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IL-1β induced protein changes in diabetes prone BB rat islets of Langerhans identified by proteome analysis

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Abstract

Aims/hypothesis. Type I (insulin-dependent) diabetes mellitus is characterized by selective destruction of the insulin producing beta cells. Interleukin-1β (IL-1β) modulates the beta-cell function, protein synthesis, energy production and causes apoptosis. We have previously shown changes in the expression of 82 out of 1 815 protein spots detected by two dimensional gel electrophoresis in IL-1β exposed diabetes prone Bio Breeding (BB-DP) rat islets of Langerhans in vitro. The aim of this study was to identify the proteins in these 82 spots by mass spectrometry and compare these changes with those seen in IL-1β exposed Wistar Furth (WF) rat islets.
Methods. The 82 protein spots, that changed expression after IL-1β exposure, were all re-identified on preparative gels of 200 000 neonatal WF rat islets, cut out and subjected to mass spectrometry for identification.
Results. Forty-five different proteins were identified from 51 spots and grouped according to function: (i) energy transduction and redox potentials; (ii) glycolytic and Krebs cycle enzymes; (iii) protein, DNA and RNA synthesis, chaperoning and protein folding; (iv) signal transduction, regulation, differentiation and apoptosis; (v) cellular defence; and (vi) other functions. Comparison of IL-1β exposed BB-DP and WF islets showed common changes in 14 proteins and several proteins influencing similar pathways, suggesting that similar routes in the two strains lead to beta-cell destruction.
Conclusion/interpretation. We demonstrate that proteome analysis is a powerful tool to identify proteins and pathways in BB-DP rat islets exposed to IL-1β. [Diabetologia (2002) 45:1550–1561]

Keywords Proteome analysis, two dimensional gel electrophoresis, mass spectrometry, cytokines, IL-1β, BB rat, diabetes, pathogenesis.

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Abbreviations: 2-DGE, Two dimensional gel electrophoresis; BB-DP, diabetes prone Bio Breeding; WF, Wistar Furth; NO, nitric oxide; iNOS, inducible NO syntase; BN, Brown Norway; LS, Lewis-Scripps; HSP, heat shock protein; HBSS, Hanks' balanced salt solution; pI, isoelectric point; TCA, trichlroacetic acid; IEF, isoelectric focusing; NEPHGE, non-equilibrium pH-gradient electrophoresis; MALDI, matrix-assisted laser desorption/ionisation; GRP, glucose regulated protein; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; NOD, non obese diabetic; PIPP, pertubation in protein patterns.

Type I (insulin-dependent) diabetes mellitus is a multifactorial polygenic autoimmune disease characterized by mononuclear cell infiltration in the islets of Langerhans (insulitis) and selective destruction of the insulin producing beta cells [1, 2, 3]. It is generally accepted that the destruction of the beta cells results from interactions between various environmental factors and immune mechanisms in genetically susceptible people [3]. The very first events initiating the destructive process have not yet been described in detail. Cytokines, in particular interleukin-1β (IL-1β), are known to be released within the islets in sufficient quantities to modulate and inhibit beta-cell function in vitro [4]. Furthermore, IL-1β influences many important cellular functions such as reducing DNA content, decreasing protein synthesis and intracellular energy

production and inducing beta-cell apoptosis. During the destructive process IL-1β, TNF-α and IFN-γ are released in the islets resulting in the production of free radicals [e.g. nitric oxide (NO^-), super oxide (O_2^-) and hydroxyl (OH^-)] in the beta cells [3]. Free radicals are normally scavenged by protective proteins [e.g. haeme oxygenases and manganese superoxide dismutase (MnSOD)] [5, 6]. Many IL-1β effects are mediated by inducing the inducible nitric oxide syntase (iNOS) and its product, nitric oxide [7]; however, NO-independent mechanisms also occur [8, 9, 10]. We hypothesize that the beta cell, when exposed to IL-1β initiates a protective response in competition with a series of deleterious events, and that in beta cells the deleterious events prevail [3]. In support of this, over-expression in islet cells of scavengers of free radicals, such as catalase and glutathione peroxidase, reduces the deleterious effects of cytokines on beta cells [11].

Our laboratories recently described in Wistar Furth (WF) rat islets exposed to IL-1β in vitro, changes in the expression of 105 out of approximately 1400 proteins identified by high resolution two dimensional gel electrophoresis (2-DGE) [8]. The majority of these proteins have been identified by mass spectrometry [8, 10] and identified proteins were classified into the following groups on the basis of their known and perceived function: (i) energy transduction and redox potential; (ii) glycolytic enzymes; (iii) protein synthesis chaperones and protein folding; (iv) signal tranduction, regulation, differentiation and apoptosis suggesting a broad variety of pathways involved in IL-1β mediated islet toxicity [10].

The diabetes prone BioBreeding (BB-DP) rat spontaneously develops a diabetic syndrome with characteristics in common with human Type I diabetes [12]. Originally the BB-DP rat strain was bred from a WF rat colony [13]. Strain-dependent variations in beta-cell sensitivity to IL-1β effects have been observed in vitro and in vivo [14, 15] with islets from Brown Norway (BN) rats being less sensitive to IL-1β compared to WF, Lewis-Scripps (LS) and BB-DP as well as diabetes resistant BB (BB-DR) rats. BB-DP rat islets produce lower protective stress responses [heat shock protein 70 (HSP70)] than BB-DR rat islets which can prime beta cells for destruction [16]. We have previously shown that there is no difference in nitric oxide (NO) production and 24-h accumulated insulin release in BB-DP and WF islets in response to exposure to 150 pg/ml IL-1β for 24 h [17]. The relative resistance to IL-1β induced inhibition of beta-cell function in vitro and in vivo in BN rat islets was associated with lower expression of inducible nitric oxide synthase (iNOS) compared to Wistar Kyoto and LS rat islets [15].

Proteome analysis applied to WF rat islets exposed to IL-1β has provided a detailed picture of beta-cell destruction at the protein level [9, 10]. Comparison of IL-1β induced pertubation of protein patterns (PIPP), i.e. expression changes and or posttranslatory modifications, identified by proteome analysis in WF and BB-DP rat islets could identify proteins or pathways generally involved in beta-cell destruction as well as some specific for beta-cell destruction in the BB-DP rat. We previously showed by 2-DGE that IL-1β induce reproducible and statistically significant changes in the expression of 82 protein spots in BB-DP rat islets in vitro out of a total of 1 815 protein spots [17].

The aim of this study was to identify the proteins in these 82 spots by mass spectrometry and to compare the findings with protein identifications from IL-1β exposed WF islets [10] and to describe briefly their known functions with respect to the pathogenesis of Type I diabetes for some of the proteins.

Materials and methods

Study design. Preparative gels of 200 000 WF islets were made to describe PIPP, which were originaly identified in BB-DP rat islets (analytical gels). For these analytical gels 150 neonatal BB-DP rat islets were incubated with 150 pg/ml recombinant human IL-1β for 24 h and labelled for 2-DGE. In the analytical gels each protein spot was assigned a percentage of the total integrated optic density (%IOD) on the gel by the BioImage computer programme. Expression changes were evaluated by a Student's *t* test and considered significant at *p* values below 0.01 [17]. For most proteins this reflects a twofold or higher change in expression. Protein spots that changed expression in BB-DP rat islets after exposure to IL-1β were all identified on preparative 2-D gels of WF rat islets (200 000 islets in total) and cut out of the preparative gels and subjected to mass spectrometry.

Reagents. RPMI 1640, Hanks' balanced salt solution (HBSS) and DMEM were purchased from Gibco, Paisley, Scotland. RPMI 1640 contained 11 mmol of D-glucose and was supplemented with 20 mmol/l HEPES buffer, 100 000 IU/l penicillin and 100 mg/l streptomycin. Recombinant human IL-1β (specific activity was 400 U/ng) was from Novo Nordisk, Bagsvaerd, Denmark. Other reagents used in the study were: 2-mercaptoethanol, foetal calf serum (BSA), normal human serum (NHS), Tris HCl, Tris base, glycine, (Sigma, St. Louis, Mo., USA); trichloracetic acid (TCA), phosphoric acid, NaOH, glycerol, n-butanol, bromophenol blue, H_3PO_4 and $NaNO_2$ (Merck, Darmstadt, Germany); filters (HAWP 0.25 mm pore size) (Millipore, Boston, Mass., USA); RNA'se A, DNA'se I (Worthington, Freehold, N.J., USA); [^{35}S]-methionine (SJ 204, specific activity: >1 000 Ci/mmol, containing 0.1% 2-mercaptoethanol), Amplify (Amersham International, Amersham, UK); urea (ultra pure) (Schwarz/Mann, Cambridge, Mass., USA); acrylamide, bisacrylamide, 4N-tetra-methyl-ethylenediamine (TEMED), ammonium persulphate (BioRad, Richmond, Calif., USA); ampholytes: pH 5–7, pH 3.5–10, pH 7–9, pH 8–9.5 (Amasham Biotech, Sweden); Nonidet P-40 (BDH, Poole, UK); ampholytes: pH 5–7 and sodium dodecyl sulphate (Serva, Heidelberg, Germany); agarose (Litex, Copenhagen, Denmark); ethanol (absolute 96%) (Danish Distillers, Aalborg, Denmark); methanol (Prolabo, Brione Le Blanc, France); acetic acid (technical quality, 99% glacial) (Bie and Berntsen, Århus, Denmark) and X-ray film (Curix RP–2) (AGFA).

Animals. BB-DP (BB/Wor/Mol-BB2) rats and pregnant inbred WF rats were purchased from M & B, Ll. Skensved, Denmark. All animal experiments were carried out according to national and international law and ethical standards. The experiments were approved by the Danish Council for Animal Welfare under the Ministry of Justice.

Isolation, culture and labelling of islets for preparative gels. Islets were isolated by collagenase digestion of the pancreata from 4 to 5 day old WF rats [18]. After 4 days of preculture in RPMI 1640+10% FCS, islets were incubated for 24 h in 37°C humidified atmospheric air in 300 µl (150 islets for [^{35}S]-methionine labelling as tracer islets) or 3000 µl (40 000 non-labelled islets for preparative gels each) RPMI 1640+0.5% normal human serum. Next islets were washed twice in HBSS. Tracer islets were labelled for 4 h at 37°C in 200 µl homemade methionine-free DMEM with 10% dialysed NHS, and 200 µCi [^{35}S]-methionine and the islets for the preparative gels were incubated in the same way in 3000 µl methionine containing DMEM without adding [^{35}S]-methionine. To eliminate 2-mercaptoethanol, [^{35}S]-methionine was freeze-dried for at least 4 h before labelling. After labelling and incubation, the islets were washed thrice in HBSS, the supernatant was removed and islets were immediately frozen at –80°C. Unlabelled islets for preparative gels were washed twice in HBSS and snapfrozen. For localization of the spots, radioactively labelled tracer islets were mixed with the non-labelled islets.

The BB-DP islets used for the analytical 2-D gels were isolated and treated as described above. During incubation period islets were exposed either to 150 pg/ml recombinant human IL-1β or served as control islets in three independent experiments [17].

Sample preparation. The frozen islets were re-suspended in 100 µl DNAseI/RNAse A solution and lysed by freeze-thawing twice. After the second thawing, the samples were left on ice for 30 min to digest the nucleic acids and then freeze dried overnight. The samples were dissolved by shaking in 120 µl lysis buffer (8.5 mol/l urea, 2% Nonidet P-40, 5% 2-mercaptoethanol and 2% ampholytes, pH range 7–9) for a minimum of 4 h.

Measurement of [^{35}S]-methionine incorporation. The amount of [^{35}S]-methionine incorporation was quantified by adding 10 µl FCS (0.2 µg/ml H_2O) as a protein-carrier to 5 µl of a 1:10 dilution of each sample in duplicate, followed by 0.5 ml of 10% TCA. This was left to precipitate for 30 min at 4°C before being filtered through 0.25 µm hydroxy appatit-WP (HAWP) filters. The filters were dried and placed into scintillation liquid for counting.

2-DGE and preparative gels. The labelled gels of WF islets were produced in the same way as the analytical gels of IL-1β exposed and control BB-DP islets, each in batches of 150 islets [17]. Preparative two dimensional gels (2-DG) were produced from a pool of approximately 200 000 neonatal WF rat islets isolated, cultured, labelled and separated on gels as described above. For localization of the spots, radioactively labelled tracer islets were mixed with the non-labelled islets.

The procedure has been described previously [19, 20, 21]. Briefly, first dimension gels contained 4% acrylamide, 0.25% bisacrylamide, ampholytes and nonidet P-40. Equal amount of protein (175–200 µg for preparative gels) and counts per min (10^6 cpm) of each sample were applied to the gels. Both isoelectric focusing (IEF; pH 3.5–7) and non-equilibrium pH-gradient electrophoresis (NEPHGE; pH 6.5–10.5) gels were made. Second dimension gels contained 12.5% acrylamide and 0.063% bisacrylamide and were run overnight. After electrophoresis, the gels were fixed and treated for fluorography with Amplify before being dried. The gels were placed in contact with X-ray films and exposed at –70°C for 3 to 40 days. Each gel was exposed for at least three time periods to compensate for the lack of dynamic range of X-ray films.

Neonatal WF rat islets were used for preparative gels because of the price and higher number of islets isolated per animal. The cost of one WF rat is approximately one tenth of the price of a BB-DP rat (approximately 100 US$ each). From each rat it was possible to isolate 150 to 200 islets. All protein spots changing expression after IL-1β exposure of BB-DP rat islets were also identified in the WF islet preparative 2-D gels. Notably, no protein spots were expressed de novo after IL-1β exposure of BB-DP islets. Hence, preparative gels from WF rat islets were suitable for mass spectrometry identification of proteins in the spots that changed expression in IL-1β exposed BB-DP islets.

Determination of M_r and pI. Molecular weight and pI for individual proteins on the gels were interpolated from landmark proteins. Landmark proteins were determined using internal standards and pI calibration kits [20]. Theoretical M_r and pI were calculated using the Compute pI and Mw tool at the ExPASy Molecular Biology Server [22].

Computer analysis of fluorographs. Computer analysis was carried out using the BioImage program 2D-Analyzer (version 6.1) [17].

Protein identification by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Briefly, the 82 protein spots of interest were obtained by cutting them out of the dried gel using a scalpel. The proteins were enzymatically digested in the gel piece as described [23, 24] with minor modifications [25]. The excised gel pieces were washed in 50 mmol/l NH_4HCO_3 and acetonitrile (60/40) and dried by vacuum centrifugation. Modified porcine trypsin (12 ng/µl, Promega, sequencing grade) in digestion buffer (50 mmol/l NH_4HCO_3) was added to the dry gel pieces and incubated on ice for 1 h for reswelling. After removing the supernatant, 20 to 40 µl digestion buffer was added and the digestion was continued at 37°C for 4 to 18 h. The peptides were extracted as described [24] and dried in a vacuum centrifuge. The residue was dissolved in 5% formic acid and analysed by MALDI mass spectrometry. Delayed extraction MALDI mass spectra of the peptide mixtures were acquired using a Bruker Reflex time-of-flight mass spectrometer (Bruker AG, Germany). Samples were prepared using α-cyano-4-hydroxy cinnamic acid as matrix [26]. Proteins were identified by in silico comparison of the theoretical peptide-mass maps in the comprehensive, non-redundant protein sequence database (NRDB, European Bioinformatics Institute, Hinxton, UK [27]) using the PeptideSearch software ([28] further developed at EMBL; Heidelberg, Germany), SWISS-PROT [29], PIR [30], NIH and GENEBANK [31]. The protein identifications were examined using the "second pass search" feature of the software and critical evaluation of the peptide mass map as described [32].

Protein information. Information about the known proteins identified and putative biological functions were found at the ExPASy Molecular Biology Server at SWISS-PROT [29] and at The National Centre for Biotechnology Information (NCBI) [31].

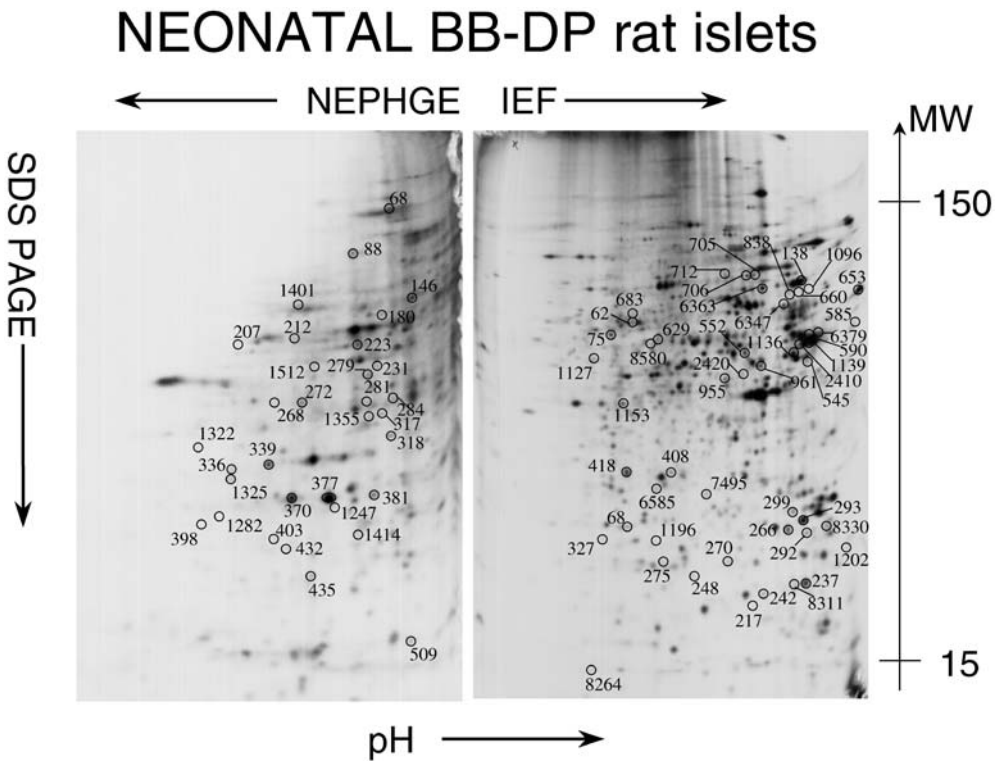

Fig. 1. Two-dimensional gel of neonatal BB-DP rat islets of Langerhans incubated for 24 h in RPMI 1640 supplemented with 0.5% NHS followed by 4 h of labelling with [^{35}S]-methionine. The gel shown is representative of three independent experiments and the marked proteins represent proteins changing expression after 24 h of exposure to IL-1β. IEF gel (pH 3.5–7) on the right side and NEPHGE gel (pH 6.5–10.5) on the left side. The numbers correspond to the proteins in Tables 1 and 2. For technical reasons a representative gel of control islets is presented, since most proteins are down-regulated after IL-1β exposure (Table 1, 2).

Results

All 82 significantly changed protein spots were re-identified in preparative gels of neonatal WF islets and could be excised from the gels for mass spectrometry identification (Fig. 1). Positive identification was obtained for a total of 45 different proteins from 51 of the 82 spots (Table 1). Six spots contained two identifiable proteins (NEPHGE match no. 339, 370 and IEF 955, 248, 1136, 660). Some proteins were present in more than one spot: three proteins were present in two spots (Heterogeneous nuclear ribonucleoproteins A2/B1, calreticulin and NADH dehydrogenase), three proteins were present in three spots [malate dehydrogenase precursor (mitochondrial), IgE binding protein and glucose-regulated protein 78 (GRP 78)] and one protein, tubulin beta-5 chain was present in four spots. The presence of the same protein in more than one spot suggests that the protein exists in different post-translatory modified or degraded forms. Positive identification was not obtained for 31 protein spots either due to spectra with no data base match (n=16) or low abundance of peptides in the excised and digested spots (n=15) (Table 2). Thus, the success rate of positively identified protein spots was 62% (51 spots out 82). A similar result, 60 spots identified out of 105 (58%) were obtained from WF rat islets exposed to IL-1β, corresponding to 57 different proteins [10]. Out of 14 proteins 12 were identified both in BB-DP and WF rat islets as identical [NEPHGE match no. 223, 231, 370, 146, 381, 377, 398 (381, 377 and 398 are all galectin-3), IEF match no. 552, 138, 660, 6347 (138, 660 and 6347 are all GRP 78), 1136, 8580, 68, 62, 6363], one was an iso-form (IEF match no. 955) and one a post translatory modified form (IEF match no. 1127) (Table 1).

Minor differences in M_r and pI values observed between proteins identified both in BB-DP and WF islets can be explained in that the gel concentrations in the two studies were different. First dimension gels were run identically for BB-DP and WF islets. However, second dimension analytical and preparative WF islet gels were run on 10% and 15% acrylamide gels in the WF islet study [10] whereas the analytical BB-DP gels were run on 12.5% acrylamide gels as were the preparative WF gels for this study. Furthermore, using the latest updated version of the protein databases and depending on the actual peptides used from the mass spectrometry spectra to identify the protein, different species can give the highest score, although it is the same protein.

In Table 1, the protein M_r's and pI's observed on the gels are presented together with the computed theoretical M_r and pI values. Differences in observed and computed values of M_r and pI could be due to posttranslatory modifications, degradation or pro- or

Table 1. Identification of proteins induced by IL-1β in BB-DP rat islets

Gel match No.	% IOD	% IOD ratio	Protein name	Function	Database acc. no.	M_r	Theo-retical M_r	pI	Theo-retical pI
Energy transduction and redox potentials									
N 318	0.165	0.56	Alcohol dehydrogenase	Redox potential	gi 1703237	42.4	36.4	7.8	6.8
N 231[b]	0.041	0.51	ATP synthase alpha chain, mitochondrial precursor	Energy transduction	gi 114523	51.1	58.8	7.9	9.2
N 370[a,b]	2.055	0.24	L-3-hydroxyacyl-CoA dehydrogenase precursor	Energy transduction, redox potential	gi 5353512	36.5	34.3	8.4	8.9
N 1247	0.084	0.20	Carbonyl reductase	Redox potential	gi 1352258	37.7	30.4	8.2	8.2
I 955[a,b]	0.104	2.41	Creatine kinase-B	Energy transduction	gi 203476	49.3	42.7	5.4	5.3
I 1136[a]	0.405	0.45	H+ transporting ATP synthase	Energy transduction	gi 92350	52.9	56.4	4.8	5.2
I 706	0.086	0.43	NADH dehydrogenase	Energy transduction	gi 4826856	86.7	79.6	5.3	5.8
I 705	0.084	0.35	NADH dehydrogenase	Energy transduction	gi 4826856	87.3	79.6	5.2	5.8
I 552[b]	0.132	0.07	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58 kD, isoform 1	Energy transduction, intracellular environment	gi 4502309	57.6	57.0	5.3	5.5
Glycolytic and Krebs cycle enzymes									
I 629	0.065	2.15	Glucose-6-phosphate dehydrogenase	Energy generation	gi 204197	61.8	59.2	6.0	6.0
I 1127[b]	0.067	0.29	Glyceraldehyde-3-phosphate dehydrogenase	Energy generation	gi 203142	54.4	35.7	6.7	8.4
N 223[b]	0.379	0.52	Pyruvate kinase M2 isozyme	Energy generation	gi 1346398	55.6	57.6	8.0	7.4
N 88	0.288	0.41	Aconitate hydratase, mitochondrial precursor	Energy generation	gi 1351857	77.8	85.4	7.3	8.1
N 272	0.433	0.48	Isocitrate dehydrogenase 2, mitochondrial	Energy generation	gi 6680343	45.9	58.7	8.4	8.9
N 1325	0.137	0.38	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	41.9	35.5	9.0	8.9
N 336	0.118	0.36	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	42.7	35.5	9.0	8.9
N 339[a]	0.830	0.28	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	40.4	35.5	8.7	8.9
Protein synthesis (incl. DNA/RNA processing and synthesis, purin/pyrimidine synthesis, amino acid metabolism) chaperones and protein folding									
N 146[b]	0.590	0.44	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	Purine/pyrimidine synthesis, DNA/RNA synthesis	gi 2541906	64.8	64.2	6.9	6.7
I 248[a]	0.030	0.48	Adenosine phosphoribosyltransferase	Purine/pyrimidine synthesis, DNA/RNA synthesis	gi 543829	21.5	19.5	5.7	6.2
I 248[a]	0.030	0.48	UMP-CMP kinase	Purine/pyrimidine synthesis, DNA/RNA synthesis	gi 5730476	21.5	22.2	5.7	5.4
N 1414	0.070	0.41	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA-processing	gi 6647752	42.1	36.0	8.0	8.7
N 339[a]	0.830	0.28	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA-processing	gi 6647752	40.4	36.0	8.7	8.7
N 1401	0.482	0.17	M4 protein deletion mutant	RNA-processing	gi 3126878	64.4	77.5	8.5	8.9
N 317	0.071	0.35	Aspartate transaminase, cytosolic	Amino acid metabolism	gi 91997	44.9	46.2	7.9	6.3
N 1322	0.054	0.15	Aspartate aminotransferase, mitochondrial precursor	Amino acid metabolism	gi 112987	47.6	47.3	9.1	9.1
I 237	0.534	0.12	60 ribosomal protein L11	Ribosome protein	gi 971761	20.9	18.8	4.4	9.9
I 653	0.722	1.52	Calreticulin	Chaperone, transcription factor	gi 6680836	120.7	48.0	3.7	4.3

Table 1 (continued)

Gel match No.	% IOD	% IOD ratio	Protein name	Function	Database acc. no.	M_r	Theo-retical M_r	pI	Theo-retical pI
I 585	0.061	2.82	Calreticulin	Chaperone, transcription factor	gi 6680836	39.9	48.0	6.0	4.3
I 138[b]	0.881	1.79	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gi 121574	78.4	72.3	4.8	5.1
I 660[a,b]	0.208	0.18	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gi 121574	70.2	72.3	4.8	5.1
I 6347[b]	0.173	0.39	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gi 121574	65.1	72.3	4.9	5.1
I 1136[a,b]	0.405	0.45	Probable protein disulfide isomerase P5 precursor	Chaperone, signal transduction	gi 2501206	52.9	47.2	4.8	5.0
I 8580[b]	0.034	0.38	Protein disulfide isomerase	Chaperone, signal transduction	gi 91897	60.2	56.6	6.0	5.9
I 68[b]	0.084	0.23	Endoplasmic reticulum protein ERP 29 precursor	Chaperone, signal transduction	gi 2507015	25.9	28.6	6.3	6.2
I 660[a]	0.208	0.18	Endoplasmin precursor (HSP90 family)	Chaperone, signal transduction	gi 119362	70.2	92.5	4.8	4.7
Signal transduction, regulation, differentiation and apoptosis									
N 381[b]	0.495	3.93	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gi 204728	36.8	27.1	7.9	8.2
N 377[b]	3.926	2.11	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gi 204728	36.9	27.1	8.3	8.2
N 398[b]	0.007	28.15	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gi 204728	31.4	27.1	8.8	8.2
N 370[a]	2.055	0.24	Voltage-dependent anion channel	Cellular transport, apoptosis	gi 4105605	36.5	32.4	8.4	8.3
I 62[b]	0.060	3.24	TOAD 64 (Dihydropyrimidinase related protein-2)	Differentiation, signal transduction	gi 1351260	69.5	62.3	6.3	6.0
I 1153	0.094	1.42	Eukaryotic initiation factor 4A-like NUK	Signal transduction	gi 729821	44.8	46.8	6.3	6.1
I 217[c]	0.005	23.63	Craniofacial development protein 1	Eukaryotic organogenesis	gi 5453567	19.9	33.6	5.2	4.8
I 293	0.686	0.24	Secretagogin	Proliferation, differentiation, signal transduction	gi 3757661	25.8	32.1	5.5	5.3
Cellular defence									
N 180	0.096	3.03	Catalase	Cellular defence	gi 115707	61.9	59.6	7.5	7.2
I 2420	0.026	0.13	Glutathione synthetase	Cellular defence	gi 1170038	51.9	52.3	5.3	5.5
Other functions									
I 1096	0.169	0.17	Neuroendocrine convertase 1	Hormone processing	gi 128001	73.8	84.1	4.7	5.8
I 6363[b]	0.229	0.12	Neuroendocrine convertase 2	Hormone processing	gi 128004	72.1	70.8	5.2	5.9
N 279	0.271	0.33	Beta-alanine oxoglutarate aminotransferase	GABA catabolism	gi 3046865	49.8	56.5	8.0	8.5
I 1202[c]	0.035	0.03	25-Dx	Progesterone receptor	gi 1518818	24.3	21.5	4.2	4.5
N 432	0.086	0.17	Amyloid beta-peptide binding protein	Hormone metabolism, apoptosis	gi 2961553	30.9	27.1	8.5	8.9
I 299	0.129	0.24	5-aminolevulinate synthase precursor	Haem synthesis	gi 599830	26.8	71.1	4.8	9.0
I 955[a]	0.104	2.41	Cytokeratine 8 polypeptide	Cellular structure	gi 203734	49.3	53.9	5.4	5.8
I 2410	0.331	0.46	Tubulin beta-5 chain	Cellular structure	gi 135471	58.1	49.7	4.6	4.8
I 6379	0.383	0.44	Tubulin beta-5 chain	Cellular structure	gi 135471	65.7	49.7	4.5	4.8

Table 1 (continued)

Gel match No.	% IOD	% IOD ratio	Protein name	Function	Database acc. no.	M_r	Theo-retical M_r	pI	Theo-retical pI
I 590	0.485	0.41	Tubulin beta-5 chain	Cellular structure	gi 135471	64.9	49.7	4.5	4.8
I 1139	0.561	0.37	Tubulin beta-5 chain	Cellular structure	gi 135471	59.5	49.7	4.6	4.8
N 1512[c]	0.038	0.14	Immediate-early protein 1 rat cytomegalovirus	Virus protein, transcription factor	gi 543613	51.6	66.7	8.4	4.7

Match numbers are arbitrary numbers given by the computer and correspond to the number of the spot in the gel. % IOD refers to the integrated optic density on the control gels. % IOD ratio refers to the ratio of integrated optical density between gels compared. Numbers below 1 indicate proteins down-regulated and numbers above 1 are up-regulated in islets exposed to IL-1β. Spots on the NEPHGE side have prefix N and prefix I on the IEF side. Protein name refers to the name found in the NCBI database (http://www.ncbi.nlm.nih.gov) through the database accession number

[a] Indicates spots whereby more than one protein was identified. Proteins mentioned more than once are found in more than one spot

[b] Proteins previously identified as changed in expression in WF rat islets exposed to IL-1β [10]

[c] Proteins that have not been described previously or supposed to be present in islets

Table 2. Unidentified proteins

Match No.	% IOD ratio	Mass spectrometry results	M_r	pI
N 1282	28.67	No spectrum	31.8	8.8
N 281	9.29	No spectrum	46.1	8.0
N 435	6.03	Weak spectrum no id	29.0	8.3
N 284	5.38	No spectrum	46.9	7.7
N 268	3.13	Good spectrum no id	47.7	8.6
N 509	1.50	Weak spectrum no id	23.9	7.9
N 207	0.38	No spectrum	57.4	8.8
N 212	0.33	Weak spectrum no id	57.0	8.5
N 403	0.31	Weak spectrum no id	31.8	8.5
N 1355	0.25	Good spectrum no id	44.1	8.0
N 68	0.12	Weak spectrum no id	89.1	6.4
I 8264	21.03	Good spectrum no id	13.7	6.6
I 683	3.36	Weak spectrum no id	72.9	6.3
I 961	2.58	Good spectrum no id	52.0	5.1
I 266	1.77	No spectrum	24.8	4.8
I 408	0.70	Weak spectrum no id	32.5	5.9
I 327	0.43	Weak spectrum no id	24.4	6.5
I 75	0.43	No spectrum	62.2	6.5
I 6585	0.42	No spectrum	30.2	6.0
I 8330	0.27	No spectrum	25.5	4.4
I 712	0.26	No spectrum	90.1	5.4
I 275	0.26	No spectrum	22.7	6.0
I 1196	0.22	No spectrum	24.4	6.1
I 270	0.22	Good spectrum no id	22.5	5.4
I 838	0.20	No spectrum	65.1	4.9
I 418	0.18	No spectrum	33.2	6.3
I 545	0.11	No spectrum	54.8	4.5
I 242	0.08	Good spectrum no id	20.6	5.0
I 8311	0.08	Weak spectrum no id	21.3	4.5
I 292	0.08	Weak spectrum no id	24.8	4.5
I 7495	0.04	No spectrum	30.2	5.6

Unidentified protein spots with statistically significant changes in expression level expressed as % IOD-ratio in neonatal BB-DP islets after IL-1β incubation in vitro compared to BB-DP islets without IL-1β. The M_r and pI obtained directly from the gels are given

pre-pro forms of proteins since the theoretical values are based on the open reading fame only. For example calreticulin is present in two spots with a M_r of respectively 39.9 and 120.7 whereas the theoretical value is 48. Nevertheless for most proteins there are only minor differences between observed M_r and pI values and theoretical values (Table 1).

In total, 45 different proteins and 12 modified forms of some of these proteins have been identified to change the expression after exposure to IL-1β. Based on known or putative functions the 45 identified proteins (with modified forms 57) have been grouped as follows (in brackets, number of different proteins): proteins involved in (i) energy transduction and redox potentials (*n*=8), (ii) glycolytic and Krebs cycle enzymes (*n*=6), (iii) protein synthesis (including purin and pyrimidine synthesis, DNA and RNA synthesis, RNA processing and amino acid metabolism), chaperoning and protein folding (*n*=14), (iv) signal transduction, regulation, differentiation and apoptosis (*n*=6), (v) cellular defence (*n*=2) and (vi) other functions (*n*=9) (Table 1).

Discussion

Proteome analysis comprising 2-DGE, mass spectrometry and bio-informatics offers the opportunity to identify different pathways involved in IL-1β induced beta-cell destruction. Mass spectrometry is at present the most powerful method for identifying proteins from 2-DG's [33].

For reasons outlined in the section "2-DGE and preparative gels", preparative gels from WF islets were used for mass spectrometry protein identification. This was mainly for financial reasons, but was feasible since all protein spots changing their expres-

sion in IL-1β exposed BB-DP islets were also identifiable in the WF 2D-gels, and no protein spots were induced to expression de novo by IL-1β in the BB-DP islets. We have shown previously that RIN-cells express essentially all proteins found to change the expression in response to IL-1β in BB-DP rat islets [34].

Fewer protein spots changed expression upon IL-1β exposure in the BB-DP islets (82 spots) compared to WF islets (105 spots). A total of 32 proteins were "specific" for BB-DP islet responses to IL-1β in vitro and 44 proteins "specific" for WF rat islets, although several are from similar pathways and have similar functions in the two strains e.g. NADH dehydrogenase (BB-DP) and NADH-cytochrome B5 reductase (WF).

Several proteins were seen in more than one spot suggesting that these proteins undergo posttranslational modifications. Posttranslational modifications, e.g. phosphorylations, methylations or glycosylations are important for the function or activation of the protein [35].

The cytotoxic effect of cytokines on islets is inhibited by inhibitors of protein synthesis [36], suggesting that beta-cell destruction is an active intracellular process requiring synthesis of proteins. Several proteins have been reported to change expression after IL-1β exposure [37] e.g. iNOS, heat shock protein (HSP) 70, 90 and MnSOD representing a protective response [38, 39]. On this basis we hypothesized that cytokines induce a race between protective and deleterious events, and that in beta cells the deleterious events prevail.

We have shown previously that IL-1β induces PIPP in islets [8, 9, 10]. PIPPs comprises up-regulation and down-regulation of protein expression and/or gel-position-shifts resulting from post-translatory modifications. Consequently, when we describe the expression of a protein as up-regulated or down-regulated, this can reflect a change of expression in the protein itself or a modified form of that protein.

The findings should be interpreted with some caution because they reflect the specific experimental conditions e.g. concentration of IL-1β, exposure time, labelling interval, and the general culture conditions. The study does not distinguish primary and secondary changes in time or in importance. To evaluate the effects of each of these factors a large series of experiments would be necessary.

Not surprisingly, results from a proteome study differ to a large extent from the findings of mRNA expression profile changes in comparable experiments [40, 41]. Generally, correlations between protein and the corresponding mRNA expression are poor [42]. The possibility to characterize the protein expression profile in a cell or tissue under specific conditions and at a given time point, reflecting the metabolic and functional status of these specific conditions and the exact time point, is the hallmark of proteome analysis [43].

Energy transduction, redox potentials, glycolytic and Krebs cycle enzymes (fourteen different proteins). IL-1β induces nitric oxide production in the beta cells which in turn nitrosylates the Fe-S complex in certain enzymes [44]. This reduces the activity of e.g. mitochondrial aconitase [45] and leads to the inhibition of the oxidation of glucose in the Krebs cycle. This is in agreement with the finding that glyceralaldehyde-3-phosphate dehydrogenase, pyruvate kinase M2 isozyme, aconitate hydratase, isocitrate dehydrogenase and malate dehydrogenase precursor (three spots) are all involved in glycolysis or Krebs cycle and are down-regulated.

Seven proteins involved in energy transduction and redox potentials are down-regulated in response to IL-1β exposure. Taken together this suggest that energy production is reduced by IL-1β in BB-DP rat islets, as was the case in WF islets [10].

Protein synthesis (including purin and pyrimidine synthesis, DNA and RNA synthesis, RNA processing, amino acid metabolism), chaperones and protein folding (fourteen proteins). Three identified proteins, 5-aminoimidazole-4-carboxamide ribonucleotide formyltranferase, UMP-CMP kinase and adenine phosphoribosyltransferase, are involved in the synthesis of purine, pyrimidine, nucleosides and nucleotides and important for RNA and DNA synthesis [46, 47] and two proteins, heterogeneous nuclear ribonucleoprotein A2/B1 and heterogeneous nuclear ribonucleoprotein M (M4 protein deletion mutant), in processing of pre-mRNA [48, 49] were all down-regulated. Down-regulation of gene transcription and protein synthesis by IL-1β has been shown in several studies [8, 38, 50, 51, 52].

Endoplasmin precursor, found with a 78 kDa glucose regulated protein (GRP78) in a down-regulated spot, belongs to the HSP 90 family [53] and has serine kinase activity that is enhanced by association with GRP78 [54]. GRP78, a member of the HSP 70 family (three spots; one up-regulated, one down-regulated and one together with endoplasmin in a down-regulated spot) is involved in the folding and assembly of proteins in the endoplasmatic reticulum [55]. Endoplasmic reticulum protein ERP 29 is involved in protein secretory events. IL-1β exposure down-regulated this protein. Protein disulfide isomerase, which is necessary for molecular chaperoning of glycoprotein biosynthesis [56] and probable disulfide isomerase P5 catalysing formation and re-arrangement of disulfidebonds in proteins, were both down-regulated. All of these proteins are able to bind the important cellular second messenger and regulator, calcium, like calreticulin, found in two up-regulated spots [57, 58, 59].

Increased HSP70 expression has been shown to diminish the inhibitory effect of IL-1β on islet insulin secretion and nitric oxide-induced mitochondrial impairment [16, 60, 61].

Signal transduction, regulation, differentiation and apoptosis (six proteins). IL-1β induces cell death through different pathways, e.g. apoptosis [62, 63, 64]. Galectin-3, which inhibits apoptosis [65, 66, 67], was up-regulated in three spots. Expression of eukaryotic initiation factor 4A-like NUK-34, involved in transcriptional and translational regulation [68], was increased suggesting activation of protein synthesis. Toad 64, present during neurogenesis and important for axon growing [69], was up-regulated after IL-1β exposure. The calcium binding protein secretagogin, specific for neuroendocrine cells that has extensive homology to the anti-apoptotic calbindin D-28 k [70] involved in cell growth and maturation [71], was down-regulated. Calreticulin, another calcium binding protein, found to be up-regulated in two spots, functions as a modulator of gene transcription via nuclear hormone receptors (glucocorticoid and androgen receptors) [72, 73].

Craniofacial development protein 1, not previously described in islets of Langerhans, could have multiple functions during embryonic development, e.g. in the developing neuroepithelium [74]. Expression of craniofacial development protein 1 was increased, suggesting a function of this protein in the plethora of proteins induced in islets by IL-1β.

Voltage-dependent anion channel protein was identified in a down-regulated spot together with L-3-hydroxyacyl-CoA dehydrogenase precursor. The voltage dependent anion channel protein forms pores in the outer mitochondrial membrane serving as the permeability pathway for metabolite flux between the cytoplasm and mitochondria [75]. Bcl2 proteins bind to the channel and regulates mitochondrial membrane potential, permeability and release of cytochrome c during apoptosis [76, 77].

Cellular defence (two proteins). Free radicals (e.g. nitric oxide and oxygen-derived radicals) possibly play a role in the destruction of beta cells [3]. Catalase, which protects cells from the toxic effects by hydrolysing hydrogen peroxide, was up-regulated in response to IL-1β exposure, suggesting the presence of hydrogen peroxide in BB-DP rat islets. The presence of the toxic peroxynitrite is a signal to higher production of catalase and glutathione peroxidase to convert peroxinitrite to oxygen and water. Overexpression of catalase, superoxide dismutase and glutathione peroxidase in rat insulinoma (RIN) cells protects against the toxicity of nitric oxide [78] as well as against cytokines [11]. The observed increased expression of catalase (threefold) in the BB-DP islets is apparently insufficient to protect against the IL-1β induced free radicals. Described strain differences in rat islet sensitivity to IL-1β and nitric oxide might be due to different capacities to mount a free radical defence [14]. Hereditary catalase deficiencies and polymorphisms in the *MnSOD* gene are reported to increase the risk for Type I diabetes [79, 80, 81].

Expression of glutathione synthetase, important for protecting cells from oxidative damage [78, 82, 83], was reduced eightfold, further suggesting that IL-1β reduces cellular defence against oxygen-derived free radicals in BB-DP rat islets.

These two defence proteins, catalase and glutathione synthetase, changed expression due to IL-1β exposure in BB-DP rats but not in WF rat islets. This indicates that the two strains respond differently in either production of free radicals or in the defence from free radicals induced by IL-1β exposure.

Other functions: hormone and neurotransmitter metabolism (five proteins). Insulin and other hormones are formed through a series of precursors that are processed by different enzymes before they become biologically active. Proinsulin is processed by the calcium dependent serine proteases neuroendocrine convertase 1 and 2. Inherited defects in prohormone processing enzymes have been found associated with metabolic syndromes and disorders [84, 85]. In IL-1β exposed islets both neuroendocrine convertase 1 and 2 are down-regulated more than fivefold explaining decreased insulin release after IL-1β exposure of islets in vitro [86]. In human islets exposed to cytokines expression of neuroendocrine convertase 1 and 2 is reduced [87].

The 25-Dx, a receptor for progesterone, was down-regulated after IL-1β exposure. 25-Dx has 71% homology with the transmembrane domain of the precursor for the IL-6 receptor [88]. Amyloid beta-peptide binding protein, involved in androgen metabolism and suggested to be involved in apoptosis and amyloid toxicity [89], was down-regulated by IL-1β.

Beta-alanine oxoglutarate aminotransferase (GABA-transaminase), responsible for the catabolism of the major inhibitory neurotransmitter gamma-aminobutyric acid (GABA), was down-regulated in IL-1β exposed BB-DP islets. GABA is synthesized by glutamic acid decarboxylase (GAD) and could be involved in islet-cell development. GAD has been associated with Type I diabetes in humans [90, 91] and in non-obese diabetic (NOD) mice [92, 93, 94]. GAD expression in islets is inhibited by IL-1β exposure [95].

Miscellaneous functions (two proteins). The 5-aminolevulinate synthase precusor is a nuclear-encoded mitochondrial enzyme in the haem biosynthetic pathway. IL-1β down-regulated its expression and might thereby increase the beta-cell susceptibility to IL-1β due to lower substrates for catalase production, since catalase is a haem containing protein.

Cytomegalovirus protein immediate-early protein 1, able to transactivate heterologous promoters [96] in rat cytomegalovirus, was down-regulated in the BB-DP islets.

The complexity of the effects of IL-1β on islets, described here by PIPP, substantiates the hypothesis, that development of Type I diabetes is the result of a collective, dynamic instability, rather than the result of a single factor [97].

Taken together, proteome analysis of IL-1β exposed neonatal BB-DP islets showed that IL-1β induced statistically significant changes in the expression of many proteins. Several proteins were identical and others were from similar pathways in both strains, suggesting that several but similar routes can lead to impaired beta-cell function and destruction in BB-DP and WF rats. Several proteins, previously not described in islets of Langerhans, were identified (Table 1).

In conclusion, we have shown that the combination of 2-DGE and mass spectrometry is a powerful method for identifying proteins involved in the cytotoxic effects after IL-1β exposure in rat islets. Whether similar changes in protein expression are seen in vivo in prediabetic BB-DP rats is currently under investigation.

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