA and BASF Germany. The EPISKIN test was conducted in the following laboratories L'Oreal (lead lab) France, Unilever UK and Sanofi-Synthelabo France. The prediction model (PM) applied in the formal SIVS validation study used the following endpoints: MMT - threshold of 50% reduction of cell viability; IL-1α release - threshold of 60 pg/ml. The results of the SIVS study proved that the sensitivity and specificity of the EPISKIN skin irritation test (MTT + IL-1α-release) were acceptable and that the method can therefore be recommended as a replacement for the Draize skin irritation test (EU Annex V B.4; OECD TG 404). Moreover, only the specificity of the EpiDerm assay (MTT) was acceptable and, therefore, the assay cannot yet be recommended as a replacement for the Draize skin irritation test but for use within a testing strategy.
[1]Federal Institute for Risk Assessment (BfR), National Centre for Alternative Methods (ZEBET), 12277 Berlin, Germany; [2]Syngenta, Macclesfield (UK); [3]Unilever, Sharnbrook (UK); [4]ECVAM/JRC Ispra (I), [5]L'Oreal, Clichy (F); [6]ECB/JRC (I), [7]Sanofi Aventis, Montpellier (F), [8]BASF, Ludwigshafen (D), [9]Inst. for In Vitro Sciences, Gaithersburg (USA).

496

METABOLIC PROFILING OF MERCAPTURIC ACIDS IN HUMAN URINE: A VALIDATION APPROACH FOR LC/MS METABONOMICS
S. Wagner, K. Simon, M. Sieber, M. Kellert, W. Völkel
Whilst for 1H-NMR techniques there already exist common quality standards, this does not yet apply for LC-MS metabolic profiling approaches. These standards are the more recommended when applying metabonomics to human biofluids, particularly urine samples, due to the high degree of biological variation compared to animals. A control study was performed and urine samples of 30 healthy male and female human subjects were collected during a 8 h overnight and 8 h daytime period for three consecutive days (n=180). Using a selective multiple reaction monitoring scan in combination with a column-switching tool for the analysis of mercapturate pattern, samples were screened for time and gender differences, the most common confounders in metabonomic studies. Data preprocessing parameters, alignment, scaling and normalization techniques were optimized by PCA, PLS-DA and OPLS models. Great care was taken in the validation process of both analytical and chemometric protocols. Additionally, a big problem of LC-MS, the combination of "different-batch" data to "one-batch" data could be solved by a batch-wise scaling procedure. Based on these results, the use of metabolic profiling via mercapturates will be feasible for the detection of disease or toxicity markers in future since mercapturates are important biomarkers of reactive metabolites known to be involved in many toxic processes.
Department of Toxicology, University of Würzburg, Versbacher Str. 9, Würzburg, Germany

497

AN INTEGRATED GC-MS METHOD FOR METABONOMICS OF BIOFLUIDS
M. Sieber, W. Dekant
Metabonomic analysis with gas chromatography coupled to mass spectrometry (GC-MS) has been widely applied in plant biology, but only sparsely in toxicological approaches. In this field, liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) dominate the area of metabolomics to predict potential toxicities. A reason for this may be the lack of an integrated, readily available GC-MS method comprising all data processing steps from sample analysis to biomarker identification. Contrary to many other published methods, the method presented here is not based on in-house programs, but only uses commercially available or open-source software. Therefore it can be implemented on any GC-MS engine able to export data in the network Common Data Form (netCDF). Thus an easy-to-use metabonomics platform can be installed on any standard personal computer without special knowledge in programming. The data generation consists of drying, oximation and silylation of the samples and subsequent GC-MS analysis with electron impact ionisation (GC-EI-MS). Data analysis is performed by combining the peak-picking and alignment software XCMS (http://metlin.scripps.edu/download/) with the SIMCA program (Umetrics, Umeå, Sweden) for multivariate statistics. Metabolites filtered by the software as potential biomarkers can be identified with the help of the US. National Institute of Standards and Technology (NIST) mass spectral data base (http://www.nist.gov/srd/nist1a.htm). The method was tested by spiking pool urine with a test mix of ten endogenous compounds. Nine out of ten spiked compounds could be identified correctly as discriminating markers between control and spiked samples.
University of Würzburg, Department of Pharmacology and Toxicology

498

DEVELOPMENT OF A SCREENING ASSAY FOR TERATOGENICITY: COMBINATION OF *DarT* WITH A MAMMALIAN METABOLIC ACTIVATION SYSTEM
F. Busquet[1], F. von Landenberg[1], S.O Mueller[1], R. Nagel[2], and T.H Broschard[1]
The assessment of teratogenic effects of chemicals is usually performed using *in vivo* assays. Following the 3R concept and public expectations, ECVAM and ZEBET promote the use of alternative methods, such as whole embryos and cell cultures (EST), to reduce the use of animals. From this perspective, we have developed for the first time, an *in vitro* test with the zebrafish *Danio rerio* embryo test (*Dar*T) combined with an exogenous mammalian metabolic activation system (MAS), able to biotransform proteratogenic compounds. The zebrafish embryos were co-cultured at 2hpf (hours post fecundation) with varying concentration of cyclophosphamide (CPA), induced male rat liver microsomes and NADPH for 90min at 32°C under moderate agitation in Tris-buffer. The negative control (CPA alone) and the vehicle control (MAS alone) were incubated in parallel. For each parameter, 20 eggs were used for statistical robustness. Afterwards fish embryos were transferred individually into 24-wells plate filled with fish medium for the next 48hours at 26°C with a 12hour-light cycle. Teratogenicity was scored using morphological endpoints. The results of two dose-response tests showed that the highest CPA concentration (57mM) combined with MAS caused ≥30% of the embryos with morphological malformations. All the controls fulfil the acceptance criteria of ≤10% impaired embryos. The severity of malformations increased with CPA concentration and we could show specific metabolised CPA's effects on the fish organs. We expect that the inclusion of the MAS will improve and refine *Dar*T as a predictive and valuable alternative method to screen teratogenic compounds.
[1]Institute of Toxicology, Molecular Toxicology, Merck KGaA, 64293 Darmstadt, Germany; [2]TU Dresden, Institute of Hydrobiology, 01062 Dresden, Germany

499

SEARCH FOR BIOMARKERS OF FURAN EXPOSURE BY METABOLITE PROFILING OF RAT URINE WITH LC-MS/MS AND PRINCIPAL COMPONENT ANALYSIS
M. Kellert[1], S. Wagner[1], J. Schlatter[2], W.K. Lutz[1]
Furan has been found in a number of food items, primarily as a product of heat treatment. It is carcinogenic in the liver of rats and mice, with a particular sensitivity of the bile ducts of F344 rats. Estimates of human exposure on the basis of concentrations measured in food are not reliable because of the volatility of furan. A biomarker approach is therefore indicated. As a first step, we searched for metabolites excreted in the urine of rats treated with furan by oral gavage. A control group received the vehicle oil only. Urine was collected over two 24-hour periods after dosing. Samples were diluted to equal specific gravity and analyzed by a column-switching LC-method coupled to a linear ion trap mass spectrometer. Data were acquired in the enhanced full scan mode (EMS) in combination with information dependent acquisition (IDA) of enhanced product ion scans (EPI). Peak areas were extracted from the chromatograms by MarkerView® software 1.1. The resulting 430 peaks defined by mass and retention time were used for multivariate analysis. Principal components clearly separated the samples of treated rats vs. controls. Variables that contributed most to the separation were extracted by the loadings plots. Using the MS information on mass and fragmentation yielded four compounds that could be attributed to putative furan metabolites. Structures were compatible with glutathione conjugation or adduct formation with lysine. The four metabolites were shown to be formed in vitro from appropriate incubations of the primary furan metabolite *cis*-2-butene-1,4-dial with glutathione, *N*-acetyl-L-lysine or *N*-acetyl-L-cysteine. Additional compounds showed fragmentation patterns known from mercapturic acids (neutral loss of 129) or glucuronides (neutral loss of 176). Our data show that biomarkers of furan exposure can be identified in urine so that further investigations on dose response in rats and search for these biomarkers in human urine are warranted.
[1]Department of Toxicology, University of Würzburg, Germany; [2]Nutritional and Toxicological Risks Section, Swiss Federal Office of Public Health, Zürich, Switzerland

500

DIFFERENCES IN THE URINARY EXCRETED PAH METABOLITE LEVELS OF THE GENERAL POPULATION OF EASTERN AND WESTERN EUROPE
A. Seidel[1], G. Dettbarn[1], A. John[1], J. Gromadzinska[2], F. Palitti[3], R. Antic[4], G. Joksić[5], J. Jacob[1]
Data on the excreted levels of metabolites of polycyclic aromatic hydrocarbons (PAH) in urine of the general population in Europe are limited. In the framework of the DIEPHY project we have investigated cohorts of nonsmoking women of the general population from Italy (N=100), Poland (N=103), and Serbia (N=100). Among the PAH metabolites naphthols and 1-hydroxypyrene have been determined which reflect the endogenous PAH body burden of each individual. Furthermore, the recently established biomarker phenanthrene-1,2,3,4-tetrol (PhenT) has been determined that reflects the metabolic activation of carcinogenic PAH (Hecht et al., 2003). The nonsmoking status of each subject has been validated by determination of the urinary cotinine level. The medianes for the sum parameter 1-+2-naphthol and 1-hydroxypyrene of the Italian cohort were 5.50 µg/L and 0.08 µg/L, respectively. In contrast, the corresponding medianes for the Polish cohort were 11.33 µg/L and 0.19 µg/L and for the Serbian cohort they were 8.80 µg/L and 0.15 µg/L. The median for PhenT was 0.48 µg/L in the Italian, 1.17 µg/L in the Polish, and 0.95 µg/L in the Serbian cohort. The concentrations of PAH metabolites found in the Italian cohort compared closely to findings in Germany and the United States. The higher concentrations of urinary excreted PAH metabolites in the Polish and Serbian cohort clearly point out that the individuals are exposed to a significant higher level of PAH in ambient air and/or contaminated food. This study is supported by the European Union (Sixth Framework Programme, STREP Project "Dietary Exposure to Polycyclic Aromatic Hydrocarbons and DNA Damage" (DIEPHY) Contract FOOD-CT-2003-505609).
[1]Biochemical Institute for Environmental Carcinogens (BIU) Prof. Dr. Gernot Grimmer-

Foundation, Lurup 4, 22927 Grosshansdorf, Germany; [2]Nofer Institute of Occupational Medicine (NIOM), 91-348 Lodz, Poland; [3]Department of Agrobiology and Agrochemistry, University of Tuscia, 01100 Viterbo, Italy, [4]The Academic Association for Research on Occupational and Public Health (AROPH), 11080 Zemum-Belgrade, Serbia; [5]Vinca Institute of Nuclear Sciences, 11001 Belgrade, Serbia

501

CHROMOSOMAL BREAKS AND LOSSES IN LYMPHOCYTES FROM WORKERS EXPOSED TO FUMES OF BITUMEN AT HIGHER PROCESSING TEMPERATURES – A CROSS SHIFT STUDY

P. Welge[1], B. Marczynski[1], M. Raulf-Heimsoth[1], A. Spickenheuer[1], A. Erkes[1], R. Bramer[1], D. Breuer[2], J.-U. Hahn[2], T. Mensing[1], B. Pesch[1], H.-U. Käfferlein[1], T. Brüning[1]

At higher processing temperatures mastic asphalt workers may be exposed to several polycyclic aromatic hydrocarbons (PAH) contained in fumes of bitumen. Little is known regarding clastogenic or aneugenic effects in bitumen-exposed workers. The cytokinesis-blocked micronucleus (CBMN) assay detects chromosome breaks and losses specifically in cells that have completed nuclear division once. CBMN has emerged as a maturing biomarker of chromosomal damage relevant to cancer. We conducted a cross-shift study in German mastic asphalt workers (n = 34) and a reference group without exposure to bitumen (n = 14). All workers were examined before and after shift. The CBMN assay was done according to the method described by Fenech (2000). For each sample, the number of micronuclei (MN) in 1000 binucleated cells (BNC) was measured. In 34 workers exposed to fumes of bitumen a median of 8.25 MN/1000 BNC (P25-P75: 6.5-11.0) was found before shift and 7.58 MN/1000 BNC (P25-P75: 5.5-10.09) after shift (P = 0.53). In lymphocytes of the reference group (n = 14) a median of 5.75 MN/1000 BNC (P25-P75: 3.5-7.73) before shift and a median of 6.92 MN/1000 BNC (P25-P75: 5.36-9.0) after shift were found (P = 0.60). The only significant difference (P = 0.03) in the micronucleus frequencies between exposed and controls before shift should be interpreted with caution because of the limited number of controls. External exposure to fumes of bitumen showed no association with the number of micronuclei after shift (P = 0.93). The micronucleus frequencies in our study were in the middle of the range reported as background frequencies in other laboratories. Efforts are under way to expand the study size and thus the statistical power to allow a more precise estimation of the bitumen-related health effects.

[1]Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin (BGFA), Institut der Ruhr-Universität, Bochum, [2]Berufsgenossenschaftliches Institut für Arbeitsschutz (BGIA), Sankt Augustin

502

GENOTOXIC EFFECTS IN WORKERS EXPOSED TO FUMES OF BITUMEN. COMPARISON WITH AMBIENT AND BIOLOGICAL MONITORING

B. Marczynski[1], M. Raulf-Heimsoth[1], K. Förster[2], A. Spickenheuer[1], P. Welge[1], T. Mensing[1], B. Pesch[1], D. Breuer[3], J.-U. Hahn[3], H.U. Käfferlein[1], R. Bramer[1], J. Angerer[2], T. Brüning[1]

At higher processing temperatures mastic asphalt workers may be exposed to several polycyclic aromatic hydrocarbons (PAHs) contained in fumes of bitumen. We conducted a cross-shift study to determine genotoxic effects, external and internal exposure in 202 bitumen-exposed workers and 55 construction workers without exposure to bitumen as reference group. Exposure to fumes of bitumen during the shift was measured by personal air monitoring. To assess internal exposure, 1-hydroxypyrene (1-OHP) and the sum of 1-,2-,3-,4 and 9-hydroxyphenanthrenes (OHPhe) were determined in pre- and pos-shift urine. 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) adducts and DNA strand breaks and alkali-labile sites were determined as biomarkers of genotoxic effects in white blood cells (WBC). Concentrations of fumes of bitumen were moderately associated with 1-OHP and OHPhe after work shift (r_s = 0.25, P < 0.001 and r_s = 0.36, P < 0.001). Significantly more 8-oxodGuo adducts and DNA strand breaks were found in bitumen-exposed workers pre- and post-shift compared with the reference group. Significantly increased 8-oxodGuo adduct levels were observed post shift in both groups (P < 0.0001). Paradoxically, decreased DNA strand break frequencies were observed after shift in both groups (P < 0.05). No dose-dependent association was observed between exposure to fumes of bitumen and genotoxic effects. However, post shift values in DNA strand break frequency were associated with 1-OHP (r_s = 0.19, P = 0.01). These findings indicate that workers exposed to fumes of bitumen had a higher level of DNA damage (8-oxodGuo and DNA strand breaks) in WBC compared to reference workers. Due to only weak association between 1-OHP and DNA strand breaks the reasons for increased DNA damage in workers exposed to fumes of bitumen remains unclear.

[1]Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin (BGFA), Institut der Ruhr-Universität, Bochum; [2]Institut für Arbeits-, Sozial- und Umweltmedizin, Universität Erlangen-Nürnberg, Erlangen; [3]Berufsgenossenschaftliches Institut für Arbeitsschutz (BGIA), Sankt Augustin, Germany

503

BENZO[*A*]PYRENE-DNA ADDUCTS IN WHITE BLOOD CELLS, AMBIENT MONITORING AND BIOMONITORING OF WORKERS EXPOSED TO FUMES OF BITUMEN

T. Mensing[1], B. Marczynski[1], M. Raulf-Heimsoth[1], A. Spickenheuer[1], P. Welge[1], K. Förster[2], B. Pesch[1], R. Bramer[1], H.U. Käfferlein[1], D. Breuer[3], J.-U. Hahn[3], J. Angerer[2], Th. Brüning[1]

Depending on the bitumen type and temperature, small quantitities of polycyclic aromatic hydrocarbons (PAH) in the order of nanograms per cubic meter are found in fumes of bitumen. Workers handling bitumen-based products may be occupationally exposed to the PAH benzo[*a*]pyrene (B[*a*]P) in fumes of bitumen. B[*a*]P shows genotoxic effects and becomes a carcinogen after metabolic activation. B[*a*]P is metabolized to (±)-*anti*- and (±)-*syn*-benzo[*a*]pyrenediol-epoxide (BPDE) which can covalently bind to DNA. There are only few studies on B[a]P-DNA adducts in workers exposed to fumes of bitumen. In order to evaluate the genotoxic effects of bitumen fumes we conducted a cross-shift study in German mastic asphalt workers. *anti*-BPDE-DNA adducts were determined as a biomarker of exposure to B[*a*]P at the workplace. Personal exposure to fumes of bitumen as wells as urinary metabolites of pyrene (1-hydroxypyrene (1-OHP)) and phenanthrene (sum of 1-,2+9-,3-,4-hydroxyphenanthrene (OHPH)) were measured. HPLC separation and fluorescence detection was used to determine the B[*a*]P-tetrol arising after acidic hydrolysis from (±)-*anti*-BPDE-DNA (limit of detection: 0.5 adducts/10^8 nucleotides). Before and after shift 40.5 and 36.3 % of the values, respectively, were above the detection limit. However, BPDE-DNA-adduct rates were found on a low level. If all values above LOD were considered, no significant differences (P=0.113) in adduct rates before (median: 0.9 adducts/10^8 nucleotides; n=98) and after shift (median: 1.3 adducts/10^8 nucleotides; n=98) could be observed. No correlation between DNA adduct rates and bitumen fume concentration (r_s=-0.003; P=0.978) and between adduct rate and urinary metabolite concentrations was observed (1-OHP: r_s=0.228, P=0.077; OHPH: r_s=0.201, P=0.107). Exposure to B[*a*]P in fumes of bitumen is not high enough to increase B[*a*]P-DNA adduct levels during an 8-hr shift in workers exposed to fumes of bitumen.

[1]Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin (BGFA), Institut der Ruhr-Universität, Bochum; [2]Institut für Arbeits-, Sozial- und Umweltmedizin, Universität Erlangen-Nürnberg, Erlangen; [3]Berufsgenossenschaftliches Institut für Arbeitsschutz (BGIA), Sankt Augustin

504

A COMMON SNP OF THE HUMAN CYP4Z1 GENE SHOWING FEMALE-SPECIFIC HARDY-WEINBERG DISEQUILIBRIUM

P.H. Roos[1], W. Weistenhöfer[1], K. Golka[1], D. Löhlein[2], S. Tschirbs[1] and C.U. Köhler[1]

Tissue-specifc expression of cytochromes P450 is probably related to site-specific cancerogenic processes. Overexpression of certain CYP enzymes is known for tumor cells and its degree can be of prognostic value. For example, CYP1B1 is considered a marker for several tumor types and CYP51 and CYP4Z1, both involved in the metabolism of endogenous compounds, show increased expression in ovarian and breast carcinomas. Since its discovery, CYP4Z1 has been largely neglected and little is known on its tissue distribution, enzymatic activities and gene polymorphisms. An important result, however, is its overexpression in breast carcinomas (Rieger et al., 2004). We found that two SNPs are located in exon 9 of the CYP4Z1 gene just in the binding region of PCR primers used in the literature. They are separated by 2 bases only. For detection of these SNPs, we developed methods using restriction endonucleases. The *Hph*I binding site covers both SNP-sites, while for *Bcc*I only the N-terminally located SNP is relevant. Both endonucleases recognize the 'wild' type gene sequence. Genotyping for the two RFLPs was performed in a control group of 141 persons, mostly West-Europeans. Not a single *Bcc*I-RFLP was detected while the *Hph*I-RFLP was rather common including 38% heterozygotes (het) and 16% homozygotes (hom). Interestingly, the genotype distribution shows gender specific differences: 47% het, 10% hom in males (n=62) and 30% het, 20% hom in females (n=79). In contrast to males, genotype distribution in females is not in Hardy-Weinberg equilibrium (Chi^2: 8,926 for females; 0,199 for males). The disequilibrium may be caused by evolutionary factors. Because the *Hph*I-RFLP is synonymous as judged from gene bank derived sequence variations, we assume that a functionally relevant SNP which is in linkage disequilibrium with the *Hph*I-RFLP will be found in the CYP4Z1 gene. Association of the new CYP4Z1-RFLP with susceptibility for certain cancer types is under investigation.

[1]Institute of Occupational Physiology at the University of Dortmund, Ardeystr. 67, 44139 Dortmund, Germany; [2]Chirurgische Kliniken, Klinikum Dortmund, Beurhausstr. 40, 44137 Dortmund, Germany

505

UDP-GLUCURONOSYLTRANSFERASE 2B7 $C_{802}T$ ($HIS_{268}TYR$) POLYMORPHISM IN BLADDER CANCER PATIENTS FROM SAXONY-ANHALTINA

A. Zimmermann[1], M. Blaszkewicz[1], G. Roth[2], T. Seidel[2], H. Dietrich[2] and K. Golka[1]

UDP-Glucuronosyltransferase 2B7 (UGT2B7) is involved in benzidine metabolism, as demonstrated by in vitro experiments with liver slices. In a study on Chinese benzidine-exposed workers an association between the homozygous mutant genotype and an elevated bladder cancer risk was reported (Lin *et al.* Toxicol. Sci. 2005 85: 502-506). This study was designed to investigate a possible impact of the UGT2B7 genotype on bladder cancer risk in Caucasians. *UGT2B7* polymorphism at locus $C_{802}T$ ($His_{268}Tyr$) was detected using a PCR-RFLP based procedure in a modification of the procedure described by Holthe *et al.* (Eur. J. Clin. Pharmacol. 2002 58: 353-356). A total of 214 bladder cancer cases and 195 controls suffering from different urological diseases and without any history of cancer were investigated. Cases and controls were matched for age and gender. In the case group, the following portions of UGT2B7 genotypes were observed: T/T: 27%, C/C 25%, CT 48%. The portions in the control group were T/T 34%, C/C 24%, C/T 44%. The portions of the alleles in the cases were 51% for the T allele and 49% for the C allele. In the control group, the portions were 55% for the T allele and 45% for the C allele. This study points to ethnic differences between Caucasian and Chinese populations regarding the investigated UGT2B7 genotype. In contrast to the investigation in benzidine-exposed Chinese bladder cancer patients, no relevant differences between the investigated group of unselected bladder cancer patients and the controls could be observed.

[1]Institute for Occupational Physiology at the University of Dortmund, Dortmund, Germany; [2]Dept. of Urology, Lutherstadt Wittenberg, Germany

506

ARSENIC-RELATED SKIN LESIONS DUE TO HIGH ARSENIC COAL AND GLUTATHIONE S-TRANSFERASE P1 $A_{1578}G$ ($ILE_{105}VAL$) POLYMORPHISM

G.F Lin[1], H. Du[2], J.G. Chen[3], H.C. Lu[2], W.C Guo[1], H. Meng[4], T.B. Zhang[4], X.J. Zhang[5], D.R. Lu[6], K. Golka[7] and J.H. Shen[1]

Arsenic-related diseases due to contaminated drinking water are a serious problem in Bangladesh and some other countries. In contrast, in a few villages in Southwest Guizhou Autonomous Prefecture, China, a total of 2,402 patients with arsenic-related skin lesions, like hyperkeratosis, hyper- or hypopigmentation or even skin cancer due to indoor combustion of high arsenic coal have been observed. This study aimed to investigate the cluster of arsenism cases and the possible relevant factors including GSTP1 polymorphism in two clans of different ethnic origin living in one village for generations. A questionnaire-based study was performed in 170 Miao clan P members, 10 of which presenting arsenic-related skin diseases and 153 Han clan G1 members, 50 of which presenting arsenic-related skin diseases. The data was checked with the registration archives since the 1980s. At the same time, arsenic concentrations in samples of coal, indoor air, drinking water, corn and chili pepper that were once baked over the stoves for desiccation, as well as in samples of urine and hair of clan members were determined. Glutathione S-transferase *P1* (*GSTP1*) $A_{1578}G$ polymorphism was genotyped by a RFLP based procedure. Arsenism morbidity in Miao clan P was significantly lower than in the neighbouring

Han clan G1 (5.9% vs. 32.7%, OR: 0.13, 95% CI: 0.06-0.27, P<0.0001). No gender differences were confirmed inside both clans. Analyses of the environmental samples indicated that Miao clan P members were exposed to higher amounts of arsenic via inhalation and food ingestion. Hair and urine samples also proved a higher arsenic body burden in ethnic Miao individuals. No corresponding differences by gender were found. Higher frequencies of combined mutant genotype G/G_{1578} and A/G_{1578} (OR=4.72, 95% CI: 2.34-9.54, P<0.0001) and of mutant allele G_{1578} (OR=3.22, 95% CI: 2.00-5.18, P<0.0001) were detected in diagnosed arsenism patients than in non-diseased subjects. The Miao individuals showed a lower percentage of combined mutant genotypes (30.6% vs. 52.7%, OR=0.40, 95% CI: 0.19-0.84, P=0.015) as well as of mutant allele G_{1578} (OR=0.46, 95% CI: 0.24-0.88, P=0.017) than their Han neighbours. Genetic predisposition influences dermal arsenism toxicity. The GSTP1 $A_{1578}G$ ($Ile_{105}Val$) status might be a susceptibility factor for arsenic-related skin lesions.

[1]Laboratory of Toxicology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai China, [2]Prefecture Center for Disease Prevention and Control, Xingyi, Guizhou, China, [3]Municipal Center for Disease Prevention and Control, Shanghai, China, [4]Department of Health Statistics, 2nd Military Medical University, Shanghai, China, [5]Division of Dermatology, Zhengyi Medical College, Affiliated Hospital, Zhengyi, Guizhou, China, [6]Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, China and [7]Institute for Occupational Physiology at the University of Dortmund, Dortmund, Germany

507

SIGNIFICANCE OF HUMAN ARNT POLYMORPHISMS IN ENVIRONMENTAL CARCINOGENESIS DUE TO CHANGES IN SOME CARCINOGEN METABOLIZING ENZYMES AND CIRCADIAN RHYTHMS

E. Reszka*, H. Zatonski*, W. Wasowicz#, F. Oesch*, B. Oesch-Bartlomowicz*

Evidence has been presented that in industrialized societies light at night, by suppressing melatonin production, poses a new risk for the development of breast cancer and, probably, other cancers as well. Melatonin, as a new member of an expanding group of regulatory factors that control cell proliferation and removal, is the only known chronobiotic, hormonal regulator of neoplastic cell growth. Key regulatory enzymes in melatonin synthesis are arylalkylamine *N*-acetyltransferase (AANAT) and adenyl cyclase (AC). These enzymes play a unique "time keeping role as a molecular interface between the environment and the hormonal signalling of time". Melatonin expression is regulated by the E-boxes on AANAT and AC. Depending on time of the day, these E-boxes mediate either transcriptional activation or repression. Transcription factors BMAL1 (brain and muscle ARNT-like protein) and CLOCK bind as a heterodimer to the E-box enhancers and activate the expression of other circadian rhythm regulating genes that encode transcriptional repressors such as the period (PER1, PER2) and cryptochrome (CRY) proteins. Similar to BMAL1 and CLOCK, ARNT (aryl hydrocarbon receptor nuclear translocator) is also a member of the basic helix-loop-helix PAS (bHLH-PAS) proteins. The homo- or heterodimers of ARNT can recognize E-box or E-box like sequences of various genes thereby mediating biochemical responses to TCDD (e.g. *CYP1A1* induction by aryl hydrocarbon receptor [AHR]/ARNT heterodimer), but also mediating the transcriptional response of cells to a variety of physiological stimuli. Genetic polymorphisms of *hARNT* have been described (www.ncbi.nim.nih.gov, SNP linked to gene, ID: 405). Of the six known *ARNT* genetic non-synonymous polymorphisms we investigated three which are located within the functionally important PAS region: pCMXhArntP200Q, pCMXhArntF363L and pCMXhArntR430Q. In the present study we already have shown, that wild type ARNT together with CLOCK increases transcriptional activity of the E-box containing *Per1* and *Per2* genes. Moreover we analyzed the genetic variation in *hARNT* with respect to their possible functional significance in individual susceptibility to chemical carcinogens (investigating the transcriptional activation of *CYP1A1* as a prototype for carcinogen metabolizing enzymes of the so-called AHR-battery) as well as in molecular clock mechanism participating genes (*Per1, Per2, AANAT, AC*) in various cell lines including human adult retinal pigmented ephitelial cells. The results obtained up to now suggest that a synergistic action of these signalling routes as well as a cross talk between them may occur at restricted temporal windows and should be taken into account for new therapeutic avenues for treating circadian rhythm disturbance-related diseases including some cancers such as breast cancer.

This work was supported by EPI-ToK (Marie Curie ToK Action) and by ECNIS, a network of excellence operating within the European Union 6th Framework Program, Contract No 513943.

*Institute of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, 55131 Mainz, Germany, #Institut of Occupational Medicine, Department of Toxicology and Carcinogenesis, Teresy 8 Str. Lodz, Poland

508

TOXICITY OF NANOPARTICLES TO *DAPHNIA MAGNA* - COMPARISON OF DIFFERENT TiO_2 PARTICLES

K. Wiench, R. Landsiedel, L. Ma-Hock, V. Hisgen, K. Radke, S. Zok, S. Schulte

Nanoparticular TiO_2 has broad spectrum UV attenuation properties and is used in sunscreen applicationsto protect the human skin against UVB radiation. It has been demonstrated that nano-TiO_2 does not penetrate human skin. However, the possible impact on the environment and its aquatic organisms has not been well examined. We studied the acute effects of different species of nano-TiO_2 (uncoated and with different coatings) in comparison with micron-sized TiO_2 pigment on *Daphnia magna* considering the OECD Test Guideline 202 (Part 1). Both, nano- and micron-sized, TiO_2–particles had none or weak acute effects on Daphnids, independent of size or coating of the material. In addition the behaviour of those nanoparticles in different media (OECD M4 media, demineralized water, surface water) was studied, showing that in OECD M4 water and under environmentally relevant conditions nanoparticles rapidly agglomerated and sedimented almost completely. Based on the low toxicity found in the acute studies, a chronic study in Daphnids was started. Results and findings of the studies will be presented and discussed.

BASF Aktiengesellschaft, Department of Product Safety, Ludwigshafen, Germany

509

TOXICITY OF PARKED MOTOR VEHICLE INDOOR AIR

J.T.M. Buters[1], W. Schober[1], J. Gutermuth[1], T. Jakob[2], A. Aguilar-Pimentel[3], J. Huss-Marp[1], C. Traidl-Hoffmann[1], S. Mair[4], S. Mair[4], F. Mayer[4], K. Breuer[4], H. Behrendt[1]

Background The interior of motor vehicles is made of a wide variety of synthetic materials, which emit volatile organic compounds (VOC). We tested the health effects of emissions from vehicles exposed to "parked in sunshine" conditions.

Methods A new and a 3 years old vehicle with identical interior were exposed to 14.000 watt of light. Indoor air was analyzed by GC-MS. Toxicity of extracts of indoor air was assayed in human primary keratinocytes, human lung epithelial A549 cell line and Chinese hamster V79 lung fibroblasts. In addition, toxicity after metabolic activation by CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6 and CYP2E1 was assayed. The effect on type I allergic reaction (IgE-mediated immune response), type IV allergic reaction (T-cell mediated immune response), and irritative potential was also evaluated.

Results A total of 10.9 and 1.2 mg/m^3 VOC were found in new and used motor vehicle indoor air, respectively. The major compounds in the new vehicle were o,m,p-xylene> C_3 and C_4-alkylbenzenes> dodecane> tridecane> methyl-pyrrolidinone. In the used vehicle they were acetone> methylpyrrolidinone> methyl-cyclohexane> acetaldehyde> o-, m-, p-xylene > ethylhexanol> toluene. No toxicity was observed in any cell line with or without metabolic activation. Neither did we find an effect on type IV sensitization or an irritative potential. A slight but statistically significant aggravating effect on IgE-mediated immune response of only the new vehicle indoor air was determined ($p<0.05$).

Conclusion The IgE-response modulating effect of indoor air might be relevant for atopic individuals. Else no direct toxicity, no toxicity after metabolic activation by cytochrome P450, and no irritative or type IV sensitizing potential of motor vehicle indoor air were found, neither from the new nor used vehicle. Our investigations indicated no apparent health hazard of parked motor vehicle indoor air.

[1]Division of Environmental Dermatology and Allergy GSF/TUM, ZAUM-Center for Allergy and Environment, Technical University Munich, Munich, Germany, [2] Klinische Forschergruppe Allergologie, University Freiburg, [3]Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy, Technical University Munich, Munich, Germany, [4]Fraunhofer-Institute for Building Physics IBP, Branch Institute Holzkirchen, Valley, Germany.

510

INVESTIGATIONS ON THE EFFECTS OF SHORT TERM DIESEL EXHAUST INHALATION IN RAT BRAIN

D. van Berlo[1], C. Albrecht[1], A.M. Knaapen[2], F.R. Cassee[3], I.M. Kooter[3], M.E. Gerlofs-Nijland[3], F.J. Van Schooten[2], A. Wessels[1], J. Krutmann[1], and R.P.F. Schins[1].

Combustion-derived nanoparticles (CDNP), such as diesel exhaust particles, have been implicated in the adverse health effects of particulate air pollution. Recent studies indicate that CDNP may translocate to the brain, either along the olfactory nerve upon nasal deposition, or via translocation from the alveoli into the blood and subsequently across the blood-brain barrier. However, the functional consequences of such translocation processes for the brain remain largely to be determined. The aim of our study was to investigate whether short-term diesel exhaust exposure elicits oxidative stress, inflammation and xenobiotic metabolizing enzyme responses in the rat brain. Male Fischer F344 rats were exposed for 2 hours (nose only) to 1.9 mg/m^3 diluted diesel exhaust from the idling (1500rpm) engine type F3M2011 (Deutz Ag, Köln, Germany) with a primary particle size of 65 nm. Animals were sacrificed 4, 18, 24, 48, and 72 hours post-exposure, brains were removed and dissected into six regions, i.e. pituitary, olfactory bulb, olfactory tubercles, hypothalamus, cerebral cortex and cerebellum. Total RNA was extracted for quantitative RT-PCR determination of the expression of Heme Oxygenase-1 (HO-1), inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase-2 (COX-2), Cytochrome P450 1A1 (CYP1A1), 8-Oxoguanosine-Glycosylase-1 (Ogg1), Apurinic/Apyrimidinic Endonuclease/Redox Factor-1 (APE/Ref-1), and DNA-polymerase-β (polβ). Preliminary findings revealed an enhanced expression of CYP1A1 and iNOS in pituitary tissue 4 hours post-treatment, indicating that short term exposure to diesel exhaust may elicit rapid gene expression responses in specific brain tissue areas. Whether these effects result from direct actions of the particulate and/or volatile component of diesel exhaust in the brain, or via indirect mechanisms remains to be elucidated.

This study is supported by grants from BMU, and Institute for Public Health and the Environment, the Netherlands.

[1]Institut für umweltmedizinische Forschung (IUF) an der Heinrich Heine Universität Düsseldorf gGmbH, Germany; [2]Maastricht University, The Netherlands; [3]National Institute for Public Health and the Environment (RIVM), The Netherlands

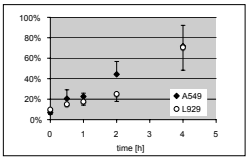
511

EFFECTS OF OZONE IN A549 AND L929 CELLS

K. Reinelt, E. Doklea, H. Mückter

Ozone (O_3) is increasingly being used in large quantities for the elimination of persistent contaminants in wastewater treatment plants. The high throughput (>10kg/h) of high O_3 concentrations (>50g/m^3) of such plants prompted us to expose A549 (lung adenocarcinoma) and L929 (mouse fibroblasts) cells to high ozone levels.

In an *in-vitro* exposure apparatus described previously (J Pharmacol Toxicol Meth 40: 63-9, 1998) we exposed A549 and L929 in standard culture flasks (50mL, 25cm^2) to graded concentrations of O_3 (0…4000ppm), delivered in carbogen carrier gas (95%O_2, 5% CO_2). Controls received carrier gas only. Cells were completely covered by the culture medium to avoid a direct gas/cell contact. Cells were exposed to O_3 over 4h, with 8 flasks being exposed simultaneously plus 4 control flasks. After 4h the cells were switched to fresh medium and ambient air. Cells in some flasks were allowed to spontaneously recover from O_3 exposure for another 20h. After 24h the incubation was terminated. Cytotoxic damage was assessed by measuring dye exclusion (TrypanBlue), total glutathione (GSX), ATP and other indicators of cytotoxicity.



The figure shows the percentage of stained A549 and L929 cells after exposure to O_3 (2800ppm for ≤4h). There was only little spontaneous recovery of exposed cells, as assessed by revovery of glutathione (GSX) and ATP contents. A549 cells, which are rich in GSX when compared to L929 cells, had no better survival chances than the fibroblasts. Our findings indicate that intracellular redox buffers cannot avert O_3 damage from exposed cells.

Walther-Straub-Institut für Pharmakologie und Toxikologie der LMU, Goethestr. 33, D-80336 München, Germany

512

DNA INTEGRITY IN LYMPHOCYTES FROM SUBJECTS EXPOSED TO OZONE *IN VIVO* DETERMINED BY FAST MICROMETHOD[+]

C. Finkenwirth[1, 2], H.C. Schröder[1], B. Rossbach[2] and A. Muttray[2]

INTRODUCTION: *In-vitro* and *in-vivo* animal studies have demonstrated that exposure of cells to ozone may cause DNA damage including single strand breaks. The question of our research is if there is an effect on DNA integrity of human lymphocytes. METHODS: The frequency of DNA single-strand breaks in healthy adult men was determined in a randomised parallel group (n=40) before, directly after and 4 hours after exposure to ozone. The "ozone group" was exposed to 0.21 ppm ozone in an exposition chamber for two hours while filtered air was used for the control group. Lymphocytes were isolated and stored in liquid nitrogen. DNA damage was determined using the Fast Micromethod DNA single-strand break assay. This microplate assay measures the rate of unwinding of cellular DNA under alkaline conditions using a fluorescent dye that binds to double-stranded DNA. The results were expressed as strand scission factor (SSF). RESULTS: There were no differences of the median SSF values determined directly after exposure (median SSF values ozone vs. control: -.00025 vs. .01388) and 4 hours after exposure (-.02069 vs. -.00159) using the Mann-Whitney-Test for comparison (p= .799 directly after exposure; p= .822 4 hours after exposure). DISCUSSION: The lack of an effect after ozone exposure could be caused by the fast repair of ozone-induced DNA damage as demonstrated in other studies. Most other studies used higher concentrations of ozone. Therefore, a further explanation might be that the ozone concentration of 0.21 ppm used in this study is too low to induce DNA damage exceeding the natural repair capacity.

[1] Institut für Physiologische Chemie und Pathobiochemie, Johannes Gutenberg-Universität, Mainz, Germany
[2] Institut für Arbeits-, Sozial- und Umweltmedizin, Johannes Gutenberg-Universität, Mainz, Germany

513

AHR PATHWAY INDUCED IN RAT HEPATOMA CELLS BY BROMINATED DIBENZOFURANS IN CONTAMINATED BDE47 SAMPLES

S. Strack, M. Wahl, R. Guenther, C. Weiss

Brominated flame retardants (BFR) and various decomposition products can be detected world wide in the environment, but also regional monitoring programs prompt concern by observed concentrations in human and wild life. Risk assessment for this novel class of persistent organic pollutants is an important premise for preventive and sustainable policies, for which reliable toxicological data bases are essential. BDE47 is an environmentally relevant tetrabromodiphenyl ether (2,2',4,4' tetra BDE) found in biota and human tissues. Structural similarities with halogenated dibenzodioxins and dibenzofurans refer to the possibility that similar toxic effects can be caused by BDE47, and indications for an involvement of the Ah receptor (AhR) would suggest a 'dioxin-like' behaviour. In our experiments we use the rat hepatoma cell line 5L and an AhR deficient sub clone BP8 to analyze on protein level possible CYP1A1 induction by our BDE47 sample in presence and also absence of the Ah receptor. The substance we use for exposures of the cells was synthesised and purified at the University of Stuttgart. GC-MS analyses of different batches did not show any dioxin impurities, however, traces of dibenzofurans could not be excluded completely. Exposure to that BDE47 sample for 24 hours leads to a dose-dependent induction of a CYP1A1 expression in 5L wt cells beginning at concentrations of 50 µM, but no induction was visible in BP8 cells. Recent HRGC/LRMS analysis made evident that the sample was contaminated by brominated dibenzofurans: 0.04 % 2,3,7,8 – TBDF and 0.03 % 1,2,3,7,8 PeBDF, suggesting that those minimal traces of contaminants in our BDE47 sample were responsible for the observed 'dioxin-like' effects.

Financially supported by BWPLUS (BWR 24013)

Forschungszentrum Karlsruhe GmbH, Institut für Toxikologie und Genetik, D-76021 Karlsruhe, Germany, e-mail: strack@itg.fzk.de

514

AHR SIGNALLING IS NOT INDUCED BY POLYBROMINATED DIPHENYL ETHER (BDE 47) IN RAT HEPATOMA CELLS

M. Wahl, R. Guenther, L. Yang, U. Strähle, C. Weiss , S. Strack

Polybrominated diphenyl ethers (PBDE) have been widely used as flame retardants in a variety of consumer goods. They were produced mainly as technical mixtures containing different brominated congeners. Especially the tetrabromodiphenyl ether congener BDE47 (2,2',4,4'-tetra BDE) accumulates in the environment as evidenced by a variety of monitoring studies. However, underlying mechanisms of toxicity are still insufficiently understood and need to be established to provide a basis for human and ecological risk assessment. Structural similarities of PBDE congeners with polychlorinated biphenyls (PCBs) and other dioxin-like compounds raised concerns about a possible activation of the aryl hydrocarbon receptor (AhR). Indeed, some reports demonstrate AhR activation by PBDE measured by induction of its target gene *cyp1A1*, whereas others could not confirm these observations. As a possible explanation for these discrepant findings, trace contaminations of PBDE samples with potent AhR ligands, such as dioxins and furans, were discussed. In the present study we used different grades of purified PBDE samples and analyzed their effects on AhR activity using not only *cyp1A1* induction as a rather limited read out but also include several other known downstream consequences of AhR activation including additional target genes as well as inhibition of proliferation. Whereas highly purified BDE47 did not trigger AhR signalling, low grade purified PBDE samples enhanced expression of multiple AhR target genes and blocked cell cycle progression in 5L rat hepatoma cells. Chemical analysis of the latter samples identified traces of tetrabrominated dibenzofuran (TBDF), which most likely were responsible for AhR activation. Indeed, treatment of 5L cells with simply the same TBDF concentration as detected in the contaminated PBDE sample resulted in identical AhR dependent responses. In conclusion, PBDE does not induce AhR signalling measured with a variety of different end points in rat hepatoma cells indicating that AhR may not be involved in PBDE toxicity. Preliminary experiments in primary rat hepatocytes as well as in the ecotoxicological model organism zebrafish also indicate no substantial effect of PBDE on AhR thus further corroborating our findings *in vivo*.

Supported by BWPLUS (BWR 24013)

Forschungszentrum Karlsruhe GmbH, Institut für Toxikologie und Genetik, D-76021 Karlsruhe, Germany, e-mail: wahl@itg.fzk.de

515

ACCUMULATION OF DIOXIN, PCB AND OTHER CONTAMINANTS IN FARMED SALMON

M.P. Magnussen[1)] and G. Vang[2)]

In recent years there has been considerable focus on the contaminants in farmed atlantic salmon. Health authorities in Europe and the USA have divergent opinions regarding the health benefits of eating fish. To protect the population against the contaminants in fish, health authorities have introduced limits for several of these contaminants, and others may be introduced in the future. Therefore, we tried to gain insight into the kinetics of the transfer of different contaminants from the fish feed into the edible portions of farmed salmon under normal rearing conditions. About 70,000 salmon (mean weight: 200 g) were transfered to an ocean pen in May 2004. Samples were taken every three months until September 2005, 467 days later (mean weight: ca. 5 kg). The batches and amounts of feed used were registered and feed samples were mixed accordingly. Following compounds resp. groups were determined: Dioxin (PCDDs/PCDFs), dioxin-like PCBs, marker PCBs, PBDEs, organochlorine pesticides, PAHs and heavy metals. Analyses were performed by ERGO Forschungsgesellschaft, Hamburg. Most of the compounds analysed accumulated during the investigation with a similar pattern, but to various extents. All concentrations were presented on wet weight and lipid basis. Accordingly, two different prediction models were developed. The "wet weight" model based on 1) wet weight data in the trimmed fillet, 2) feed conversion rate, 3) mean concentrations in the feed, 4) number of days of feeding, and 5) mean fillet fraction, and the "lipid data" model based on 1) mean lipid data in the feed, 2) mean ratio between lipid data in the trimmed fillet and the feed, and 3) lipid concentration in the fillet. Compared to actual data (367 - 494 days after starting the feeding), the two models gave fairly good predictions (www.havsbrun.fo), deviating only marginally from found data. All concentrations found in the trimmed fillet were far below EU limits. However, the prediction models presented are built only on this investigation. Time will show how good they are in practice and if they must be made with time more reliable.

[1)]Food, Veterinary and Environmental Agency, FO-100 Tórshavn, Faroe Islands (present address Faroe Marine Products, FO-520 Leirvík, Faroe Islands) and [2)]P/F Havsbrún, FO-530 Fuglafjørður, Faroe Islands

516

IDENTIFICATION OF THREE DIFFERENT FUNCTIONAL DOMAINS IN THE ACTIVATOR PROTEIN ActA FOR 3α-HYDROXYSTEROID DEHYDROGENASE/CARBONYL REDUCTASE EXPRESSION IN *COMAMONAS TESTOSTERONI*

G. Xiong, S. Jin, C. Glockner, U. Hansen, S. Zander, E. Draus, K, Gerwien, C. Freudenhammer and E. Maser

3α-Hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is a key enzyme in the degradation of steroid compounds and polycyclic aromatic hydrocarbons in *Comamonas testosteroni*. We previously reported the cloning and isolation of an activator protein (ActA) which regulates gene expression of 3α-HSD/CR and which undergoes proteolytic cleavage to an active ActA protein from an inactive pre-activator protein. In this work, it was found that ActA binds to ^{3}H-testosterone. Antibodies against ActA were prepared and an ELISA method for ActA determination established. We could show that ActA overexpression resulted in an increase in 3α-HSD/CR expression. Apparently, ActA itself consists of three domains with different function: an N-terminal ^{3}H-testosterone binding domain of 49 amino acids, a central DNA binding domain, and a C-terminal RNA polymerase binding domain. The C-terminal RNA polymerase binding domain was characterized in the present study with purified RNA polymerase from *E. coli*. To this end we hypothesize that upon proteolytic ActA activation, the N-terminus is cleaved from the pre-activator protein such that the central parts binds to the promoter of 3α-HSD/CR and attracts RNA polymerases via affinity to its C-terminus. The gene fragments coding for the three different ActA domains were cloned into pET-15, expressed in BL21 cells and purified with His-Tag columns. ^{3}H-testosterone, DNA and RNA polymerase binding activities for each of these three protein parts are currently in progress.

Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswiker Str. 10, D-24105 Kiel, Germany

517

CHARACTERIZATION OF FUNCTIONAL DOMAINS OF THE TEIR PROTEIN WHICH REGULATES TESTOSTERONE-INDUCED EXPRESSION OF 3α-HYDROXYSTEROID DEHYDROGENASE/CARBONYL REDUCTASE IN *COMAMONAS TESTOSTERONI*

E. Maser, G. Trentmann, S. Paulsen, A. Göhler and G. Xiong

A new steroid-inducible gene, *teiR* (testosterone inducible regulator), necessary for testosterone degradation in *Comamonas testosteroni* was suggested to be a LuxR-type transcription factor

being involved in part by a quorum-sensing regulatory mechanism. In the present work, we constructed a TeiR-GFP fusion protein to elucidate the subcellular location of TeiR within *Comamonas testosteroni* cells. Fluorescence microscopy revealed TeiR to be a membrane-associated protein with an exclusively polar localization of the bacterium. Twitching and swimming tests showed that TeiR serves as a sensor protein for a variety of different steroid compounds. Cotransformation with plasmids harbouring the 3α-HSD/CR gene and the TeiR gene, respectively, into *E. coli* cells yielded an increase in 3α-HSD/CR gene expression. TeiR-knockout mutants of *Comamonas testosteroni* did not respond to any steroid compound which otherwise induced high levels of 3α-HSD/CR in wild type cells. A more detailed analysis of TeiR suggested the presence of different functional domains at the *N*-terminus and the *C*-terminus. Preliminary results indicate that testosterone binds to the *C*-terminal domain. Plasmids containing different gene fragments of the domains were prepared and are currently used to proof their function in steroid dependent gene regulation in *Comamonas testosteroni*.

Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswiker Str. 10, D-24105 Kiel, Germany

518

QUANTITATIVE HUMAN HEALTH RISK CHARACTERISATION – A NEW APPROACH FOR BIOCIDES

R. Solecki, M. Herzler, R. Pfeil, M. Rauch, V. Ritz, H. Schneider, C. Sieke, D. Westphal, L. Wollenberger and U. Gundert-Remy

To assist both applicants and authorities in implementing the EU-Biocide Directive, a series of Technical Notes for Guidance has been issued by the European Chemicals Bureau in 2002. However, a variety of different approaches for Quantitative Human Health Risk Characterisation were applied so far. Therefore, we have taken the initiative to further develop the existing risk characterisation procedures. We suggest putting special emphasis on all different routes of exposure and to take into account aggregate and cumulative exposures. For all relevant exposure scenarios characterised by duration, frequency, route, and the (sub-)population exposed, the most appropriate end-points to be used as Points of Departure in risk characterisation must be identified. Moreover, the new approach have to be able to identify the relevant animal model to derive NOAELs for specific (sub)populations. Consequently, we propose the derivation of acute, medium-term and long-term Acceptable Exposure Levels (AELs) as general health-based reference values for the general and specific (sub)populations. Systemic AELs should be derived for acute, medium-term, and long-term exposure via all routes applicable based on the systemic toxicity of the active substance using default Assessment Factors. In the absence of chemical-specific data, a default 100-fold factor is applied when assessing risks to the general population. Local effects at the portal of entry should be dealt with separately. Considering the combined exposures (aggregate and cumulative) from all representative uses facilitates the establishment of limit values for active substances used in several areas such as biocidal products, veterinary medicinal products, plant protection products, and in industrial chemicals. The new approach, specifically developed for biocides, might also be applicable as a general tool for Risk Assessment under other EU substance regulations.

Federal Institute for Risk Assessment, Thielallee 88-92, D-14195 Berlin, Germany

519

CHEMTEST PHASE I PROJECT: EVALUATION OF EXPOSURE DATA FOR CONSUMER PRODUCTS

K. Schneider[1] T. Platzek[2], G. Heinemeyer[2], M. Doser[3], M. Lahaniatis[4], D. Papameletiou[4]

The EIS-ChemRisks Toolbox has been established by the Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC). It was developed over the period 2003 – 2005 and represents an infrastructure to systematically support the exchange of information on exposure issues among stakeholders. It provides exposure data, tools and scenarios to facilitate harmonised and up-to-date exposure assessments for consumer products. Valuable exposure data (chemical content, release, migration) will be compiled for the Toolbox in the ChemTest-Phase I Project* ("Compilation, analysis and evaluation of data and methodologies to qualitatively and quantitatively characterise human exposure to chemicals released from consumer products/articles", July 2006 - April 2007). Exposure data and test methodologies on chemicals released from the following types of consumer products are collected and evaluated: apparel/clothing and home textiles, carpets, automotive textiles, toys and non-woven personal care products (e.g. diapers, feminine hygiene liners). These goals are achieved by an extensive literature search, by a Europe-wide questionnaire survey, by developing evaluation criteria for data quality, and by conceptional contributions to the further development of the toolbox. Literature search and questionnaire survey identified hundreds of data sets on chemical exposures from these product categories. The questionnaire survey included contacts to industrial associations (Euratex, Edana, Toy Industries of Europe, among others), authorities and other stakeholders and served also to establish a network of experts for exposure assessments for these types of consumer products. Proposed evaluation criteria for data quality are structured in 4 groups with the following key aspects: 1. description of (the type of) data; 2. description of the procedure for obtaining/measuring data; 3. Internal consistency and plausibility of report/data; 4. Other aspects of quality - formal criteria (e.g. certification, good laboratory praxis).

* http://www.jrc.cec.eu.int/eis-chemrisks/chemtest_phaseI.cfm

[1] FoBiG, Forschungs- und Beratungsinstitut Gefahrstoffe GmbH, Freiburg, Germany; [2] BfR, Bundesinstitut für Risikobewertung, Berlin, Germany; [3] ITV, Institute of Textile Technology and Process Engineering, Denkendorf, Germany; [4] European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Ispra, Italy

520

STUDIES ON THE CYTOTOXICITY AND GENOTOXICITY OF SELECTED VOLATILE ORGANIC COMPOUNDS (VOC) USING A NEW DEVELOPED CHAMBER EXPOSURE SYSTEM (BIKAS): AN APPROACH FOR RISK ASSESSMENT OF VOLATILE INDOOR AIR CONTAMINANTS

Richard Gminski, Tao Tang, Volker Mersch-Sundermann

A method for evaluating cells exposed to airborne, inhalable compounds from indoor air is desired as a more rapid and physiological alternative to exposing cells with the compounds in culture medium. The most promising approach is based on a biphasic cell culture technique, where cells are grown on microporous membranes at an air-liquid interface. Here the cells were supplied with culture medium from the basal side of the membrane whereas the apical part with the cultivated cells is in direct contact with the gaseous compounds. To mimic more closely the physiological environment, we cultured A549 cells (type II-like human cell line) on microporous membranes (Falcon Transwells®), which were placed in an exposure chamber (CULTEX®) with culture medium provided only on the basolateral surface. This alveolar type II cells are the metabolically active cells of the deep lung and are potentially important targets of inhaled compounds. The apical surface of the cells were than exposed to gaseous volatile organic compounds (VOC) generated in a BIKAS-Chamber (Biologisches Kammer-Expositions-System, 0.25 m^3) by vaporisation of the liquids with filtered und humidified air (flowrate: 2.2 ml/min, humidity 50%, air change 0.5 h^{-1}) at a temperature of 22°C. The concentration of VOC in the gas phase were evaluated by sampling the air on Air Toxic Tubes® (sampling rate: 1 ml/min) followed by Thermal-Desorption-Capillary GC-MS analysis. The air was passed over the cell surface at a flow rate of 5 ml/min per transwell. Cells were exposed for 1 hour at different concentrations of selected VOC. Analyzed responses include cleavage of water soluble tetrazolium salts (WST-1) as a measure of cell numbers and metabolic activity, erythrosine red assay as a measure of viability and comet assay as an indicator of genotoxicity. The combination of this new cultivation and exposure technique with cytotoxicity assays and comet assay provides a realistic and efficient model for sensitive detection of cytotoxicity and DNA damage induced by airborne and inhalable compounds such as VOC in indoor air.

Institute of Indoor- and Environmental Toxicology (IIUT), Fachbereich Medizin, Uniklinikum Giessen, Germany

521

A NEW SAMPLING METHOD FOR DETERMINING VOLATILE TOXIC COMPOUNDS IN LABORATORIES

W. Marquardt[1], M. Seiss[2], A. Oxynos[2], W. Spahl[3], R. Hickel[1], F.-X. Reichl[1]

A new sampling method based on "Solid Phase Micro-Extraction" (SPME) was developed for a fast screening and quantification of volatile toxic compounds at workplaces in laboratories. The new method was tested in dental laboratories to investigate the exposure of dental technicians to various volatile methacrylates. The methacrylates methyl methacrylate (MMA), ethyleneglycol dimethacrylate (EGDMA), and triethyleneglycol dimethacrylate (TEGDMA) are used as monomers and comonomers in many dental materials. From dental personnel dental technicians are most strongly exposed to these substances. The air levels of these methacrylates were measured at different times and different workplaces in three dental laboratories in Munich, Germany. Short-term air sampling (15 min) was performed using SPME fibers. The SPME fibers were coated with carbowax/divinylbenzene to enrich the analytes. For analysis the analytes were thermical desorbed from the fiber and subsequently analyzed directly by gas chromatography-mass spectrometry. MMA, EGDMA and TEGDMA were identified in the air of dental technicians' workplaces. The exposure levels of these methacrylates varied at the workplaces. The maximum concentrations found were 30 mg/m^3 for MMA, 186 µg/m^3 for EGDMA, and 41 µg/m^3 for TEGDMA. Air levels of MMA up to 1.1 mg/m^3 occurred in all laboratories in the morning, before the dental technicians started to work. The maximum levels of MMA measured in this study were 4-14 times lower than the toxicologically relevant maximum allowable concentrations defined in various countries. Due to the high allergenic potential of methacrylates, their exposure levels should be kept as low as possible to reduce health risks in dental technicians.

[1] Department of Operative Dentistry and Periodontology, Ludwig-Maximilians-University of Munich, Goethestr. 70, 80336 Munich, Germany; [2] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Goethestr. 31, 80336 Munich, Germany; [3] Department of Organic Chemistry, Ludwig-Maximilians-University of Munich, Butenandtstr. 5-13, 81377 Munich, Germany

522

THE NEED FOR A NEW PARADIGM FOR DOSE SELECTION IN REPRODUCTIVE TOXICOLOGY STUDIES

Anderson J. M. Andrade, Simone W. Grande, Christine Gericke, Chris E. Talsness, Konstanze Grote, Ibrahim Chahoud

The use of animal bioassay data is essential for the hazard identification process of chemicals. Most regulatory guidelines usually require that a control and three doses be used. In developmental and reproductive toxicology studies, the highest dose is selected to produce maternal or adult toxicity. However, given the advancements made by chemical industries, very high doses are usually needed to induce even slight maternal toxicity. The lowest dose tested is generally selected to yield the no observed adverse effect level (NOAEL) for adult and/or offspring effects. As a consequence, the spacing of experimental dosage levels is in most cases very large. Several aspects of the NOAEL approach have been criticized and the use of the benchmark dose (BMD) method has been proposed as an alternative model. This method can be applied to different types of dose-response curves and allows the calculation of a dose at a specified response level. However, once the BMD is estimated, high-to-low-dose extrapolation procedures similar to those applied to the NOAEL are needed. Here, we use the data of an extensive dose-response study with the plasticizer di-(2-ethylhexyl)-phthalate (DEHP) to compare the NOAEL and BMD methods. Two wide ranges of DEHP doses (low and high) were used. For the endpoints which the BMD method could be applied, we noted little, if any, difference between the two methods, *i.e.,* the NOAEL and BMD values were very similar. More importantly, our results show that both the NOAEL and BMD approaches are not satisfactory when dealing with nonmonotonic dose-responses, *i.e.*, biphasic responses are overlooked when only high doses are tested. We conclude that the main problem with current toxicity testing protocols is not related to the approach used to estimate risks, but rather to the selection of doses. We propose that widening the range of doses tested to include human relevant levels will considerably reduce the uncertainties associated with quantitative risk assessment.

Charité University Medical School Berlin, Institute of Clinical Pharmacology and Toxicology, Germany

523

COMPARATIVE GENOMICS AND PHYLOGENETICS OF CYP3 FAMILY

H. Qiu[1], S. Taudien[2], H. Herlyn[3], M. Platzer[2], L. Wojnowski[1]

CYP3A enzymes play an important role in drug therapies, since they metabolize an estimated 50% of currently prescribed drugs. Our understanding of the physiological roles of the individual CYP3A genes and of their regulation is incomplete. Using genomic sequences from 16 species we investigated the evolution of Cyp3 genes and their genomic loci over a period of 450 million years. More than one Cyp3 locus was found in the frog, as well as in all fish genomes available. Cyp3b and Cyp3c genes were found exclusively in some fish species. Avian, non-eutherian mammalian, and some ray finned fish Cyp3a loci reside in an ancestral location. In contrast, Cyp3a genes of eutherian mammals are found in a derivative locus in a different genomic environment. Despite superficial similarities, Cyp3a loci of different lineages arose through independent, multiple gene duplication events. Orthologs of human CYP3A genes can be identified only within other primates. Cyp3a in new world monkeys such as the common marmoset evolved substantially differently from catarrhine primates. The marmoset contains a duplicated Cyp3a5, a Cyp3a21 gene, but no Cyp3a43. In contrast, catarrhine primates, besides single copies of Cyp3a5 and Cyp3a43, have a higher number of Cyp3a21-related genes (Cyp3a4, Cyp3a7, and Cyp3a67). The hominid-specific Cyp3a67 has been lost in humans. Maximum likelihood estimates of the dN/dS ratio suggest that Cyp3a7 has evolved under particularly strong positive selection following Cyp3a7/67-duplication and before hominid speciation. Taken together, our results provide a framework for studies of CYP3A function and regulation using phylogenetic data. The utility of the marmoset as an animal model of the entire human CYP3A gene family appears to be limited. CYP3A7, the dominant fetal CYP3A in humans, may have acquired some catalytic functions specific to hominids.

[1] Institut für Pharmakologie, Johannes Gutenberg-Universität Mainz; [2] Leibniz Institute for Age Research - Fritz Lipmann Institute, Jena; [3] Institut für Anthropologie, Johannes Gutenberg-Universität Mainz

524

PHARMACOGENETICS OF REVERSE TRANSCRIPTASE INHIBITORS: PREDICTIVITY OF *CYP2B6* GENOTYPE FOR PHARMACOKINETICS OF EFAVIRENZ IN AIDS-PATIENTS

U.M. Zanger[1], M. Rotger[2], H. Tegude[1], S. Colombo[3], M. Cavassini[4], H. Furrer[5], L. Décosterd[3], J. Blievernicht[1], T. Saussele[1], H.F. Günthard[6], M. Schwab[1], M. Eichelbaum[1], A. Telenti[2], and the Swiss HIV Cohort Study.

The non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) is often used as first-line treatment in patients with acquired immunodeficiency syndrome (AIDS). Elevated plasma concentrations of EFV, a substrate of CYP2B6, are associated with CNS toxicity. To assess the impact of *CYP2B6* allelic diversity on EFV pharmacokinetics, we performed extensive genotyping in 169 AIDS patients recruited from the genetics project of the Swiss HIV Cohort Study (SHCS, www.shcs.ch; Rotger et al., *Pharmacogenet Genomics,* 2005) using a recently developed MALDI-TOF assay for 15 SNPs (Blievernicht et al., *Clin Chem* 2007). Seventy-seven (45.5%) individuals carried known (*CYP2B6*6,*11,*15,*18),* or new loss/diminished-function alleles. Re-sequencing of *CYP2B6* defined two new loss-of-function alleles: allele **27* (593T>C [M198T]), that results in 85% decrease in enzyme activity as shown by transient transfection of COS1 cells; and allele **28* (1132C>T), resulting in protein truncation at arginine 378. Median AUC levels were 188.5 µg*h/ml for individuals homozygous for a loss/diminished-function allele, 58.6 µg*h/ml for carriers, and 43.7 µg*h/ml for non-carriers ($p<0.0001$). Individuals with a poor metabolizer genotype had a likelihood ratio of 35 (95% CI, 11-110) of presenting very high EFV plasma levels. These data show that *CYP2B6* genotypic variation explains to a large extent EFV pharmacokinetics thus emphasizing the usefulness of *CYP2B6* genotyping for adapting dosage to avoid toxicity and to prevent viral failure.

Supported by the Swiss National Science Foundation (grant No 324-11655/1), the Swiss HIV Cohort Study (Swiss National Science Foundation, grant 3345-062041), the Santos Suarez Foundation, the H.W. &. J. Hector Foundation (Mannheim), and the Robert-Bosch Foundation (Stuttgart, Germany).

[1]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, and University of Tübingen, Germany; [2]Institute of Microbiology, University of Lausanne; [3]Division of Clinical Pharmacology, University Hospital, Lausanne, [4]Division of Infectious Diseases, University Hospital, Lausanne, [5]Division of Infectious Diseases, University Hospital, Bern, [6]Division of Infectious Diseases, University Hospital, Zurich, Switzerland.

525

A POLYMORPHISM ADJACENT TO THE INSIG2 GENE AND THE RISK FOR OBESITY – MECHANISTIC ASPECTS

A. Bornhorst, C. Rimmbach, A. Kayser, A. Krüger, G. Tessmann, I. Geissler, C. Schwahn, H. Völzke, H.K. Kroemer, D. Rosskopf

The obesity epidemic is a major contributor to the global burden of chronic disease and disability. A polymorphism (rs7566605) adjacent to the INSIG2 gene has recently been reported to control approximately 10 % of the BMI increase in obesity (Science, 2006). INSIG 2 (just like INSIG1) is an endoplasmic reticulum protein acting as an anchor for proteins with a sterol-sensing domain, e.g. SCAP and HMG-CoA-reductase. We analyzed the effect of the rs7566605 polymorphism on obesity in 4310 individuals of the Study of Health in Pomerania, a cross-sectional population-based survey for the epidemiologic analysis of common diseases. In contrast to the original report we did not observe an increased risk for obesity in homozgous carriers of the rs7566605 C-allele. When we re-addressed this question in subjects with a BMI of > 25, i.e. already overweight individuals, we observed a consistent effect of the rs7566605 C-allele on the risk to become obese (OR 1.32; 95%CI 1.02 – 1.72; p = 0.0378). The mean BMI of CC carriers was 0.73 ± 0.25 kg/m^2 higher than in the other genotypes (p = 0.0034). This result suggests that the INSIG2 polymorphism acts as a disease-modifying factor in individuals on their way to obesity. To unravel the mechanisms for this association, we analyzed several other polymorphisms in the INSIG2 gene and detected four other polymorphisms highly linked to the rs7566605 C-allele. Furthermore, we analyzed the relative expressions of INSIG2 (and of INSIG1) transcripts by quantitative RT-PCR in several human tissues from different donors stratified by their genotype. Interestingly, the genotype-specific regulation of INSIG2 transcripts differed in liver from other tissues not involved in metabolic regulation (lymphoblasts, fibroblasts). We hypothesize that the rs7566605 C-allele affects INSIG2 transcript expression either directly or via tightly linked genetic variants.

Institut für Pharmakologie & Institut für Epidemiologie und Sozialmedizin – Ernst-Moritz-Arndt Universität Greifswald

526

FUNCTIONAL CHARACTERIZATION OF MRP4/ABCC4 PROTEIN VARIANTS IN *XENOPUS LAEVIS* OOCYTES

T. Lang[1], D. Janke[1], S. Mehralivand[1], U. Gödtel-Armbrust[1], A. Habermeier[1], U. Gradhand[5], C. Fischer[4], D. Strand[2], M. Toliat[3], K. Klein[4], U.M. Zanger[4], M. Schwab[4], M.F. Fromm[5], E.I. Closs[1], and L. Wojnowski[1]

The importance of genetic variants of drug transporters for drug disposition is less well understood in comparison to variants of drug-metabolising enzymes. We have undertaken a comprehensive functional characterization of protein variants of the multiple drug resistance protein 4 (MRP4, ABCC4) identified by direct sequencing of Caucasian DNA samples. MRP4 protein belongs to the C subfamily of the ATP-binding cassette (ABC) transporter superfamily (ABCC) and participates in the transport of diverse antiviral and chemotherapeutic agents. We identified and examined 11 *MRP4* missense genetic variants (non-synonymous SNPs) for their effect on the expression, localization, and function of the transporter. All MRP4 variants were expressed in *Xenopus* oocytes in-frame with a green fluorescent protein. All variants were found to be expressed predominantly in the oocyte membrane. Four variants (Y556C, E757K, V776I, T1142M) exhibited a 20 to 40% reduced expression level compared to wild-type. Efflux studies showed that the anti-cancer drug 6-mercaptopurine is transported by MRP4 in an unmodified form. Compared to wild-type MRP4, the variant V776I revealed a significantly lower activity in 6-mercaptopurine transport, while variant Y556C displayed a significantly higher transport of 9-(2-phosphonyl methoxyethyl) adenine PMEA. The transport properties of the other variants were comparable to wild-type MRP4. Our study shows that *Xenopus* oocytes are an experimental system well-suited to characterize the human MRP4 protein and its protein variants. Carriers of the rare MRP4 variants Y556C and V776I may have altered disposition of certain MRP4 substrates.

[1]Institute of Pharmacology, University of Mainz, Mainz, Germany; [2]First Department of Internal Medicine, University of Mainz, Mainz, Germany; [3]Cologne Center for Genomics, University of Cologne, Köln, Germany; [4]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany; [5]Institute of Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Erlangen, Germany.

527

ALLELIC FREQUENCIES OF 5 NON-SYNONYMOUS GENETIC VARIANTS IN THE HUMAN *SLCO1B1* GENE AND *IN VITRO* ANALYSIS OF SNP 1929A>C

A. Seithel[1], K. Klein[2], U.M. Zanger[2], M.F. Fromm[1], J. König[1]

There is increasing evidence that polymorphisms in the *SLCO1B1* gene (encoding the human organic anion transporting polypeptide OATP1B1) can be associated with alterations in transporter properties, e.g. loss of transport activity or miss-trafficking of the protein to the membrane. Therefore, the pharmacokinetic of drugs which are transported by OATP1B1 may be affected by these polymorphisms. In this study we determined the allelic frequencies of 5 genetic variations (452A>G, 1007C>G, 1454G>T, 1628G>T, 1929A>C) in the *SLCO1B1* gene. These variations have been first described in Japanese. Since we could not find the four variations 452A>G, 1007C>G, 1454G>T, and 1628G>T in the investigated 285 Caucasian DNA donors, these variations apparently have no relevance for the Caucasian population. 25 subjects were heterozygous and one subject was a homozygous carrier of 1929A>C giving an allelic frequency of 4.7 %. Therefore, we focused our *in vitro* analysis on SNP 1929A>C. Samples which were homozygous or heterozygous for SNP 1929A>C were analyzed on their hepatic OATP1B1 protein expression. Immunoblot experiments demonstrated no difference in expression level in 1929A>C samples compared to wild-type samples. To analyze the possible consequences of SNP 1929A>C on the transport mediated by OATP1B1, we established HEK cells stably expressing the mutated OATP1B1 protein (HEK-OATP1B1-Leu643Phe). We performed uptake experiments using sulfobromophthalein, taurocholic acid, and estradiol-17-β-D-glucuronide as established substrates. The uptake rates and kinetics were similar between HEK-OATP1B1-Leu643Phe cells and HEK-OATP1B1 wild-type cells. In conclusion, we demonstrated that the frequent SNP 1929A>C had no effect on the transporter protein expression level or on the transport properties for the investigated substrates.

Supported by the Deutsche Forschungsgemeinschaft (DFG KO 2120/1-3).

[1]Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-University Erlangen-Nuremberg, [2]Dr. M. Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany

528

GENETIC VARIATION IN RENAL ORGANIC ANION TRANSPORTERS (OATs) AND THE RENAL EXCRETION OF FRUSEMIDE

SV Vormfelde, D Sehrt, I Meineke, J Brockmöller

We investigated the renal clearance of bumetanide, frusemide and torsemide in a three-period cross-over study in near 100 healthy male volunteers. The individual results were correlated with genetic variation in the genes encoding the basolaterally located organic anion transporters OAT1 and OAT3 and the luminally located OAT4 (*SLC22A6*, *SLC22A8* and *SLC22A11*). The renal frusemide clearance was log-normally distributed with a median of 84.3 ml/min (interquartile range 33.5, range 41 – 160 ml/min). The renal frusemide clearance was associated with one OAT1/OAT3 haplotype having an allele frequency of 10.5 % and an OAT4 haplotype with 7.7 % allele frequency in single and multiple stepwise linear regression analysis. Carriers of no allele of both haplotypes had a mean renal frusemide clearance of 79.9 ml/min, compared to a clearance of 131.3 ml/min in subjects homozygous for both haplotypes in the multiple linear regression analysis. In comparison to the renal clearance of bumetanide and torsemide, the renal frusemide clearance was five times the mean torsemide clearance and in the order of the mean bumetanide

clearance. It was only weakly associated with the renal clearances of bumetanide and torsemide with r^2 = 8.5 % and 10.0 %, respectively. This is much lower than the correlation between the creatinine clearances on the three study days with r^2 = 26.3 – 30.0 %. Only the OAT4 haplotype associated with the renal frusemide clearance was also associated with one other renal clearance (bumetanide). Taken together, the renal clearances of bumetanide, frusemide and torsemide differed in a number of aspects including the genotype associations. These results imply that different mechanisms determine the active renal excretion of these three loop diuretic drugs.
Department of Clinical Pharmacology, University Medical Centre, Göttingen

529

ALTERATIONS OF THE TRANSCRIPTIONAL ACTIVITY OF THE HUMAN IGF1 PROMOTER CAUSED BY A SNP ARE ASSOCIATED WITH HYPERTENSION AND BLOOD PRESSURE

R. Telgmann[a], E. Brand[b], C. Dördelmann[a], V. Nicaud[c], C. Rüßmann[a], F. Cambien[c], L. Tiret[c], M. Paul[d], S-M. Brand-Herrmann[a]

IGF1 is a pleioptropic growth factor exerting important functions in the cardiovascular system. A well-characterized case-control study for essential hypertension (PEGASE: n=745 hypertensives [HT]; n=769 normotensives [NT]) was genotyped for a common hIGF1 promoter variant (major allele [1], minor allele [2]). There was a significant difference in genotype frequency between HT and NT (P=0.019), with an odds ratio for hypertension of 0.73 ([95%CI 0.58-0.91], P=0.006) associated with allele 2. In NT there was a significant decrease in blood pressure level with the number of alleles 2. *In silico* analyses suggested the presence of several transcription factor binding sites (TFBS). EMSAs demonstrated that the genetic variant interacts with nuclear proteins from HepG2 cells in an allele-specific pattern, being differentially altered by stimulation with cAMP or PMA. SuperShifts showed that the region constitutes a complex enhancer module, whose binding protein constituents were identified as AP-1 binding proteins, members of the C/EBP family and interferon-consensus site binding proteins (ICSBP, IRF). Reporter gene assays revealed that the region is a functional *cis*-active transcriptional unit, identifying it as a true enhancer region of the hIGF1 gene. Alleles displayed a significantly different, cell type-specific activity with partly dramatic consequences for gene expression. Cotransfections proved a high specificity for the functional recruitment of nuclear proteins to the enhancer. In the present analysis, we characterized the molecular functionality of an enhancer region of the hIGF1 promoter and identified allele-specific proteins responsible for the alteration in transcriptional activity introduced by a genetic promoter variant, which was highly associated with essential hypertension as well as blood pressure in NT.

[a]Leibniz-Institute for Arteriosclerosis Research, Department of Molecular Genetics of Cardiovascular Disease, Muenster; [b] Internal Medicine D, University Clinic Muenster; [c]Inserm U525, Paris, France; [d]Charité, University Medicine Berlin, Institute of Clinical Pharmacology and Toxicology, Berlin.

530

NUMBER OF FUNCTIONAL CYP2D6 ALLELES AND ADVERSE CARDIOVASCULAR EVENTS IN THE PERI- AND POSTOPERATIVE PERIOD

R. Maas[1], M. Haddad[2], A. Runge[1], E. Schwedhelm[1], W. Thoms[3], L. Dentz[1], T. Klöss[3], T. Standl[4], R.H. Böger[1]

The number of functional *CYP2D6 alleles* is a major determinant of interindividual differences in plasma concentrations of frequently used cardiovascular drugs. Impaired metabolism may increase the risk for adverse drug reactions while extensive metabolism may be associated with reduced cardioprotective effects. It was the aim of the present study to investigate the impact of *CYP2D6* genotype on peri- and postoperative cardiovascular events in patients undergoing non-cardiac surgery.

Methods: In 298 Patients scheduled for elective non-cardiac surgery (61% high risk procedures) we determined polymorphisms, deletions and duplications of the CYP2D6 gene (accounting together for >98% of alterations with known functional relevance). All patients were followed for 30 days post surgery for a composite cardiovascular endpoint.

Results: In 9 (3%) patients we observed CYP2D6 gene duplications, 148 (50%) patients carried two fully functional alleles, 103 (35%) had only one and 35 (12%) no functional allele. Three patients could not be classified. Event rates were 3%, 6%, 12% and 11% for patients carrying 0, 1, 2 and >2 fully functional CYP2D6 alleles, p<0.05. In the 43 patients taking betablockers (thereof 35 (81%) metoprolol) metabolized by CYP2D6 for cardiovascular protection the event rate increased from 0% in patients without functional CYP2D6 allele to 50% in patients carrying gene duplications, p<0.05. The number of functional CYP2D6 alleles was inversely correlated with the plasma concentration of metoprolol (rho= -0.341, p<0.05) and the metoprolol/α-OH-metoprolol ratio (rho= -0.508, p<0.01).

Conclusion: The frequency of adverse events in the peri- and postoperative period increased with the number of fully functional CYP2D6 alleles. This association was more pronounced in patients taking beta blockers for cardiovascular protection. Lower drug concentrations in patients with extensive metabolizer genotype resulting in a reduced therapeutic efficacy of cardioprotective drugs could be a possible explanation.

[1]Institute of Experimental and Clinical Pharmacology and Toxicology, [2]Department of Clinical Chemistry, [4]Center of Anaesthesiology; University Hospital Hamburg-Eppendorf, Hamburg, Germany.
[3]Asklepios Klinik Harburg, Hamburg, Germany.

531

IDENTIFICATION AND FUNCTIONAL ANALYSES OF MOLECULAR HAPLOTYPES OF THE HUMAN OSTEOPROTEGERIN GENE PROMOTER IN SAOS2 OSTEOSARCOMA CELLS

C Hagedorn,[1] R Telgmann,[1] E Brand,[2] V Nicaud,[3] S Kurtz,[4] J Schönfelder,[4] C Dördelmann,[1] B Knipper,[5] P Baumgart,[5] P Kleine-Katthöfer,[6] F Cambien,[3] M Paul,[4] SM Brand-Herrmann[1]

Osteoprotegerin (OPG), a member of the TNF receptor superfamily, plays an emerging role in cardiovascular disease (CVD); some studies reported associations between OPG SNPs and CVD phenotypes including plasma OPG levels. By use of OPG gene scanning in 190 chromosomes from high-risk MI patients, we identified 2 exonic, 3 intronic and 5 variants in the 5'-regulatory gene portion (T-957C, A-943G, G-897A, T-861G, T-156C). After resequencing of 64 additional chromosomes, we identified three common distal molecular haplotypes by subcloning procedures (OPG-wt, OPG-Haplo2 [-943G], OPG-Haplo4 [-957C, -943G, -897A and -861G]), which we introduced into the reporter gene vector pGL3prom. Transient transfection analyses in SaOs2 under basic and stimulatory conditions with TNFα (2 ng/mL; 6hrs), 17β-Estradiol (E2) (10^{-8}M; 24hrs), and both, revealed that stimulation with E2 and E2/TNFα resulted in a mildly but significantly lesser transcriptional activity of OPG-Haplo2 (P=0.023 and P=0.028, respectively). Irrespective of stimulation, OPG-Haplo4 consistently displayed a significantly higher activity compared to OPG-wt ($P=4.88x10^{-6}$); both results were confirmed in kinetic analyses over a ~14h period. We characterized the functionality of molecular OPG promoter haplotypes under different stimulatory conditions and suggest that gene expression and transcriptome analyses are now warranted to show their differential respective impacts on phenoptypes.

[1]Leibniz-Institute for Arteriosclerosis Research, Department of Molecular Genetics of Cardiovascular Disease, University of Muenster, Germany; [2]Department of Internal Medicine D, Nephrology and Hypertension, University Clinic of Muenster, Muenster, Germany. [3]Inserm U525, Faculté de Médecine Pitié-Salpêtrière, Paris, France, [4]Charité, University Medicine Berlin, Campus Benjamin Franklin, Institute for Clinical Pharmacology and Toxicology, Berlin, Germany; [6]Clemens hospital, Clinic of Internal Medicine I, Muenster, Germany; [6]St. Franziskus hospital, Medical Clinic III - Cardiology, Muenster, Germany;

532

DOPAMINE D2, SEROTONIN 2A, AND 2C RECEPTOR POLYMORPHISMS IN AFRO-CARIBBEAN INPATIENTS: DIFFERENCES IN ASSOCIATION WITH PARKINSONISM, TREMOR, AND RIGIDITY

A.F.Y. Al Hadithy[1], B. Wilffert[1,2], R.E. Stewart[3], N.M.G. Looman[1], R. Bruggeman[1,4], J.R.B.J. Brouwers[1,2], G.E. Matroos[5], J. van Os[6], H.W. Hoek[4,7] and P.N. van Harten[4,8]

Background: Dopamine D2 receptor (DRD2), serotonin 2_A (HTR2A) and 2_C (HTR2C) receptor genes are plausible candidates for the study of the pharmacogenetics of PK.

Aims: To investigate the association between PK and the polymorphisms -141CIns/Del, *Taq*IA, *Taq*IB, *Taq*ID, 957C>T, Ser311Cys of DRD2; -1438A>G, 102T>C, and His452Tyr of HTR2A; and -759C>T and Cys23Ser of HTR2C in Afro-Caribbeans treated with antipsychotics.

Methods: PK was measured with the UPDRS. Data were dichotomized in presence/absence of PK, rigidity (RIG), and tremor at rest (TREM). Associations were analyzed with Chi-square test. For continuous data, like age, the *t*-test was applied.

Results: 126 patients (99 males and 27 females; mean ages: 47.5 and 55.4 years, respectively) were included [*Schizophr.Res.*1996;19:195]. There were no differences in age, chlorpromazine, and benztropine equivalents between patients with and without PK, RIG, or TREM. There was, however, a significant association between PK and *23Ser*-allele carriership (p=0.0208;φ=0.2), the frequency of PK in *23Ser*-allele carriers being 1.7 times higher. Furthermore, there was a significant association between RIG and *-141CDel*-allele carriership (p=0.0046;φ=0.2) the frequency of RIG in *-141CDel*-allele carriers being 9.5 times higher. TREM, however, was not associated with any of the 11 polymorphisms.

Conclusion: Our findings do support in Afro-Caribbeans a genetic association between RIG and -141CIns/Del (DRD2) and between PK and Cys23Ser (HTR2C) polymorphisms, but not between TREM and any of the 11 polymorphisms studied. In this population PK and its more specific symptoms RIG and TREM seem therefore to differ pharmacogenetically.

[1]Dept. of Pharmacotherapy and Pharmaceutical Care, University of Groningen, the Netherlands (NL); [2]Dept. of Clinical Pharmacy, Zorggroep Noorderbreedte and De Tjongerschans, Leeuwarden, NL; [3]Dept. of Public Health, University Medical Center Groningen, NL; [4]Dept. of Psychiatry, University Medical Center Groningen, NL; [5]Dr. D.R. Capriles clinic, Curacao, Netherlands Antilles; [6]Dept. of Psychiatry and Neuropsychology, Maastricht University, NL; [7]Parnassia Psychiatric Institute, The Hague, NL and [8]Symfora Group Psychiatric Centre, Amersfoort, NL.

533

INFLUENCE OF SEX, HORMONES AND GENOTYPE ON THE EXPRESSION OF THE SEROTONIN TRANSPORTER IN HUMAN BLOOD CELLS

A. Seeringer, S. Pagani, K. Pitterle, J. Kirchheiner

The serotonin transporter (5-HTT) is responsible for the reuptake of the neurotransmitter serotonin in the central nervous system. It represents the binding site for many antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs). Previous data indicated differences in the 5-HTT expression in dependence of response to antidepressant drugs. Since premenopausal women are reported to show a better response to treatment with SSRIs than men and postmenopausal women, we studied differences in the 5-HTT expression in blood in dependence of sex, genotype, intake of oral contraceptives and pre- or postmenopausal state. 5-HTT expression was mesured by quantitative RT-PCR (TaqMan) in whole blood, collected in PAXgene® tubes. We quantified mRNA and determined 5-HTT ΔCT values using Tatabox Binding Protein as control. A significant lower 5-HTT expression was detected in men versus women with mean ΔCT of 6.42 (standard deviation 1.19) in men (n=10) and 7.78 (standard deviation 1.55) in women (n=12, p=0.04). Interestingly the effect seems to be more pronounced in women taking hormonal contraceptives (mean ΔCT 8.18, versus 7.23 in women not taking external hormones, and 6.42 in men, p=0.06). Postmenopausal women (n=3) showed nearly the same ΔCT as men (6.64 vs 6.42). It is known that 5-HTT expression is regulated by a promotor polymorphism of the 5-HTT gene creating either a short (S) or a long (L) allele. A trend towards lower 5-HTT expression in carriers of the LL genotype might be visible but was not statistically significant in our sample. Differences in 5-HTT expression in premenopausal versus postmenopausal women and men might be due to hormonal regulation of 5-HTT expression. The higher 5-HTT expression might be one of the reasons why younger women respond better to SSRIs than men and older women. We only measured mRNA levels using quantitative RT-PCR but of course these findings should be confirmed on protein level. The influence of gene polymorphisms on the 5-HTT expression remains vague.

Institute of Pharmacology of Natural Science & Clinical Pharmacology, University of Ulm, D-89081 Ulm, Germany

534

APPLICATIONS OF A MALDI-TOF MASS SPECTROMETRIC GENOTYPING METHOD FOR CYP2B6

J.K. Blievernicht, E. Schaeffeler, K. Klein, T. Saussele, M. Eichelbaum, M. Schwab, U.M. Zanger

CYP2B6 is a polymorphic enzyme involved in the metabolism of drugs including the antidepressant bupropion, also used in smoking-cessation and the reverse-transcriptase inhibitor, efavirenz. Matrix Assisted Laser Desorption Ionization – time of flight (MALDI-TOF) mass spectrometry (MS) is an efficient and accurate tool for the detection of single nucleotide polymorphisms (SNPs). We developed a MALDI-TOF method for the detection of 15 *CYP2B6* SNPs with functional relevance (Blievernicht et al., *Clin Chem* 2007). The final assay consists of five multiplex incubations and the results allow to genotype for *CYP2B6* alleles **4,*5,*6,*7,*8,*9,*11,*12,*13,*14,*15,*16,*17,*18,*19,*21,*22.* Additional single-plex assays were developed for promoter polymorphism -750T>C and Intron 3 SNP g.15582C>T, suggested to be associated with changes in gene expression (Lamba et al., *JPET* 2003). Here we describe several applications of this assay:

1) We extended our genotype-phenotype correlation analysis in our human liver bank by fully genotyping 269 samples. *CYP2B6* protein content varied 407–fold (0.51 pmol/mg to 210 pmol/mg). Compared to *CYP2B6*1/*1*, significantly lower protein amount and activity was found for genotypes *CYP2B6*1/*5*, **1/*6, *6/*6* and **5/*6*. Bupropion hydroxylation as a marker of *CYP2B6* catalytic activity varied 246-fold (5.6 to 1370 pmol/mg/min). *CYP2B6*1/*6, *6/*6,* and **1/*15* genotypes showed significantly decreased activity compared to wild type. In contrast, SNPs -750T>C and g.15582C>T were not associated with changes in either protein or activity.

2) We analyzed whether *CYP2B6* polymorphisms could be a risk factor for *Parkinson's Disease (PD)* based on a patient cohort consisting of 95 patients with Parkinson's disease and 106 controls previously analyzed for *MDR1* polymorphisms (Furuno et al., *Pharmacogenetics* 2002). No significant differences were found between patients and controls with respect to any of the *CYP2B6* polymorphisms.

3) We are currently applying the MALDI-TOF MS assay to the molecular haplotyping of various DNA samples with unclear *CYP2B6* allelic association.

Supported by the BMBF (HepatoSys grant 0313080D) and by the Robert Bosch Foundation, Stuttgart, Germany.

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstr. 112, D-70376 Stuttgart, Germany.

535

DOPAMINE RECEPTOR TYPE-1 GENE AND RENAL SODIUM REGULATION

S. Hasenkamp[1], JA. Staessen[2], H. Zhang[3], M. Bochud[4], J. Westerkamp[1], T. Richart[2], L. Thijs[2], T. Kuznetsova[2], X. Li[3], SM. Brand-Herrmann[5], E. Brand[1]

Activation of type-1 dopamine receptors (DRD1) reduces renal sodium reabsorption. In a family-based random sample of 611 Caucasians (women, 45.0%; mean age, 38.6 years), we assessed renal sodium handling by measurement of the clearance of endogenous lithium in relation to three SNPs in the DRD1 promoter (A-48G, G-94A, and C-800T). For each pair of SNPs, D' (Lewontin's disequilibrium coefficient) was ≥0.86 (P<0.0001). The frequent haplotypes were AGC (51.2%), GGT (33.2%), and AAC (14.6%). Family-based analyses did not reveal population stratification, except for the AGC (P=0.021) and AAC (P=0.027) haplotypes in relation to diastolic pressure. While standardizing to mean sodium excretion (8.7 mmol/hour), both before and after adjustment for covariates, the fractional distal sodium reabsorption (RNadist) was lower in DRD1 -94GG homozygotes than -94A allele carriers (adjusted effect size, -0.95%; P=0.005), whereas the opposite was the case for the fractional excretion of sodium (FENa) (+0.084%; P=0.014). In similarly adjusted analyses, AGC carriers (adjusted effect size, -0.91%; P=0.012) and AAC carriers (+1.00%; P=0.005) had different RNadist compared to the corresponding non-carriers. Furthermore, FENa was lower in AAC carriers than non-carriers (–0.084%; P=0.019). Transmission of the AGC haplotype was associated with lower systolic (-2.64 mm Hg; P=0.054) and diastolic (-2.24 mm Hg; P=0.019) blood pressures, a slight decrease in RNadist (-0.68%; P=0.099) with an opposite trend for plasma renin activity (+16.9%; P=0.088). In conclusion, RNadist, FENa and blood pressure are associated with genetic variation in the DRD1 promoter, whereas the underlying functional mechanisms remain to be determined.

[1]Department of Internal Medicine D, Nephrology and Hypertension, University of Münster, Münster; [2]Division of Hypertension and Cardiovascular Rehabilitation, Department of Cardiovascular Diseases, University of Leuven, Leuven; [3]Department of Cardiology, Nanjing Medical University, Nanjing; [4]Division of Hypertension and Cardiovascular Medicine, University of Lausanne, Lausanne; [5]Leibniz Institute for Arteriosclerosis Research, Department of Molecular Genetics of Cardiovascular Disease, University of Münster, Münster

536

CYTOKINE POLYMORPHISMS ASSOCIATED WITH CAROTID INTIMA-MEDIA THICKNESS IN STROKE PATIENTS

C. Dördelmann[1], J. Schönfelder[2], J. Labreuche[3], O. Poirier[4], P.-J. Touboul[3], C. Combadière[5], F. Cambien[4], M. Paul[2], P. Amarenco[3], S.-M. Brand-Herrmann[1]

Carotid intima-media thickness (IMT) reflects generalized atherosclerosis and is predictive of future vascular events. Evidence exists that carotid IMT is heritable and genetic studies can provide clues in the pathogenesis of atherosclerosis. We recruited 470 Caucasian ischemic stroke patients, measured common carotid artery IMT and analyzed 54 polymorphisms with suspected roles in atherosclerosis. Among the polymorphisms tested, the angiotensin-converting enzyme insertion/deletion (ACE I/D), osteopontin (OPN) T-443C, monocyte chemoattractant protein-1 (MCP-1) G-927C, and MCP-1 A-2578G polymorphisms were associated with CCA-IMT in age-gender-adjusted analysis. In multivariate analysis, the association between the OPN and MCP-1 polymorphisms remained significant. The OPN-443C allele was associated with increased IMT in the dominant model (0.053 mm for the TC and CC genotypes, p=0.001). The MCP-1-927C allele was associated with increased IMT in the additive model (0.040 mm for each C allele, p=0.001), and the MCP-1-2578 G allele was associated with decreased IMT in the recessive model (0.088 mm for the GG genotype), p=0.002). The OPN and MCP-1 genes, coding for two cytokines with known roles in atherosclerosis, may contribute to increased carotid IMT and warrant further functional studies.

[1]Leibniz-Institute for Arteriosclerosis Research, Department of Molecular Genetics of Cardiovascular Disease, [2]Charité, University Medicine Berlin, Campus Benjamin Franklin, Institute for Clinical Pharmacology and Toxicology, Berlin, Germany, [3]Department of Neurology and Stroke Centre, Bichat University Hospital, Denis Diderot University and Medical School, Paris, France, [4]INSERM 525, Pitié-Salpêtrière Medical School, Paris, France, [5]INSERM U543, Pitié-Salpêtrière Medical School, Paris, France

537

ACTUAL NECESSITY OF PREDICTIVE CYP2D6-GENDIAGNOSTICS IN PSYCHIATRIC THERAPY

S. Röhm[1], A. K. Frobel[1], E. Tot[1], S. Läer[1]

Purpose: Psychiatric pharmacotherapy is often accompanied with adverse events leading to a high percentage of treatment attrition. CYP2D6 dependent neuroleptics and antidepressants may enhance these adverse events in poor metabolizers. Our aim was to determine the prescription prevalence of those drugs in an outpatient setting. In addition, we defined a collective of poor metabolizers that might be protected from a higher risk of adverse events based upon collected prescription data. Methods: From two independent sites, one outpatient psychiatrist/ neurologist clinic and a pharmacy in Cologne (Germany), we analysed data over a period of three weeks. 172 patient files of the doctor's clinic were documented including age, gender and pharmacotherapy. Diagnoses were assorted using the International Classification of Diseases (ICD-10) code. 1706 prescriptions from the pharmacy were documented for the same criteria-excluding diagnoses. Analyses: Antidepressants and neuroleptics were classified according to the Anatomical Therapeutic Chemical Classification System (ATC-Code). The drugs were subdivided according to a CYP2D6 dependent metabolism and already published reports of adverse events in poor metabolizers that indicated a clinical relevance. Results: Out of 172 patients 118 were identified as psychiatric patients (median age 67 years, 66% women). 36 of these patients received CYP2D6 dependent drugs (7 antidepressants and 7 neuroleptics). Out of the 1706 prescriptions in the pharmacy 66 were psychiatric patients (median age was 57 year, 73% women). 49 of these received CYP2D6 dependent drugs (8 antidepressants and 4 neuroleptics). Based on the fact that 8% of the Caucasian population are poor metabolizers we deduced from our analysis that 3 poor metabolizers would appear in 3 weeks. This means that statistically one patient in each collective who receives CYP2D6 dependent psychotropic drugs may be a poor metabolizer. Conclusion: CYP2D6 dependent psychiatric drugs are often prescribed in this region. We assume a prevalence of one poor metabolizer per week in the outpatient setting. Thus, predictive gendiagnostics for a CYP2D6 polymorphism might help to prevent severe adverse events for these patients.

[1]Department of Clinical Pharmacy and Pharmacotherapy, Heinrich-Heine-Universität Düsseldorf, Germany

538

PHARMACOGENETICS OF TRIPTANS IN CLUSTER HEADACHE

G. Tessmann, I. Geissler, M. Schürks, D. Rosskopf

Cluster headache (CH) is a rare primary headache disorder characterised by severe strictly unilateral pain attacks occurring in 'cluster' episodes. CH is responsive to triptans with responder rates of 50 to 70%. We have collected more than 250 CH patients from all over Germany organized in the CH self help group. The characteristics of the disease and the response to different acute and prophylactic therapeutic regimes were assessed by a standardized questionnaire. Carriers of a common variant in the gene for the orexin/hypocretin receptor 2 (HCRTR2) have a higher risk for CH (Schürks et al., Neurology, 2006). In a first study, we examined whether this polymorphism in HCRTR2 – a receptor implicated in central pain modulation – affects therapeutic responses. There was no difference in allele and genotype distributions between triptan responders and non-responders. Triptans are agonists at $5HT_{1B/D}$ receptors, typical G protein-coupled receptors. While genetic polymorphisms in $5HT_{1B/D}$ receptors have not been characterized so far, we focused on a common polymorphism in the G protein β3 subunit (C825T), the next step in triptan signal transduction. Gβ3 is expressed in the brain and available evidence suggests that the alleles of this polymorphism may differentially affect serotoninergic mechanisms. 180 CH patients used triptans of whom 71% reported therapeutic response. The odds ratio for heterozygous carriers of the GNB3 C825T polymorphism to respond to triptans was 2.96 (95%CI 1.34 – 6.56; p = 0.0074) compared to CC carriers. The number of homozygous carriers of the T allele was too small 8 to analyze for potential gene-dose effects. Alleles of the GNB3 C825T polymorphism did not affect responder rates to acute (oxygen) or prophylactic treatments (glucocorticoids, verapamil). Interestingly, targets of these drugs do not primarily involve coupling to G proteins. Together, we conclude that pain relief by triptans is significantly modulated by a common genetic GNB3 variant.

Institut für Pharmakologie – Ernst-Moritz-Arndt Universität Greifswald
Neurologische Klinik – Universitätsklinikum Essen

539

GENETIC POLYMORPHISM IN THE *CYP 1A2* AS SUSCEPTIBLE FACTOR IN CERVICAL CARCINOGENESIS?

K. Tröger[1], A. Balogh[1], R. Vollandt[4], G. Wagner, R. Schlösser[3], M. Dürst[2], I. Runnebaum[2]

Objective: CYP1A2 primarily activates aromatic and heterocyclic amines. Single nucleotide polymorphisms (SNPs) in *CYP1A2* are associated with altered enzyme inducibility, which results in altered rates of mutagen activation. The most important single nucleotide polymorphism is –163C > A (*CYP 1A2*F*).Therefore the genotype with high in vivo CYP1A2 activity (A/A or A/C) was suggested to be a susceptiblity factor for cervical carcinogenesis. Methods: In this study, we analyzed the distribution of three *CYP1A2*F* genotype A/A,A/C or C/C in 199 patients (aged between 24 and 80 years) with cervical cancer in comparison to 276 volunteers (aged between 19 and 88 years). Results: Following genotype frequencies of *CYP 1A2*F* were found:

0.487 A/A, 0.407 A/C and 0.106 C/C in patients and 0.449 A/A, 0.449 A/C and 0.10 C/C in volunteers.The distribution is in accordance with the frequencies expected, when applying the Hard-Weinberg principle based on the experimentally determined frequency of the C/C genotype in both groups. There was a slight, but not significant (chi-square test p=0.652) over-representation of genotype A/A (48.7%) in patients in comparison to the group of subjects without tumors (44.9%). Conclusion: Our results suggest that polymorphism of *CYP1A2*F* is possibly not a risk factor for human papillomavirus-associated tumorgenesis. The data will be stratified according to age, histological grading and lymph node involvement.

[1]Inst. of Clinical Pharmacology, [2]Gynecological Molecular Biology of the Dept. of gynecology,[3]Dept. of Psychiatry, Dept. of Medical Informatics of the Friedrich Schiller University Jena, Germany.

540

GLUTATHIONE-S-TRANSFERASE, N-ACETYLTRANSFERASE 2 AND UDP-GLUCURONOSYLTRANSFERASE 1A1 ALLELE FREQUENCIES IN CATARACT OR GLAUCOMA PATIENTS COMPARED TO CONTROLS

U. D. Kuhn[1], A. Schlaegel[1,2], J. Dawczynski[3], E. Königsdörffer[3], T. Lorenzen[4], H. Thude[4], D. Barz[4], M. Hippius[1], A. Lupp[2]

Glutathione-S-transferase (GST), N-acetyltransferase 2 (NAT2) and UDP-glucuronosyltransferase (UGT) enzymes play an important role in the detoxification of xenobiotics. Genetic polymorphisms of these enzymes might exert some influence on the development also of ophthalmic diseases such as cataract or glaucoma. Due to scarce data, GST or NAT2 pheno- or genotype as a risk factor for cataract incidence still needs further investigation. With respect to UGT1A1 polymorphism (Gilbert's syndrome) no such reports exist at all. Neither NAT2 nor UGT polymorphism have been investigated as risk factors for glaucoma so far. 197 consecutive patients (182 age-related cataract, 90 glaucoma, 75 both) from the University Eye Hospital and 210 healthy volunteers (blood donors from the Institute of Transfusion Medicine) serving as control population were genotyped with respect to GSTP1 Ile(105)Val and GSTP1 Ala(114)Val polymorphism, GSTM1 and GSTT1 null genotype, NAT2 (slow/rapid acetylator) genotype, and UGT1A1*28 polymorphism (TATA box repeat). The prevalence of the GSTP1, GSTM1 and GSTT1 gene polymorphisms investigated in the present study was not different between cataract or glaucoma patients and the control population. Also with respect to NAT2 phenotype similar distributions were observed with both groups of patients and controls. A significant difference, however, was seen when investigating the number of cases carrying one or two of certain NAT2 alleles, with patients displaying higher NAT2*5 and lower NAT2*7B allele frequencies. UGT analysis revealed an association between the genotype and cataract or glaucoma development, with patients having a lower UGT1A1*28 allele frequency. Since bilirubin at low concentrations is a well known potent antioxidant, possibly, the somewhat higher serum bilirubin concentrations associated with one or two UGT1A1*28 mutations exert some protection also of ocular tissues against oxidative stress and thus cataract or glaucoma development.

[1]Institute of Clinical Pharmacology, [2]Institute of Pharmacology and Toxicology, [3]University Eye Hospital, [4]Institute of Transfusion Medicine, Friedrich Schiller University, 07743 Jena, Germany

541

NO EFFECT OF A COMMON POLYMORPHISM IN THE GENE OF THE G PROTEIN SUBUNIT Gβ3 ON THYROID FUNCTION AND MORPHOLOGY IN A LARGE POPULATION-BASED STUDY

A. Krüger, A. Bornhorst, C. Rimmbach, H. Völzke, D. Rosskopf

G protein-mediated signalling is of key importance for the regulation of thyroid function by TSH. A common polymorphism (GNB3 C825T) has been associated with enhanced signal transduction by heterotrimeric G proteins. Two recent reports described associations of GNB3 alleles with the risk for oncocytic tumors (J Pathol, 2007; 211; 60 – 66), thyroid adenomas and carcinomas (J Pathol, 2005, 207, 430 – 435). Here, we investigated whether this polymorphism affects parameters of thyroid function (iodide and thiocyanate excretion) and morphology (thyroid ultrasound) in 4310 participants of the cross-sectional population-based SHIP study (Study of Health in Pomerania), a cohort that has been extensively analysed with regard to various aspects of thyroid morbidity under conditions of iodine deficiency. The data were analysed separately for men and women. GNB3 genotypes or alleles did not affect the risk for goiter or thyroid nodules. They were not associated with mean thyroid volumes. Likewise, we did not observe effects of the GNB3 genotypes and alleles on iodine excretion. Together, these data do not support a relevant role of GNB3 variants for the pathogenesis of goiter or thyroid nodules in a population-based survey under conditions of moderate iodine deficiency.

Institut für Pharmakologie & Institut für Epidemiologie und Sozialmedizin – Ernst-Moritz-Arndt Universität Greifswald

542

EXPRESSION OF ORGANIC ANION TRANSPORTING POLYPEPTIDES (OATP) IN THE HUMAN SMALL INTESTINE

H. Glaeser, C. Willmer, J. König, M.F. Fromm

Drug metabolizing enzymes and drug transporters can be major determinants of the bioavailability of drugs. In comparison to the liver, little is known about the role of intestinal OATPs for the bioavailability and disposition of drugs. Therefore, we investigated the expression of several human OATPs in the human intestinal samples using semiquantitative Real-Time PCR and Western Blot analysis. A qualitative analysis of the OATP expression revealed that OATP1A2, OATP2B1, OATP3A1 and OATP4A1 mRNA were detectable in human intestinal samples (n=10). The subsequent semiquantitative Real-Time PCR showed the highest expression for OATP4A1 followed by OATP2B1, OATP3A1 and OATP1A2 when normalized to the enterocyte-specific expressed villin. OATP1A2 mRNA expression was characterized by the most pronounced variability (129%) compared to OATP2B1 (100%), OATP4A1 (29%) and OATP3A1 (14%). Furthermore, a tendency towards higher mRNA expression was observed in female subjects compared to male subjects for OATP2B1, OATP3A1 and OATP4A1, but not for OATP1A2 mRNA. In addition, the Western Blot analysis of OATP2B1 and OATP4A1 showed also a marked variability. In conclusion, the knowledge about the expression of OATPs in the human small intestine may improve the understanding of drug absorption and bioavailability.

Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany; Supported by the DFG grants GL 588/2-1 and KO 2120/1-3

543

FUNCTIONAL INTERACTION OF OATP2B1 WITH DIFFERENT DRUGS

M. Grube, K. Köck, S. Oswald, G. Jedlitschky, W. Siegmund and H. K. Kroemer

The organic anion transporting polypeptide (OATP) family plays a prominent role in the uptake of pharmacological important drugs. While some of the best characterized OATPs like OATP1B1 and OATP1B3 are mainly expressed in the sinusoidal membrane of hepatocytes, other OATPs have a wide tissue distribution. OATP2B1 for example is not only expressed in liver but also in brain, heart, placenta, intestine and further tissues. Thus, this transporter may be important for drug uptake not only into hepatocytes but also from intestine and into target structures. We therefore studied the interaction of OATP2B1 with a wide variety of different drugs. Using our OATP2B1 overexpressing MDCKII cells, we first identified a variety of compounds interacting with the OATP2B1-mediated estron-3-sulfate uptake, including several statins, hormones, cytostatics and antimycotics. In particular, most prominent inhibition was observed for atorvastatin (IC_{50} = 0.4 µM), paclitaxel (IC_{50} = 8.9 nM) and ketoconazole (IC_{50} = 2.8 µM). To further analyse the interaction of OATP2B1 and statins, we directly studied transport of atorvastatin and simvastatin into OATP2B1-overexpressing cells. Thereby, we could demonstrate a high affinity uptake of atorvastatin (K_m = 0.2 µM) by OATP2B1, while simvastatin was not transported at all. Moreover, additional analyses revealed a reduced atorvastatin uptake in the presence of the OATP2B1 substrates estrone-3-sulfate and glibenclamide. Furthermore, atorvastatin uptake was enhanced in acidic environment (4 fold higher at pH 6.0 compared to pH 7.4). Taken together our data demonstrate an important interaction of OATP2B1 with drugs of different classes and indicate atorvastatin as a high-affinity OATP2B1 substrate. Moreover, these findings may be of special interest for orally administered drugs because of the intestinal and hepatic expression of OATP2B1.

Dept. of Pharmacology, Ernst Moritz Arndt University, Greifswald, Germany

544

METAMIZOLE IS A POTENT INDUCER OF HUMAN CYP2B6 AND CYP3A4

T. Saussele[1], O. Burk[1], J.K. Blievernicht[1], K. Klein[1], A. Nussler[2], N. Nussler[2], M. Eichelbaum[1], M. Schwab[1], U.M. Zanger[1]

To systematically investigate drug metabolism in human liver we have established a large liver bank. Comprehensive clinical documentation which comprised age, gender, medication and diagnosis of the liver donors allows us to analyze the impact of drug treatment on liver expression and function of drug metabolizing enzymes. Here we report on the effects of metamizole (dipyrone), which is used as an analgesic, antipyretic and antispasmodic drug, it's metabolite 4- amino-antipyrine (AA) and derivative antipyrin. In this study we investigated the influcence of metamizole on the RNA and protein expression of different cytochrome P450 enzymes, nuclear transcriptions factors, NADPH:P450-oxidoreductase (POR) and ABCB1 in human liver microsomes and in human primary hepatocytes. Microsomal CYP2B6 and CYP3A4 expression revealed a significantly higher (3.8-fold and 2.8-fold, respectively) protein expression as well as 2.9-fold higher bupropion hydroxylase activity and 1.3-fold higher verapamil-N-demethylase activity in patients treated with metamizole compared to untreated subjects. In human primary hepatocytes treatment with metamizole (100 µM) for 72 h resulted on average (N=5) in 7.8-fold and 2.4–fold, increased CYP2B6 and CYP3A4 mRNA expression, respectively. Similarly enhanced expression was observed by the derivatives AA and antipyrine. The protein expression of CYP2B6 and CYP3A4 was also induced up to 3.1-fold and 2.9-fold, respectively. Other investigated enzymes, including CYP2C9, CYP2C19, and CYP2D6, as well as the nuclear receptors PXR and CAR, and the transporter Pgp and POR werde not influenced by either metamizole or its derivatives. Using reporter gene assays we show that metamizole is not acting as a direct ligand to either PXR or CAR, suggesting a phenobarbital-like mechanism of induction. These data warrent further studies to elucidate the drug-interaction potential of metamizole, especially in patients with long-term treatment.

Supported by the German Federal Ministry of Education and Research ("HepatoSys" grant 0313080D) and by the Robert Bosch Foundation, Stuttgart, Germany.

[1]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstr. 112, 70376 Stuttgart, Germany
[2]Department of Surgery, Campus-Virchow-Clinic, Humboldt University, Berlin, Germany

545

HUMAN BREAST CANCER PROTEIN (BCRP/ABCG2): INTERACTION WITH CARDIOVASCULAR DRUGS

O. Zolk, T.F. Solbach, M.F. Fromm

Previous studies demonstrated that the ABCG2 drug efflux transporter is expressed in the human heart. Moreover, our own studies showed that ABCG2 was markedly up-regulated at the mRNA (3.8-fold; p<0.001) and protein level (2.0-fold; p<0.05) in failing left ventricles compared to non-failing control myocardium. This raises the possibility that ABCG2 might be relevant for the cardiac disposition of drugs. To study the interaction of ABCG2 with cardiovascular active compounds, we expressed human ABCG2 in HEK293 cells and in the polarized MDCKII cell line. In transepithelial transport experiments, translocation of the ABCG2 test substrate [^{14}C]PhIP was increased in the apical direction in MDCK-ABCG2 cells compared to vector control cells (transport ratio 13.2 vs. 1.1; p<0.0001). The ABCG2 inhibitor fumitremorgin-C completely abolished the asymmetry in translocation of [^{14}C]PhIP, indicating high expression and activity of human ABCG2 in MDCK-ABCG2 cells. In this cell line we tested aldosterone, propranolol, metoprolol and bisoprolol. As indicated by similar apically and basolaterally directed transport in MDCK-ABCG2 cells these compounds were not transported by ABCG2. Translocation in the apical direction was observed for digoxin, which was also observed in MDCK control cells and was abolished by the P-glycoprotein inhibitor PSC-833. This indicates that digoxin is transported by endogenous canine P-glycoprotein, but is not a substrate of the human BCRP efflux pump. In contrast, the significant transport of rosiglitazone in the apical

direction (P_{app} [basal-apical] 36.0±1.2 cm/s * 10^{-6}; P_{app} [apical-basal] 23.4±1.2 cm/s * 10^{-6}; n=5, p<0.0001) was completely abolished by fumitremorgin-C but not by PSC-833, suggesting that translocation of rosiglitazone was ABCG2 dependent. Furthermore, rosiglitrazone increased the accumulation of the fluorescent ABCG2 substrate pheophorbide A in HEK cells stably transfected with human ABCG2. The maximum inhibitory effect of rosiglitazone on ABCG2 (related to the effect of the reference inhibitor fumitremorgin-C set at 100%) amounted to 83% (IC_{50} 14.3 µM). In conclusion, neither digoxin nor the most commonly used beta-blockers propranolol, metoprolol and bisoprolol were transported substrates of ABCG2 and, thus, ABCG2 is not relevant for the cardiac disposition of these drugs. In contrast, rosiglitazone, which is used to treat type 2 diabetes, but might also have protective effects against ischemia-reperfusion injury of the heart, interacts with ABCG2 as a substrate and inhibitor.

Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-University, D-91054 Erlangen

546

FRET-BASED METHOD FOR DETECTION OF β_1-ADRENERGIC RECEPTOR ACTIVATING AUTOANTIBODIES AS A TEST FOR EFFICACY OF THERAPEUTIC STRATEGIES IN HEART FAILURE

V.O. Nikolaev[1], V. Boivin[1], S.Störk[2], C.E. Angermann[2], M.J. Lohse[1], and R. Jahns[1,2]

Activating autoantibodies against extracellular epitopes of the human β_1-adrenergic receptor (β_1-Abs) play a causative role in pathogenesis of dilated cardiac myopathy (DCM) [1,2]. Recently, we have developed a new microscopic method for their detection in patients, which is based on sensors that use fluorescence resonance energy transfer (FRET) as a tool to measure cAMP [3]. Using this new technique, we analyzed IgG fractions from n=50 healthy controls (which did not affect cAMP levels) and from n=55 previously described DCM patients [2], which varied in their capacity to activate cAMP synthesis. IgG from these patients fell into 3 distinct groups: "high-activators" (50±5% of isoproterenol response, 20 patients), "low-activators" (31±7% of isoproterenol, 20 patients) and negative (no effect on cAMP, 15 patients). The effects of β_1-Abs on cAMP-levels were affected both by peptides from the receptor and by β-blockers: "high-activator" β_1-Abs were blocked more by a peptide derived from the 2nd extracellular receptor loop (EL_{II}, -40%), than by EL_I-peptide (+7%); the reverse was true for "low-activator" β_1-Abs (-45% EL_I vs. +9% EL_{II}). Among commonly used beta-blockers, β_1-selective (bisoprolol, metoprolol) and nonselective drugs (alprenolol, carvedilol) only partially (52-70%) blocked cAMP responses. In contrast, cAMP-signals from "low-activator" β_1-Abs were completely reversed. However, when IgG preparations from "high-activators" were treated with EL_{II}-peptide, bisoprolol could completely block the stimulatory effects. This suggests that an add-on therapy with beta-blocker and receptor peptide might be beneficial for DCM treatment. In summary, our FRET-based approach allows not only to detect β_1-abs in DCM patients, but also to test for efficacy of conventional and novel therapeutic strategies for treatment of heart failure.

[1] R. Jahns et al. (2004) *J. Clin. Invest.* 113: 1419-29
[2] R. Jahns et al. (1999) *Circulation* 99: 649-654
[3] V.O. Nikolaev et al. (2004) *J. Biol. Chem.* 279: 37215-37218

[1] Department of Pharmacology, University of Würzburg, Germany
[2] Medical Clinic and Policlinic, Cardiology, University of Würzburg

547

EFFECTS OF THE β1-SUBUNIT OF THE BK CHANNEL ON THE DEVELOPMENT OF NITRATE TOLERANCE AND ON ANGIOTENSIN II INDUCED OXIDATIVE STRESS

J. Debrezion[1], M. Oelze[1], P. Wenzel[1], M. Hortmann[1], S. Schuppan[1], J. Müller[1], S. Schuhmacher[1], L. Steinhoff[1], M. Brand[1], A. Daiber[1] and T. Münzel[1]

Introduction: Nitric oxide (NO)-induced vasorelaxation involves activation of large conductance Ca^{2+}-activated K^+ channels (BK). A regulatory BKβ1 subunit confers Ca^{2+}-, voltage- and NO/cGMP-sensitivity to the BK channel. The deletion of BKβ1 causes endothelial dysfunction by increasing O_2^- formation via increasing activity and expression of the vascular NADPH oxidase. We investigated to which extent oxidative stress, endothelial function and NO/cGMP signalling is affected by induction of nitrate tolerance and angiotensin II induced hypertension in BKβ1-knockout mice (BKβ1-/-).

Methods and results: Nitrate tolerance and angiotensin II hypertension were induced via implantation of osmotic minipumps with either nitroglycerin (NTG, 60 nmol/kg/d for 4 days) or angiotensin II (AT-II, 0.8 mg/kg/d for 7 days) in Wildtype (WT) and BKβ1-/- mice. In both treatment group's endothelial-dependent (against acetylcholin) as well as -independent (against NTG) relaxation was significantly more affected in BKβ1-/- then in WT determined by isometric tension studies. These findings were coupled with potentiated degradation of NO/cGMP-signalling (VASP-serin 239 phosphorylation). In addition we measured increased NADPH-oxidase activity particularly in heart membrane fractions of AT-II treated BKβ1-/- (Lucigenin enhanced chemiluminescence, 5 µM) and enhanced production of reactive oxygen species particularly in heart mitochondria from NTG treated BKβ1-/- (L-012 enhanced chemiluminescence, 100 µM). In contrast to NTG treatment, protein and mRNA expression of the NADPH-oxidase subunit Nox1 (Western blotting and realtime RT-PCR) was significantly increased only in AT-II treated BKβ1-/- versus WT and untreated BKβ1-/- (BKβ1-/-+AT-II 4.5-fold, untreated BKβ1-/- 2-fold and WT+AT-II 2.5-fold versus untreated WT).

Conclusions: The results of the present study demonstrate a crucial role of the BKβ1 subunit during development of oxidative stress, not only on enhanced activity and expression of NADPH-oxidases, but also on other sources of oxidative stress such as mitochondria as primarily observed in the phenomenon of nitrate tolerance.

[1] Klinikum der Johannes Gutenberg-Universität Mainz, II. Medizinische Klinik, Kardiologie, Mainz, Germany

548

CELECOXIB HAS THE STRONGEST ANTIPROLIFERATIVE EFFECT AMONG THE COXIBS: EVIDENCE FOR AN INTRACELLULAR ACCUMULATION OF CELECOXIB

S. Schiffmann, T. J. Maier, A. Janssen, R. Schmidt, H. Corban-Wilhelm, C. Angioni, G. Geisslinger and S. Grösch

Celecoxib, a COX-2-selective inhibitor (coxib), is the only NSAID which has been approved for adjuvant treatment of patients with familial adenomatous polyposis. To investigate, whether or not the antiproliferative effect of celecoxib also holds true for other coxibs, we compared the antiproliferative potency of all coxibs currently available. All experiments were conducted using COX-2-overexpressing (HCA-7) and COX-2-negative (HCT-116) human colon cancer cells, to elucidate if the antiproliferative potency depends on COX-2 inhibition. Cell survival was assessed using the WST proliferation assay. Apoptosis and cell cycle arrest was determined using flow cytometric and western blot analysis. The *in vitro* results were confirmed *in vivo* using the nude mouse model. Intracellular concentrations of the coxibs were measured using LC-MS/MS. Among all coxibs tested only celecoxib and methylcelecoxib (a non COX-2-inhibiting celecoxib derivative) clearly decreased cell survival by induction of cell cycle arrest and apoptosis in human colon carcinoma cells and significantly reduced the growth of tumor xenografts in nude mice. Measurement of intracellular drug concentrations revealed that celecoxib and methylcelecoxib display five to ten fold higher intracellular concentrations than the other coxibs in both cell lines. In line with this, HCA-7 cells were more sensitive to celecoxib and methylcelecoxib and showed higher intracellular concentrations of both drugs as compared to HCT-116 cells. It can be concluded, therefore**,** that the antiproliferative effect of celecoxib is unique among coxibs. Intracellular accumulation seems to be an important mechanism by which celecoxib and methylcelecoxib reach sufficient concentrations to exert their antiproliferative effects also acting through COX-2-independent targets.

pharmazentrum frankfurt, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany

549

INHIBITION OF TH1 AND TH17 EFFECTOR FUNCTIONS BY CALCITRIOL AND SIMULTANEOUS UPREGULATION OF REGULATORY T CELLS IN COLITIS

C. Daniel1#, N.A. Sartory1#, N. Zahn1, J.M. Stein1#§, H.H. Radeke2#§

Epidemiological studies of human autoimmune diseases including type 1 diabetes, multiple sclerosis and rheumatoid arthritis, animal models of autoimmunity and in vitro studies in immune cells testify that calcitriol alone or in combination with corticosteroids exerts strong immunomodulatory activity. Here, we studied the effects of the combined application of calcitriol and dexamethasone in Th1-mediated trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. The combination of these steroids reduced the clinical and histopathologic severity of TNBS colitis. As a first analytical approach to decipher these clinical observations in our subsequent experiments we could clearly demonstrate that Th1 related parameters were down- while Th2 markers like IL-4 and GATA3 were up-regulated. Unlike steroids alone specifically calcitriol promoted regulatory T cell functions as indicated by a marked increase of IL-10, TGFß, FoxP3 and CTLA4. To better understand these complex immunomodulatory effects of calcitriol we expanded our investigation to dendritic cell mediators responsible for a pro-inflammatory differentiation of T cells and found a significant reduction of members of the IL-12 family, IL-12p70, and IL-23p19 as well as IL-6 and IL-17. Thus, our data not only confirm the rationale for a steroid-sparing, clinical application of calcitriol derivatives in inflammatory bowel disease, but also prove that early markers indicative of inflammatory dendritic cell differentiation and Th17 development represent new target molecules for calcitriol and promising highly selective immune modulating vitamin D analogs.

1First Department of Internal Medicine, 2pharmazentrum frankfurt, #ZAFES, JWG University of Frankfurt/Main. §HHR and JMS contributed equally to this work.

550

FTY720 AMELIORATES TH1-MEDIATED COLITIS IN MICE BY DIRECTLY AFFECTING THE FUNCTIONAL ACTIVITY OF $CD4^+CD25^+$ REGULATORY T CELLS[1]

C. Daniel*‡, N.A. Sartory*‡, N. Zahn*, G. Geisslinger†‡, J.M. Stein*‡§, H.H. Radeke†‡§

Following the present concepts the synthetic sphingosine analog of myriocin FTY720 alters migration and homing of lymphocytes via sphingosine1-phosphate receptors. However, several studies indicate that the immunosuppressive properties of FTY720 may alternatively be due to tolerogenic activities via modulation of dendritic cell differentiation or based on direct effects on $CD4_+CD25_+$ regulatory T cells (Treg). As Treg play an important role for the cure of inflammatory colitis, we used the Th1-mediated TNBS colitis model to address the therapeutic potential of FTY720 in vivo. A rectal enema of TNBS was given to Balb/c mice. FTY720 was administered i.p. from day 0-3 or 3-5. FTY720 substantially reduced all clinical, histopathologic, macroscopic and microscopic parameters of colitis analyzed. The therapeutic effects of FTY720 were associated with a down-regulation of IL-12p70 and subsequent Th1 cytokines. Importantly, FTY720 treatment resulted in a prominent up-regulation of FoxP3, IL-10, TGFβ and CTLA4. Supporting the hypothesis that FTY720 directly affects functional activity of $CD4_+CD25_+$ Treg, we measured a significant increase of CD25 and FoxP3 expression in isolated lamina propria $CD4_+$ T cells of FTY720-treated mice. The impact of FTY720 on Treg induction was further confirmed by concomitant in vivo blockade of CTLA4 or IL-10R which significantly abrogated its therapeutic activity. In conclusion, our data provide clear evidence that in addition to its well established effects on migration FTY720 leads to a specific down-regulation of pro-inflammatory signals while simultaneously inducing functional activity of $CD4_+CD25_+$ Treg. Thus, FTY720 may offer a promising new therapeutic strategy for
the treatment of IBD.

1First Department of Internal Medicine, 2pharmazentrum frankfurt, #ZAFES, JWG University of Frankfurt/Main. §HHR and JMS contributed equally to this work.

551

THERAPEUTIC EFFECTS OF FTY720 IN A MODEL OF T HELPER TYPE 2 COLITIS VIA THE IL-1 RECEPTOR RELATED PROTEIN T1/ST2

C. Daniel[ab], N.A. Sartory[ab], N. Zahn[a], R. Schmidt[bc], G. Geisslinger[bc], J.M. Stein[bc§*], H.H. Radeke[bc§]

The sphingosine-1-phosphate analogue FTY720 is known to alter migration and homing of lymphocytes via sphingosine1-phosphate receptors. However, several studies indicate that its mode of action is more complex and that FTY720 may also directly influence cytokine effector functions. Therefore we studied the effect of FTY720 in T helper type (Th2)-mediated oxazolone-induced colitis in mice. Following rectal oxazolone instillation, Th2-cells producing IL-13 induce a progressive colitis resembling human ulcerative colitis. A rectal enema of oxazolone [90 mg/kg body weight] was applied to Balb/c mice. FTY720 was administered i.p. from day 0-3 or from day 3-5 following the instillation of the haptenating agent. Assessment of severity of colitis was performed daily. FTY720 plasma levels were detected using LC-MS/MS analysis. Colon tissue was analyzed macroscopically and microscopically, myeloperoxidase activity as well as cytokine levels of lamina propria $CD4_+$ T-cells and T1/ST2-expression were determined. Treatment with FTY720 prominently reduced the clinical and histopathologic severity of oxazolone-induced colitis, abrogating body weight loss, diarrhea, and macroscopic and microscopic intestinal inflammation. The therapeutic effects of FTY720 were associated with a prominent reduction of the key effector Th2 cytokines IL-13, IL-4 and IL-5. Strikingly, FTY720 also distinctly inhibited T1/ST2 expression a member of the IL-1 receptor family, which is specifically involved in Th2 effector functions. Our data provide the first evidence that FTY720 exhibits beneficial prophylactic as well as therapeutic effects in Th2 mediated experimental colitis by directly affecting Th2 cytokine profiles probably by reducing T1/ST2, thus offering a new auspicious therapeutic instrument for the treatment of human ulcerative colitis.

[a]First Department of Internal Medicine, [b]ZAFES, [c]pharmazentrum frankfurt, JWG University of Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main §HHR and JMS contributed equally to this work.

552

CHARACTERIZATION OF CALCITRIOL ($1,25(OH)_2D_3$) AS A POTENT IMMUNE REGULATOR IN TYPE 1 DIABETES

E. Ramos-Lopez[1], K. Badenhoop[1], U. Christen[2], J. Pfeilschifter[2] and H.H. Radeke[2]

$1,25(OH)_2D_3$ is the most active natural vitamin D metabolite and possesses immune modulatory properties apart from its role in calcium and bone homeostasis. In humans, low serum levels of $1,25(OH)_2D_3$ are correlated with an impaired function of the immune system and autoimmune diseases such as type 1 diabetes. Here we like to report about an ongoing effort to investigate immune modulation by calcitriol with three different approaches For the first part, serum levels of $1,25(OH)_2D_3$ were measured in type 1 diabetes patients (n= 173) and the mRNA expression of the CYP27B1 in type 1 diabetes patients (n=33) and healthy controls (n=23). Secondly, we are determining the functional activity of $1,25(OH)_2D_3$ on cytokine production of human monocytes as well as for the development of a RIP-LCMV-GP and a RIP-LCMV-NP animal model of type 1 diabetes. Patients with type 1 diabetes had a lower expression of CYP27B1 mRNA in comparison with healthy controls (1.7165 vs. 1.7815, $P = 0.0268$). Additionally, patients carrying the genotype *CC* of the -1260 (C/A) polymorphisms possessed a reduced amount of CYP27B1 mRNA compared to healthy controls (1.6855 vs. 1.8107, respectively, $P = 0.0220$). The heterozygosity rate of the -1260 C/A polymorphism was more frequent in patients with normal levels of $1,25(OH)_2D_3$ (≥19.9 pmol/ml) than in whose with a level of less than 19.9 pmol/ml (46.7% vs. 22.2%, $P = 0.0134$). *In vitro* our preliminary results showed that human GM-CSF-primed and LPS / type 1 IFN treated monocytes, which were incubated with $1,25(OH)_2D_3$ released more IL-10 than without $1,25(OH)_2D_3$. Finally, preliminary *in vivo* data reveal that mice (RIP-LCMV-GP und RIP-LCMV-NP), which received $1,25(OH)_2D_3$ exhibited a delayed time onset of type 1 diabetes in comparison to control mice. So far, these findings supported by *epigenetics*, *in vivo* and *in vitro* experiments indicate that $1,25(OH)_2D_3$ is indeed an important co-factor for the onset and progression of type 1 diabetes.

[1] Internal Medicine I, Endocrinology, Diabetes and Metabolism, [2] pharmazentrum frankfurt, Clinic of JWG University, Frankfurt/M, Germany

553

INFLUENCE OF BUSULFAN ON BLOOD COAGULATION PARAMETERS IN ECV304 CELLS AND ASSOCIATION WITH VENO-OCCLUSIVE DISEASE (VOD)

J. Reimer[1], S. Ciecholewski[2], S. Bien[1], U. Völker[2], H. K. Kroemer[1], and C. A. Ritter[1,3]

High dose chemotherapy with busulfan in combination with cyclophosphamide is a common conditioning regime prior to haematopoietic stem cell transplantation. A typical conditioning-related toxicity is hepatic veno-occlusive disease (VOD). Endothelial cells within the liver sinusoids have been identified as the structure initially damaged with subsequent activation of procoagulative parameters.To elucidate the underlying busulfan dependent mechanisms ECV304 cells were treated with 300 µM busulfan for 96 h, initially followed by Affymetrix GenChip analysis to determine busulfan-dependent alterations in global gene expression. Clusters of different biological processes and molecular functions were generated. Focusing on blood coagulation parameters, eight genes were found to be induced: activin A, PAI-1, TGF-β1, fibronectin, urokinase receptor and tissue factor. In contrast, three genes were repressed with a minimum fold change of 1.5 ($p<0.05$) including protein S. Regulation of these genes may result in an increased fibrin accumulation, an important pathophysiological parameter of VOD. We could verify these array results using real-time RCR analyses on mRNA and ELISAs on protein level. Moreover, treatment of ECV304 cells with recombinant activin A and TGF-β1 are followed by enhanced PAI-1 production, which is also a predictive marker of VOD. In addition, TGF-β1 contributed to the induction of other important parameters for blood coagulation such as tissue factor, fibronectin and the urokinase receptor, as determined by real-time PCR. Finally, treatment of ECV304 cells with a combination of busulfan and the specific activin A/TGF-β1 inhibitor SB431542 suppressed the induction of PAI-1 substantially leading to the assumption that busulfan induces PAI-1 via upregulation of TGF-β1 and activin A. In summary, we present evidence for the influence of busulfan on blood coagulation regulating parameters. These results are important for a better understanding of busulfan-induced molecular mechanisms leading to VOD and may help to prevent this serious side effect.

[1]Research Center of Pharmacology and Experimental Therapeutics, Department of Pharmacology, [2] Interfacultary Center of Functional Genomics (ICFG),[3]Institute of Pharmacy, Ernst Moritz Arndt University, Greifswald, Germany

554

ROLE OF CXCR3 AND CXCR4 FOR TRANSMIGRATION OF IMMUNE CELLS IN CHRONIC INFLAMMATION

O. Giegold, J. Will, R. Kinkel, J. Pfeilschifter, H. H. Radeke

Chronic inflammatory diseases like rheumatoid arthritis are characterized by an increased infiltration of Th1 and dendritic cells (DC) into affected tissues. Typical chemokine receptors of Th1 cells are CXCR3 and CXCR4. Conventionally, CXCR3 is known as an inflammatory and CXCR4 as a homeostatic receptor. However, convincing evidence was presented that during chronic inflammation CXCR4 may act differentially due to an interaction with CXCR3. Therefore at first we performed static chemotaxis assays focusing on gradient dependent transmigration of Th1 cells induced by ligands of CXCR3 (CXCL9, -10, -11) and CXCR4 (CXCL12). Our data revealed that both CXCL9 AND CXCL12 augmented chemotaxis of Th1 cells, however the percentage of migrating cells with a CXCL12 gradient was strongly reduced. Careful inspection of the filter membranes revealed that unlike CXCL9, CXCL12 led to an accumulation of adhering Th1 cells readily explaining the reduced number of migrating cells. To get closer to in vivo conditions we started to examine these differential effects of CXCR3/-4 in a dynamic endothelial cell (EC) flow chamber system assessing rolling, adhesion and transmigration of Th1 cells. First results with a monolayer formed by b.End3 (brain microvascular EC, kindly provided by D. Vestweber) showed comparable to the static assay an increased adhesion after CXCL12. Interestingly, when we used AMD3100, a CXCR4 antagonist, we found that Th1 cells transmigrated through the endothelial monolayer. Therefore, our data confirm a major role of CXCL12/CXCR4 for adhesion, however blocking CXCR4 surprisingly did not lead to a reduction of transmigration but rather an enhancement. Because our aim is to prevent the invasion of inflammatory cells, we will carefully try to reproduce these findings with different microvascular EC and also using alternative CXCR4 inhibitors like vMIPII.

pharmazentrum frankfurt / ZAFES, Clinic of JWG University, Frankfurt/Main, Germany

555

11β-HYDOXYSTEROID DEHYDROGENASE TYPE 1 INHIBITORS WITH OLEANAN AND URSAN SCAFFOLDS

A. Blum, E. Maser

Pentacylic triterpenes of the ursan and oleanan type are widespread in the plant kingdom and many of them have been described as physiological active compounds. Clinical interests in ursan and oleanan type triterpenes relate to their anti-inflammatory, hypoglycemic, anti-virial and apoptosis inducing properties. 18β-Glycyrrhetinic acid, a compound with an oleanan scaffold, is known as strong non-selective inhibitor of 11β-hdroxysteroid dehydrogenase activity. It could be shown in the last decade that 11β–hydroxysteroid dehydrogenase type 1 (11β-HSD1), an enzyme that converts cortisone to cortisol and which acts as a cellular switch to mediate glucocorticoid action in tissues, plays a crucial role in the onset of type 2 diabetes. Inhibition of the second isoform, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which only acts as a deydrogenase by inactivating cortisol to cortisone, results in serious conditions associated with inappropriate mineralocorticoid receptor activation by glucocorticoids, like sodium retention, hypokalaemia and hypertension. Therefore, a selective inhibitor of 11β-HSD1 has considerable potential as a drug directed against glucocorticoid related metabolic disorders. Due to its non-selectivity, 18β-glycyrrhetinic acid cannot be used as a drug. Here, we present the results of structure/function investigations concerning 11β-HSD inhibition of oleanan and ursan triterpenoids. Furthermore, we present new compounds with an oleanan or ursan scaffold which selectively inhibit 11β-HSD1 in the nM range, whereas 11β-HSD2 activity is affected only in µM concentrations of the inhibitor. We discuss the structural determinants that confers 11β-HSD1 specificity to these compounds and show new approaches to synthesize selective 11β-HSD1 inhibitors with oleanan or ursan scaffold. Selective 11β-HSD1 inhibitors with an oleanan or ursan scaffold should have the advantage of low toxicity, the possibility of modification of the scaffold to influence pharmacokinetic and –dynamic properties, and sufficient sources in nature to get enough starting material for chemical modification.

Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswiker Straße 10, 24105 Germany

556

CBR1 AND AKR1C3 CONVERT DOXORUBICIN TO DOXORUBICINOL IN THE HUMAN LIVER

N. Kassner[1], H.J.Martin[2], E. Maser[2], T.M. Penning[3], L. Wojnowski[1]

Doxorubicin (DOX) is a highly effective antineoplastic drug but its clinical use is associated with cardiac toxicity. The latter one has been proposed to be caused by doxorubicinol (DOX-OL), the product of the two electron reduction of the carbonyl group of DOX. The identity of enzyme(s) converting DOX to DOX-OL remains unclear. We investigated the reduction of DOX to DOX-OL by 9 candidate aldo-keto reductases (AKR) and short-chain dehydrogenases/ reductases (SDR). AKR1C1, AKR1C2, and CBR3 showed none or very little (<0.02 nmol min^{-1} mg^{-1} at 100 µM DOX) conversion to DOX-OL. Moderate conversion was observed with AKR1A1, AKR1B10 and AKR1C4, with Vmax values of 1.2, 2.8 and 1.2 nmol min^{-1} mg^{-1}, respectively. AKR1B1 showed no saturation up to 250 µM DOX but a specific activity at 100 µM DOX similar to AKR1A1. Km values for all these enzymes were >240 µM. The highest activities and lowest Km values were measured for CBR1 and AKR1C3, and they were 20.6 and 183.5 nmol min^{-1} mg^{-1}, and 167 µM and 128 µM, respectively. In agreement with previous data, we found the reaction to be catalyzed predominantly in cytosols from human livers, kidneys, and small intestines, with Km values of 163, 140, and 86 µM, and Vmax values of 337, 127 and 198 pmol min^{-1} mg^{-1}, respectively. Four enzymes, including CBR1 and AKR1C3, were expressed in human tissues capable of DOX conversion to DOX-OL. Taken together, these results suggest that the conversion of DOX to DOX-OL in human livers is predominantly catalyzed by CBR1 and AKR1C3.

[1]Department of Pharmacology, Johannes Gutenberg University, D-55101 Mainz, Germany
[2]Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig Holstein, D-24105 Kiel, Germany
[3]Center of Excellence in Environmental Toxicology, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084, USA

557

NORMALIZATION OF ENDOTHELIAL DYSFUNCTION AND VASCULAR OXIDATIVE STRESS BY CHRONIC TELMISARTAN TREATMENT IN A RAT MODEL OF STREPTOZOTOCIN-INDUCED *DIABETES MELLITUS* (TYPE I)

M. Oelze[1], P. Wenzel[1], J. Debrezion[1], M. Hortmann[1], S. Schuppan[1], J. Müller[1], S. Schuhmacher[1], L. Steinhoff[1], M. Brand[1], A. Daiber[1] and T. Münzel[1]

Introduction: Endothelial dysfunction in diabetes mellitus was shown to be secondary to increased vascular reactive oxygen species (ROS) production. Studies in diabetic animals and patients pointed towards NADPH oxidases, uncoupled nitric oxide synthase, mitochondria and xanthine oxidase as potential superoxide sources. In the present study we sought to determine whether chronic treatment with telmisartan normalizes vascular oxidative stress and endothelial dysfunction in a rat model of streptozotocin (STZ)-induced *diabetes mellitus* (type I).

Methods and results: STZ-(single intravenous injection, 60 mg/kg, for 7 weeks) induced endothelial dysfunction which resulted in a significant right-shift of the acetylcholine concentration-relaxation-curve as observed by isometric tension studies was completely normalized by co-therapy with telmisartan. Based on measurements of lucigenin-enhanced chemiluminescence in isolated aortic rings we observed a significant increase in vascular ROS in response to STZ which was completely normalized by telmisartan. A similar picture was obtained in aortic cryo sections which were subjected to oxidative fluorescent microtopography using dihydroethidine fluorescence for superoxide detection. In contrast, ROS formation in response to NADPH-dependent stimulation of heart membrane fractions (reflecting NADPH oxidase activity) was not decreased by telmisartan treatment. Furthermore telmisartan therapy did not modify the induction of mRNA-levels of the NADPH oxidase subunits Nox1 and Nox2 which are both increased in diabetic rats.

Conclusions: Telmisartan normalized STZ-induced endothelial dysfunction and increased vascular oxidative stress. This beneficial effects were independent of NADPH oxidase-driven ROS formation. From the results of the present study it may be hypothesized that the antioxidative properties of telmisartan are due to more secondary effects, e.g. eNOS or superoxid-dismutase activity, then direct inhibition of superoxide sources like NADPH oxidases. Furthermore it is also likely that other superoxide sources such as uncoupled NO-synthases, mitochondria and xanthine oxidases may play a more dominant role than NADPH-oxidases.

[1] Klinikum der Johannes Gutenberg-Universität Mainz, II. Medizinische Klinik, Kardiologie, Mainz, Germany

558

PANIC ATTACKS, DEPRESSION AND SUICIDAL IDEATION FOR 5 MONTHS AFTER ONLY ONE DOSE OF PROPHYLACTIC MEFLOQUINE

I. Püntmann[1], I. Bobis Seidenschwanz[1], H. Wille[1], B. Kreusler[2], B. Mühlbauer[1]

Introduction: Mefloquine (MQ), a widely used effective and, in general, well-tolerated agent for chloroquine-resistant malaria, is known to cause various psychiatric adverse drug reactions (ADR).

Case: A 58-year-old woman with thalassaemia minor and migraine started a prophylaxis with 250 mg MQ for a journey to South Africa, a chloroquine resistant area. Two days after the first dose the patient, who was without any psychiatric history, experienced panic attacks. No further MQ was taken. One month later additional depression and suicidal ideation appeared which was treated with paroxetine (40 mg per day) and lorazepam (1 mg on demand). Slow psychiatric recovery was observed within 3 months allowing stepwise dose-reduction of paroxetine. The antidepressive and anxiolytic medication was stopped 5 months after occurence of the first psychiatric symptom. No further psychiatric disorders occured.

Research: Since 1985 MQ has been used extensively. Moderate to severe neuropsychiatric ADR, even fatal cases (due to suicide) have been reported ever since. The precise mechanism is unknown. The incidence during therapeutic intake, with 1 in 215 to 250, is approximately 60-fold higher than in prophylactic use. The recovery period of 5 months, as observed in this case, might be explained by the elimination half-life of MQ (2-4 weeks) but also shorter and longer recoveries have been reported. Compared with other antimalarial compounds MQ does not exert a higher risk for neurologic/psychiatric ADR.

Conclusion: Only a few cases with such a severe and prolonged depressive ADR after a single dose of MQ have been reported. The risk of malarial infection and the efficacy of MQ to prevent malaria should be weighed against the risk of ADR. Malaria prophylaxis with MQ should be restricted to journeys to regions with chloroquine-resistant falciparum malaria, to prevent the development and distribution of MQ-resistance. Regarding the ADR profile of other antimalaria medications, any decision for/against travel and prophylaxis has to be made with prudence.

[1] Institut für Pharmakologie, Klinikum Bremen-Mitte, D-28177 Bremen
[2] Praxis für Innere Medizin und Allgemeinmedizin, D-28219 Bremen

559

PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE (PACAP) AFFECTS HOMEOSTATIC SLEEP REGULATION IN HEALTHY VOLUNTEERS

A. Steiger, H. Murck, R.M. Frieboes, I. A. Antonijevic

Pituitary adenylate cyclase activating peptide (PACAP) is involved in autonomous regulation including time keeping by its action on the suprachiasmatic nucleus (SCN) and on neuroendocrine secretion, energy metabolism and transmitter release. In particular the interactions between PACAP and the glutamatergic system are well recognized. In rats PACAP promotes rapid-eye-movement sleep. The effects of PACAP on sleep electroencephalogram (EEG) and nocturnal hormone secretion are unknown. We compared the effect of intravenously administered PACAP to that of placebo in 8 young healthy male subjects. PACAP in a concentration of 4 x 12.5 µg was administered in a pulsatile fashion hourly between 22.00 h and 1.00 h via long catheter. Sleep EEG was recorded from 23.00 h to 10.00 h, which was also the time, when the subjects were allowed to sleep. Blood samples were taken every 20 minutes between 22.00 h and 07.00 h for the determination of ACTH, cortisol, growth hormone (GH) and prolactin. PACAP administration led to no changes in the macro-sleep structure as assessed according to standard criteria. EEG spectral analysis revealed a significant reduction in the theta-frequency range in the first 4 hour interval and of the spindle frequency range in the second 4 hour interval of the registration period. This was accompanied with a reduction in the time-constant of the physiological delta-power decline in the course of the night, i.e. a less pronounced dynamic of the reduction of delta power with time. Prolactin secretion decreased by trend during the first 4 hour period of the night. No other changes in endocrine secretion were observed. Conclusion: PACAP leads to a reduction of the dynamic of homeostatic sleep regulation and prolactin secretion. Both effects are opposite to those seen after sleep-deprivation but similar to the changes after napping, i.e. a reduced sleep propensity. PACAP might be involved in homeostatic sleep regulation.

Max Planck Institute of Psychiatry, Munich, Germany

560

OSTEONECROSIS OF THE JAWS IN TWO PATIENTS TREATED WITH ZOLEDRONIC ACID

A. Jackisch-Riemann, I. Püntmann, I. Bobis Seidenschwanz, H. Wille, B. Mühlbauer

Introduction: Bisphosphonates (Bph) have been approved, amongst other indications, for the treatment of bone lesion by multiple myeloma (MM) and solid tumor configuration. Zoledronic Acid (ZA) is a nitrogene-containing aminobisphosphonate of high potency.

Case: We report two cases of osteonecrosis of the jaws (OJ) in patients with MM. Patient 1 is a 54 year old female. Because of a malignant sacrum bone lesion local radiation therapy, chemotherapy, corticosteroids and Bph were administered. She developed mandible osteonecrosis after a treatment with ZA for nine months. Patient 2 is a 81 year old male with a history of MM since January 2001. After application of ZA for more than five years osteonecrosis of the mandible was diagnosed. Both patients were treated with debridement and sequestrectomy and received antibiotic therapy, the ZA application was discontinued.

Research: The incidence of OJ depends on type and total dose of Bph. On the strength of past experience the risk for OJ is highest in patients receiving ZA and increases over time, probably because of both long half-life and high potency of ZA. Another predisposing factor for developing OJ appears to be oral trauma, dental surgery or infection evoking damage of the thin mucosa and the underlying periosteum, causing bone necrosis.

Conclusion: Before Bph therapy the dental and mucosal state should be emended to avoid jaw infection. No evidence against or for withdrawal of Bph after diagnosing OJ exists. Minor debridement, infection management and pain control is recommended.

Institut für Pharmakologie, Klinikum Bremen-Mitte gGmbH, Sankt-Jürgen-Strasse 1, 28177 Bremen (financial support by the Stiftung Arzneimittelsicherheit)

561

METAMIZOL ELICITS SUBSTANTIAL INHIBITION OF PERIPHERAL CYCLOOXYGENASES IN HUMAN VOLUNTEERS

O. Cheremina, J. Bachmakov, K. Brune and B. Hinz

Objective: The mode of action of the nonacidic analgesic metamizol is currently not entirely clear. This study therefore addressed the impact of its metabolites 4-methyl-amino-antipyrine (MAA) and 4-amino-antipyrine (AA) on peripheral cyclooxygenases (COX). Methods: In vitro investigations were performed with human whole blood. Pharmacokinetics of metabolites and ex vivo COX inhibition were assessed in five volunteers receiving metamizol at single oral doses of 500 or 1000 mg. Coagulation-induced thromboxane B_2 formation and lipopolysaccharide-induced prostaglandin E_2 synthesis were measured ex vivo in human whole blood as indices of COX-1 and COX-2 activity. Results: In vitro, metabolites elicited no substantial COX-1/COX-2 selectivity with MAA (IC_{50} = 2.55 µmol/L for COX-1; IC_{50} = 4.65 µmol/L for COX-2) being about 8.2- or 9-fold more potent than AA. Following administration of metamizol, MAA plasma levels remained above the IC_{50} values for each isoform for at least 8 h (500 mg) and 12 h (1000 mg) post-dose. Inhibition of both enzymes showed a strong correlation with MAA plasma levels (ex vivo IC_{50} values of 1.03 µmol/L [COX-1] and 0.87 µmol/L [COX-2]). By contrast, plasma peak concentrations of AA after the 1000-mg dose were 2.8- and 6.5-fold below its IC_{50} values for COX-1 and COX-2, respectively. Maximal inhibitions of COX-1 and COX-2 were 94% and 87% (500 mg), 97% and 94% (1000 mg). A greater than 95% COX-1 inhibition was observed between 0.75 and 3 h after dosing with 1000 mg metamizol, suggesting a short-term suppression of platelet function only. Conclusions: Metamizol elicits a substantial and virtually equipotent inhibition of COX isoforms via MAA. Given the profound COX-2 suppression by metamizol - that was considerably above COX-2 inhibition by single analgesic doses of celecoxib and rofecoxib (Hinz et al., Arthritis Rheum 2006;54:282-91) - a significant portion of its analgesic action may be ascribed to peripheral mechanisms. In view of the observed COX-1 suppression, physicochemical factors (lack of acidity) rather than differential COX-1 inhibition may be responsible for the better gastrointestinal tolerability of metamizol as compared to acidic COX inhibitors.

Department of Experimental and Clinical Pharmacology and Toxicology, Friedrich Alexander University Erlangen-Nürnberg, D-91054 Erlangen, Germany

562

FREEZE LESION: PHARMACOLOGICAL AND HISTOPATHOLOGICAL CHARACTERIZATION OF A HUMAN MODEL OF HYPERALGESIA FOR TESTING THE EFFECTS OF ANALGESICS IN HEALTHY VOLUNTEERS

K. Altis [1], M. Burian [1], R. Schmidt [1], M. Podda [2], G. Geisslinger [1], A. Schmidtko [1]

For the evaluation of analgesic drugs various experimental human models of pain and hyperalgesia using different methods of pain induction and stimulation have been developed over the last decades. Almost all of these models are sensitive to opioid analgesics, but only a few models can be effectively used to evaluate the antihyperalgesic properties of traditional non-steroidal anti-inflammatory drugs (NSAIDs). In this study we have characterized the NSAID-sensitive 'freeze lesion' model, in which a freezing injury is induced on human skin, on the biomolecular level and we assessed the sensitivity of this model towards selective

cyclooxygenase (COX)-2 inhibitors. In five healthy subjects, biopsies were taken from normal skin and the area of freezing injury. Histochemical and Western blot analysis of skin biopsies revealed a strong upregulation of COX-2, a slight decrease of COX-1 and activation of nuclear factor kappa B (NF-κB) in the area of the freezing injury. In eight additional healthy subjects a freeze lesion was induced and mechanical pain thresholds (MPT) were tested for 5 h following administration of the non-selective COX inhibitor diclofenac (75 mg), the COX-2-selective inhibitor parecoxib (40 mg) or placebo in a randomized, double-blind cross-over study. Induction of the freeze lesion resulted in hyperalgesia expressed by a decrease of MPT after 24 h. Diclofenac and parecoxib, but not placebo, statistically significantly elevated MPT. These findings indicate that the freeze lesion model is sensitive to NSAIDs including selective COX-2 inhibitors, and that NF-κB-dependent COX-2 upregulation might contribute to the hyperalgesia in this model.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ge 695/2-2).

[1] pharmazentrum frankfurt / ZAFES, Institut für Klinische Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

[2] Zentrum der Dermatologie und Venerologie / ZAFES, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

563

CASE REPORT: SEVERE GASTROINTESTINAL INFLAMMATION RELATED TO PERSISTENT HHV-6B INFECTION IN A 10-MONTH OLD CANCER PATIENT

A.Breddemann[1], S. Läer[1], K.G. Schmidt[2], M. Harjes[2], R. Adam[3], A. Ludwig[4], T.Niehues[5] and D. T. Schneider[5]

Background: Due to underlying disease and immunosuppressive therapy especially children with cancer or stem cell transplantation are at considerable risk to develop life threatening viral infections. These infections will become an even greater problem in the future, since immunosuppressive treatment regimens are expected to be used increasingly. Nevertheless, evidence-based and age-appropriate pharmacotherapeutic regimens are rarely available, so that the therapy remains experimental. Approach: We report on a 10-month-old boy with an anaplastic astrocytoma and an acute Human Herpesvirus-6 (HHV-6) variant B infection, that caused or triggered a severe gastrointestinal inflammation with intractable diarrhoea and failure-to-thrive over several months. The efficacy of antiviral treatment was controlled with a PCR-based determination of the systemic and local HHV-6 virus load. An initial therapy with ganciclovir and the following therapy with foscarnet remained ineffective. Subsequently a low-dose cidofovir therapy was initiated, but the infection persisted. Result: Ultimately a combined antiviral and anti-inflammatory treatment with dose-intensified cidofovir and prednisolone controlled the HHV-6 infection and enabled resolution of clinical symptoms. This dose intensification had to be performed on a hypothetical basis: we assume cidofovir, which is exclusively renally eliminated, to show a higher bodyweight normalised clearance in infants compared to adults. Conclusions: Acute viral infections in immunocompromised children may result in severe clinical manifestations and require an effective and age-appropriate antiviral treatment. The development of a paediatric dosing schedule for cidofovir, that proved to be effective in this case, could assist others who need to consider similar therapeutic regimes. A fundamental prerequisite for the development of such a schedule is the realisation of a pharmacokinetic study in peadiatric patients.

The complete manuscript to this abstract is accepted for publication in HERPES *14:1* 2007

Departments of [1]Clinical Pharmacy and Pharmacotherapy, [2]Paediatric Cardiology and Pulmonology, [3]General Paediatrics, [4]Virology and [5]Paediatric Oncology, Haematology and Immunology,Heinrich-Heine-University of Düsseldorf, Germany.

564

HIGH RATE OF OFF-LABEL USE IN CARDIOVASCULAR PAEDIATRIC PHARMACOTHERAPY REQUIRES NEW FOCUS IN RESEARCH

L. Hsien[1], A. Breddemann[1], A.K. Frobel[1], A. Heusch[2], K. Schmidt[2], S. Läer[1]

Purpose: In paediatric patients, off-label use, i.e. the use of drugs outside the terms of their approved label, is associated with a higher rate of adverse drug reactions and inefficacy of therapy. We performed a study to identify the drug groups which are associated with the highest relevance of off-label use among pediatric inpatients. Method: Between January and June 2006, prescriptions of 351 patients in a pediatric ward at the University Hospital in Düsseldorf were collected and documented. Diagnoses of patients were obtained from the discharge reports and were classified in groups according to the International Classification of Diseases (ICD-10). The approved labels of drugs were obtained from the website of *Fachinformation* (summery of product characteristics). Drugs groups were classified according to the Anatomical Therapeutic Chemical Classification System (ATC-Code). Results: We analysed 1540 prescriptions of 191 different drugs. We found that 193 patients (55%) were given at least one off-label prescribed drug. Of all analysed prescriptions, 388 (25%) were off-label. The percentages of off-label use among the most prescribed drug groups were as follows: 56% of the drugs of cardiovascular system (ATC-Code C and B01: 100 from total 197 prescriptions), 41% of anti-infectives (ATC-Code: J, A07 and S01: 157 from 385), 25% of drugs of respiratory system (ATC-Code R: 72 from 283), 14% of drugs of alimentary tract and metabolism (ATC-Code A: 33 from 232) and 3% of analgetics and antipyretics (ATC-code: N02 and M01; 6 from 236). Conclusion: Authorization of paediatric drugs is required especially in the domain of cardiology. Since the new EU-Regulation on paediatric medicines will lead to a strong increase in paediatric studies in the near future, this result gives an important impulse, as it shows up targets for paediatric clinical studies.

Department of Clinical Pharmacy and Pharmacotherapy[1], Department of Paediatric Cardiology and Pneumology[2], Heinrich-Heine-University of Düsseldorf, Germany.

565

BIOCHEMICAL EFFECTIVENESS OF ALLOPURINOL MONOTHERAPY AND ALLOPURINOL-PROBENECID COMBINATION THERAPY IN PREVIOUSLY BENZBROMARONE-TREATED GOUT PATIENTS

Reinders MK[1,2], Van Roon EN [1,2], Houtman PM[3], Wilffert B[1,2], Brouwers JRBJ [1,2], Jansen TLThA[3].

Objectives: In 2003 the uricosuric benzbromarone was (temporarily) withdrawn from the market. The proposed alternative, allopurinol, is reported to be less potent than benzbromarone in lowering serum uric acid [Perez-Ruiz F. Ann Rheum Dis 1998;57:545-9]. Purpose of this study was to (1) compare the efficacy of allopurinol (standard dosage) and the previous benzbromarone treatment; (2) investigate the combination therapy allopurinol-probenecid as an alternative treatment compared with previous benzbromarone treatment. Methods: Prospective, open study of 32 gout patients who discontinued benzbromarone therapy because of market withdrawal. Patients were given 200-300 mg allopurinol (phase 1). When allopurinol failed to attain target serum urate levels <0.30 mM, probenecid 500 mg b.i.d. was added (phase 2). Results: Treatment with benzbromarone monotherapy (range: 100-200 mg/day) resulted in 91% of patients reaching target serum urate levels with a decrease of serum urate levels of 59[±11]%. Treatment with allopurinol monotherapy resulted in 13% of patients reaching target levels with a decrease of serum urate levels of 34[±10]% ($p<0.001$ compared to benzbromarone). No differences were found between underexcretor-type and overproducer-type gout patients. Fifteen patients received allopurinol-probenecid combination therapy which provided 87% of patients achieving target serum urate levels. Decrease of serum urate levels was 53[±9]%, which was a non-significant difference compared to benzbromarone treatment ($p=0.18$). Discussion**:** Benzbromarone is a very effective antihyperuricemic drug with 91% success in attainment of target serum urate levels. Allopurinol 200-300 mg/day shows to be a less potent alternative. In patients failing on allopurinol monotherapy, allopurinol-probenecid combination therapy proves to be provide an additive serum urate lowering effect.

[1] Department of Clinical Pharmacy & Pharmacology, Medisch Centrum Leeuwarden, Leeuwarden,

[2] Groningen University Institute for Drug Exploration (GUIDE), Subdivision Pharmacotherapy and Pharmaceutical Care,

[3] Department of Rheumatology, Medisch Centrum Leeuwarden, Leeuwarden, The Netherlands.

566

NITROFURANTOIN-INDUCED PULMONARY FIBROSIS RESULTING IN DEATH DUE TO ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

I. Bobis Seidenschwanz[1], C. Breier-Wolski[1], J. Flehmig[2], A. Lison[2], B. Muehlbauer[1]

Introduction: One out of 750 long-term users of nitrofurantoin develops chronic lung reactions serious enough to require hospitalization.

Case: A 57-year-old woman with hypertension, gout, adiposis and retinopathy pigmentosa received nitrofurantoin for 1 year because of recurrent urinary tract infections. In December 2005, the patient developed increasing dyspnoe, so an antibiotic treatment was started (acithromycin, ciprofloxacin). 2 weeks later the patient needed hospitalization due to extreme pyrexia and cough. A CT-scan of the the lungs showed massive structural abnormalities in the interstitium as signs of pulmonary fibrosis. Only 2 days later, the patient required respiratory ventilation. Treatment with high-dose corticosteroids was initiated. The patient then presented recurrent pulmonary infections and a progredient septic shock including the symptoms of ARDS. Despite the treatment with high-dose catecholamines and antibiotics (piperacillin, sulbactam, ciprofloxacin, fosfomycin/cefepime, vancomycin) septic events and pulmonary function deteriorated continuously and were complicated by an increasing renal insufficiency. 2 weeks after initial hospitalization and only 1 month after occurrence of the first symptoms the patient died due to pulmonary failure.

Research: Only a few case reports have been published with nitrofurantoin suspected to cause death owing to pulmonary failure. There were no cases reporting such a rapid fatal progression.

Conclusion: In this case most likely long-term-administration of nitrofurantoin caused pulmonary fibrosis resulting in death. No other factors (e.g. concomitant diseases or drugs) could be detected. Nitrofurantoin, causing rare severe pulmonary adverse effects, is not indicated for any sort of urinary tract infection.

[1]Institut fuer Pharmakologie, [2]Medizinische Klinik III, Klinikum Bremen-Mitte, Sankt-Juergen-Strasse 1, 28177 Bremen (support by Stiftung Arzneimittelsicherheit)

567

PLATINUM SERUM LEVELS OF CHEMOTHERAPY PATIENTS DETERMINED BY TOTAL REFLECTION X-RAY FLUORESCENCE (TXRF)

E. D. Greaves[1,2], M. Angeli-Greaves[3,4], K. Golka[4], M. Truss[5] and H.J. Knopf[5]

The therapeutic window for platinum-bearing drugs used in the treatment of cancer is quite narrow. As the detection of Pt serum levels is very laborious, we present a method which only requires 100 µl blood. 100 µl blood is spun down at 10,000 rpm. Serum is stored at -4°C. An aliquot of 3 µl is transferred to a sample carrier and dried for 20 min. Serum Pt concentrations can be detected without further sample treatment by total reflection X-ray fluorescence (TXRF). Detection limit is 0.2 µg/ml. In a pilot study based on 40 blood samples of 7 cancer patients of a local department of urology, the applicability of the method in a hospital was demonstrated. In general, the detected serum Pt levels were between 1-2 µg/ml. The increase of the Pt serum levels prior to every further Pt application could be clearly demonstrated. Although the preparation of the samples is minimal, the presented method provides useful serum Pt determinations from 100 µl blood.

[1]ISAS-Institute for Analytical Sciences, Dortmund
[2]Universidad Simón Bolívar, Caracas, Venezuela
[3]Universidad Central de Venezuela, Caracas, Venezuela
[4]Institut für Arbeitsphysiologie an der Universität Dortmund, Dortmund
[5]Department of Urology, Klinikum Dortmund, Dortmund

568

DRUG METABOLIZING CYTOCHROMES P450 IN HUMAN LIVER - A COMPREHENSIVE ANALYSIS

K. Klein, T. Saussele, C. Toscano, J. Blievernicht, M. Eichelbaum, M. Schwab, U.M. Zanger

A major determinant for drug efficacy and adverse reactions is the individual expression and function of drug metabolizing enzymes. To study in detail the factors that contribute to intra- and interindividual variability of cytochrome P450 expression we established a large human liver bank consisting of surgical tissue samples with extensive patient documentation. The most important drug metabolizing cytochromes P450 (CYPs 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4/5) were quantitatively characterized using Western blotting and/or activity measurements. The spectrally determined average liver microsomal P450 content was 420 pmol/mg (± 200 pmol/ml) which is in good agreement with the average content as reflected by the sum of measured apoprotein (430 pmol/mg). The major P450 forms were 2C9 (35%), 3A4 (22%) 1A2 (20%), and 2E1 (15%). The minor forms comprising 2D6, 2A6, 2B6, and 3A5 collectively contributed about 16% to the hepatic P450 content. Thus, in contrast to previous estimations (Shimada et al., 1994), CYP3A4 was the major P450 in only about 15% of samples where it contributed about 38% of the total P450 content. For CYP1A2 we could confirm the influence of smoking such that expression in smokers was higher than in nonsmokers ($p=0.075$) whereas for 3A4 no difference was seen between these groups. Further data analysis with respect to gender revealed a clear but opposite influence on 3A4 and 1A2 expression. For 3A4 we found higher levels in females ($p=0.0053$) as previously described (Wolbold et al., 2003) whereas 1A2 was higher expressed in male liver. Interestingly, after stratification into smokers and nonsmokers, we found significantly lower levels of CYP1A2 in nonsmoking females as compared to nonsmoking males ($p=0.047$). Gender had no influence on expression levels of 2B6, 2D6 or 2E1.

Supported by the German Federal Ministry of Education and Research (HepatoSys grant 0313080D) and by the Robert Bosch Foundation, Stuttgart, Germany

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology; Auerbachstr. 112,
D-70376 Stuttgart, Germany

569

ASSESSING THE INCIDENCE OF ADVERSE DRUG EVENTS BY DATA-BASE RESEARCH: CHANCES AND LIMITATIONS

B. Mühlbauer[1], C. Hagemeier[1], I. Langner[2], I. Püntmann[1], W. Ahrens[2], I. Pigeot[2], J. Timm[1]

Rofecoxib (Rfxb), a cyclooxygenase 2-selective nonsteroidal anti-inflammatory drug (NSAID), was recently withdrawn from the market since cardiovascular adverse events (cvAE) were detected in randomized controlled trials (RCT). In contrast to clear-cut data from RCTs, incidence estimations of the Rfxb-induced cvAE in the everyday life situation remained inconsistent. We examined in a large data sample (1.3 million individuals, 2000 through 2003) of 3 German health insurance companies the rate of cvAE leading to hospital admittance in patients whilst on (current use), 1-6 months after (recent use), and more than 6 months after (previous use) Rfxb prescription. Data were compared with users of unselective NSAIDs and non-users. Similar to several database studies published recently we found the risk for cvAE increased (hazard ratio, HR, 1.66, 95% confidence interval, CI, 1.35-2.04) for current Rfxb use which remeined elevated in the previous phase (1.36; CI 1.06-1.74). Risk was also increased in current NSAID users (HR 1.20; CI 1.09-1.33) but was below that of non-users in the previous phase (0.89; CI 0.81-0.97). In addition, the Rfxb-induced risk elevation was lower (HR 1.20; not significant) when only cvAEs in short-time users, defined as having received less than 30 tablets of any NSAID during the entire time period, were considered. The risk for cvAE appears to rise with chronic Rfxb use. Considering the HR for previous use, the Rfxb users observed here might have had a higher basal risk. Thus, HR estimate of 1.66 during current use needs to be reconsidered using a more sophisticated approach. Routine data evaluation is a valuable tool to estimate AE incidence in large populations, but its results have to be interpreted with caution considering potential confounders.

Supported by the Stiftung Arzneimittelsicherheit.

1) Kompetenzzentrum für Klinische Studien Bremen,
2) Bremer Institut für Präventionsforschung und Sozialmedizin