Insulin signalling in hepatocytes of humans with type 2 diabetes: excessive production and activity of protein kinase C- ι (PKC- ι) and dependent processes and reversal by PKC- ι inhibitors

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Abstract

Aims/hypothesis We examined the role of protein kinase C- ι (PKC- ι) in mediating alterations in the abundance of enzymes in hepatocytes of type 2 diabetic humans that contribute importantly to the development of lipid and carbohydrate abnormalities in type 2 diabetes.

Methods We examined (1) insulin signalling in isolated hepatocytes of non-diabetic and type 2 diabetic humans and (2) the effects of two newly developed small molecule PKC- ι inhibitors on aberrant signalling and downstream processes.

Results In contrast with PKC- ι deficiency in diabetic muscle, which diminishes glucose transport, PKC- ι in diabetic hepatocytes was overproduced and overactive, basally and after insulin treatment, and, moreover, was accompanied by increased abundance of PKC- ι -dependent lipogenic, proinflammatory and gluconeogenic enzymes. Heightened PKC- ι activity most likely reflected heightened activity of IRS-2-dependent phosphatidylinositol 3-kinase (PI3K), as IRS-1

levels and IRS-1/PI3K activity were markedly diminished. Importantly, insulin-stimulated PKC- ι abundance and its overabundance in diabetic hepatocytes was reversed in vitro by both insulin deprivation and PKC- ι inhibitors; this suggested operation of an insulin-driven, feed-forward/positive-feedback mechanism. In contrast with PKC- ι , protein kinase B (Akt2) activity and activation by insulin was diminished, apparently reflecting IRS-1 deficiency. Treatment of diabetic hepatocytes with PKC- ι / λ inhibitors diminished abundance of lipogenic, proinflammatory and gluconeogenic enzymes.

Conclusions/interpretation Our findings suggest that a vicious cycle of PKC- ι overactivity and overproduction exists in hepatocytes of humans with type 2 diabetes and contributes importantly to maintaining overactivity of lipogenic, proinflammatory and gluconeogenic pathways, which underlies the lipid and carbohydrate abnormalities in type 2 diabetes.

Keywords Hepatocytes · Protein kinase C- ι · Type 2 diabetes

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Abbreviations

ACC	Acetyl-CoA carboxylase
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
aPKC	Atypical protein kinase C
ATM	Aurothiomalate
FAS	Fatty acid synthase
FOXO1	Forkhead box O1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6Pase	Glucose-6-phosphatase
ICAPP	1H-imidazole-4-carboxamide, 5-amino-1-{2,3-dihydroxy-4-[(phosphono-oxy)methyl]cyclopentyl-[1R-(1a,2b,3b,4a)]}
NF κ B	Nuclear factor κ B

PDK1	Phosphoinositide-dependent kinase-1
PDK2	Phosphoinositide-dependent kinase-2
PI3K	Phosphatidylinositol 3-kinase
PIP ₃	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMSF	Phenylmethanesulfonyl fluoride
SREBP-1c	Sterol receptor element binding protein-1c

Introduction

Alterations in hepatic metabolism in type 2 diabetes mellitus lead to overproduction of glucose and lipids, which in turn abet development of glucose intolerance and dyslipidaemias. Hepatic glucose overproduction is understandable, as insulin resistance in type 2 diabetes is expected to diminish the ability of insulin to repress abundance of gluconeogenic enzymes. On the other hand, lipid overproduction in type 2 diabetes is not readily explained by insulin resistance, as insulin increases the abundance of lipogenic enzymes and resistance to this effect of insulin should diminish lipogenesis. Elucidation of the signalling aberrations underlying these paradoxical alterations in gluconeogenesis and lipogenesis is essential for developing new therapeutic approaches for type 2 diabetes.

Insulin controls metabolic processes largely by activating protein kinase B (Akt) and atypical protein kinase C (aPKC), which function distally to IRS-1- and/or IRS-2-dependent phosphatidylinositol 3-kinase (PI3K). Germane to the above-stated paradox, in rodent forms of obesity and diabetes, hepatic aPKC activation by insulin is fully conserved or heightened, even when hepatic Akt activation is markedly diminished [1–3]. Divergence of the Akt and aPKC pathways in diabetic liver [1, 3] most likely reflects downregulation of IRS-1/PI3K, which is of major importance for hepatic Akt activation [4–7], as opposed to conserved activation of IRS-2/PI3K, which, without IRS-1, controls hepatic aPKC [4, 6, 7]. In contrast with liver, in muscle, IRS-1/PI3K controls aPKC as well as Akt [5, 6], which together control glucose transport, and all three signalling factors are generally downregulated in obesity and diabetes [8].

Conserved hepatic aPKC activation in obesity and type 2 diabetes is problematic, as hyperinsulinaemia inordinately activates hepatic aPKC and aPKC-dependent processes by: (1) abundance/activation of sterol receptor element binding protein-1c (SREBP-1c), which transactivates multiple lipogenic genes, including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [2, 3, 9, 10]; and (2) activation of inhibitor of κ -B kinase- β (IKK β), which phosphorylates I κ B β , the inhibitor of nuclear factor κ B (NF κ B), thus releasing

NF κ B for nuclear uptake and transactivation of proinflammatory cytokine genes, including TNF- α and IL-1 β [2, 3, 10].

Activation of hepatic aPKC, SREBP-1c and NF κ B in hyperinsulinaemic states apparently contributes importantly to the development of hepatosteatosis, hypertriglyceridaemia, hypercholesterolaemia, impaired insulin signalling in muscle and systemic insulin resistance. Indeed, tissue-selective inhibition of hepatic aPKC by adenoviral-mediated production of kinase-inactive aPKC or knock-down of IRS-2 diminishes aPKC activity and activation of SREBP-1c-dependent lipogenic and NF κ B-dependent proinflammatory pathways and, moreover, reverses or improves clinical abnormalities in several rodent obesity and type 2 diabetes models [2, 3]. In addition, adenoviral-mediated inhibition of hepatic aPKC diminishes production of gluconeogenic enzymes, PEPCK and glucose-6-phosphatase (G6Pase) [2].

At present, we have only limited knowledge about insulin signalling mechanisms in human hepatocytes, particularly in hepatocytes obtained from diabetic patients, and particularly in which insulin effects on both aPKC and Akt have been examined. Here, we examined insulin signalling to aPKC, Akt, IRS-1 and IRS-2, and alterations in the abundance of downstream factors that regulate lipogenic, proinflammatory and gluconeogenic pathways in hepatocytes isolated from non-diabetic and type 2 diabetic humans. We tested the role of PKC- ι in provoking aberrations in these pathways in hepatocytes of type 2 diabetic humans by examining the effects of two newly developed, highly specific small molecule inhibitors of PKC- ι . We found that both inhibitors diminished these aberrations in hepatocytes of type 2 diabetic humans.

Methods

Human hepatocytes Cryo-preserved hepatocytes (70–90% viability; purchased from Zen-Bio Corp, Research Triangle, NC, USA) were prepared from collagenase-perfused livers of non-diabetic (two female and six male; age 43–60 years, 51 $\pm$ 3 years [mean $\pm$ SEM]; BMI 30 $\pm$ 2 kg/m²) and type 2 diabetic (two female and four male; age 46–68 years, 60 $\pm$ 4 years; BMI 27 $\pm$ 2 kg/m²) patients who were being maintained on life support as transplant donors after irreversible brain damage caused by traumatic injuries, anoxia or cerebral vascular accidents. The diagnosis of type 2 diabetes in these patients was made by the attending physicians, apparently (as per supplier's information) historically, on the basis of adult onset, lack of history of ketoacidosis and, in some cases, a history of maintenance on oral agents. All diabetic patients were undergoing active insulin treatment for hyperglycaemia until their demise, but other clinical and laboratory data are limited in transplant donors.

Hepatocyte incubations As per supplier's instructions, hepatocytes were incubated (10^6 cells/100 mm plate) overnight (16–20 h) in DMEM containing 5% fetal calf serum, 100 U/ml sodium penicillin, 100 µg/ml streptomycin sulphate, 1 µmol/l insulin and 2 µmol/l dexamethasone, then for 2 h in William's E medium (Sigma, St Louis, MO, USA) containing GlutaMAX (Invitrogen, Carlsbad, CA, USA), 100 U/ml sodium penicillin, 100 µg/ml streptomycin sulphate, 10 µmol/l insulin, 100 nmol/l dexamethasone, 25 mg/ml transferrin and 0.25 µg/ml sodium selenite, then for 3 h in similar but insulin-free medium, and finally for 30 min with or without aPKC inhibitor before incubation for either 15 min with or without 100 nmol/l insulin in acute signalling studies, or for 4–6 h with or without 1 µmol/l insulin in abundance studies. These concentrations of insulin in the final incubations were used to ensure that (1) insulin actions were maximal in both acute signalling and longer abundance studies, and (2) insulin-dependent diabetic abnormalities were maintained throughout all incubations. In this regard, note that diabetic abnormalities involving heightened activity of aPKC and aPKC-dependent processes (i.e. heightened abundance of lipogenic, proinflammatory and gluconeogenic enzymes) were comparable in hepatocytes analysed directly without incubation and in hepatocytes in which the insulin concentration in the final 6 h incubation was either 1 µmol/l, as presently used, or 100 nmol/l, as used in a subsequent unpublished study (M. Sajan and R. Farese). Also note that effects of PKC- ι inhibition on diabetic abnormalities were comparable regardless of whether 100 nmol/l or 1 µmol/l insulin was present in the 6 h incubation.

In some cases, hepatocytes were analysed directly (i.e. without overnight preincubation) or incubated overnight in insulin-free medium. As shown below, the presence of insulin in the overnight preincubation medium was essential for maintaining the type 2 diabetic cellular phenotype. After incubation, cells were sonicated in homogenising buffer.

Human muscle samples Vastus lateralis muscle biopsy samples were obtained from non-diabetic and type 2 diabetic humans as described [11]. All experimental procedures involving humans, including muscle biopsy studies, were approved by the institutional review board of the University of South Florida College of Medicine and the James A. Haley Veterans Administration Medical Center Research and Development Committee, Tampa, FL, USA, and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

PKC- ι inhibitors We used two PKC- ι inhibitors: aurothiomalate (ATM; Myochrisine, Taylor Pharmaceuticals, Decatur, IL, USA; see references [12–15]); and 1H-imidazole-4-carboxamide, 5-amino-1- $\{2,3$ -dihydroxy-4-[(phosphono-oxy)methyl]cyclopentyl-1R-(1a,2b,3b,4a)] $\}$ (ICAPP; Southern Research, Birmingham, AL, USA; see reference [16]).

Tissue and immunoprecipitate preparations As described previously [1–3, 10, 11], hepatocytes and tissues were homogenised in ice-cold buffer containing 0.25 mol/l sucrose, 20 mmol/l TRIS/HCl (pH 7.5), 2 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l phenylmethanesulfonyl fluoride (PMSF), 20 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/l Na_3VO_4 , 2 mmol/l NaF and 1 µmol/l microcystin, and then supplemented with 1% Triton X-100, 0.6% Nonidet and 150 mmol/l NaCl, and cleared by low-speed centrifugation.

PKC, Akt2, IRS/PI3K and AMP-activated protein kinase (AMPK) assays As described previously [1–3, 10, 11], aPKCs were immunoprecipitated from lysates with rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), which recognises C-termini of PKC- ζ and PKC- λ/ι (PKC- ι is the human homologue of mouse PKC- λ [98% homology]; note that, whereas human muscle contains primarily PKC- ι and small amounts of PKC- ζ , human liver contains substantial amounts of both PKC- ζ and PKC- λ/ι [M. Sajan and R. Farese, unpublished results]). Immunoprecipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies) and incubated for 8 min at 30°C in 100 µl buffer containing 50 mmol/l TRIS/HCl (pH 7.5), 100 µmol/l Na_3VO_4 , 100 µmol/l $\text{Na}_4\text{P}_2\text{O}_7$, 1 mmol/l NaF, 100 µmol/l PMSF, 4 µg phosphatidylserine (Sigma), 50 µmol/l [γ - ^{32}P]ATP (NEN Life Science Products, Beverly, MA, USA), 5 mmol/l MgCl_2 and, as substrate, 40 µmol/l serine analogue of the PKC- ϵ pseudosubstrate (Millipore, Bedford, MA, USA). After incubation, ^{32}P -labelled substrate was trapped on P-81 filter paper and counted.

Table 1 Activity and levels of aPKCs and aPKC-dependent production of lipogenic, proinflammatory and gluconeogenic enzymes in uncultured hepatocytes of non-diabetic and type 2 diabetic humans

Variable	Non-diabetic	T2DM	<i>p</i> value
aPKC activity	1±0.14	2.53±0.23	<0.001
PKC- ι mRNA	1±0.23	3.54±0.72	<0.01
PKC- ι protein	1±0.36	2.41±0.67	<0.05
<i>SREBP-1c</i> mRNA	1±0.17	3.08±0.70	<0.05
<i>FAS</i> mRNA	1±0.26	3.85±1.15	<0.05
<i>ACC</i> mRNA	1±0.26	2.77±0.61	<0.05
<i>TNF-α</i> mRNA	1±0.26	2.55±0.59	<0.05
<i>PEPCK</i> mRNA	1±0.23	2.87±0.61	<0.05
<i>G6Pase</i> mRNA	1±0.22	3.32±0.89	<0.05
PKC- ζ mRNA	1±0.20	0.80±0.19	NS

Hepatocytes from five non-diabetic and five type 2 diabetic (T2DM) patients were analysed directly, i.e. without overnight culturing, and results for each patient were expressed relative to the mean value of all non-diabetic patients, set at unity. Values are mean±SEM from four or five determinations. *p* values were determined by ANOVA

Table 2 Effects of overnight culturing in the presence or absence of insulin on activity, levels and production of aPKC and aPKC-dependent abundance of lipogenic, proinflammatory and gluconeogenic enzymes in hepatocytes of type 2 diabetic and non-diabetic humans

Variable	Cultured without insulin (T2DM/non-diabetic)	Cultured with insulin (T2DM/non-diabetic)	<i>p</i> value
aPKC activity	0.98±0.34	1.85±0.24	<0.05
<i>PKC-ι</i> mRNA	1.14±0.23	4.48±0.72	<0.05
PKC- ι protein	0.98±0.19	2.01±0.14	<0.001
<i>SREBP-1c</i> mRNA	0.84±0.20	2.90±0.53	<0.001
<i>FAS</i> mRNA	1.08±0.52	2.76±0.37	<0.05
<i>IL1β</i> mRNA	1.99±0.54	3.78±0.97	NS
<i>TNF-α</i> mRNA	3.02±0.41	8.39±1.87	<0.05
<i>PEPCK</i> mRNA	1.26±0.23	2.37±0.97	NS
<i>G6Pase</i> mRNA	1.91±0.88	3.03±1.24	NS

Hepatocytes from five non-diabetic and five type 2 diabetic (T2DM) patients were cultured overnight in insulin-containing or insulin-free, but otherwise identical, medium (see [Methods](#)), and T2DM values in each medium were compared as the ratio to the mean non-diabetic value in the same medium. Note that aPKC activity and abundance of PKC- ι and aPKC-dependent enzymes in non-diabetic hepatocytes were not significantly different after overnight (20–24 h) culturing in insulin-containing or insulin-free medium, followed by 3 h incubation in insulin-free medium (data not shown). Values are mean±SEM from four or five determinations

aPKC activation was also assessed by immunoblotting for phosphorylation of the auto(trans)phosphorylation site Thr-555/560 in PKC- ι/ζ , required for, and reflective of, activation [8].

For assays of recombinant aPKCs, PKC- ι and PKC- ζ , 10 ng (BioVision, Mountain, CA, USA) aPKC was incubated in the standard aPKC assay with 10 fmol/l phosphatidylinositol 3,4,5-trisphosphate (PIP₃; Matreya, Pleasant Gap, PA, USA) to maximally activate and define aPKC activity.

To examine effects of PKC- ι inhibitors on conventional and novel PKCs, we used baculovirus/Sf9 insect cell-derived highly purified conventional and novel PKCs (gift from L. Ballas, Sphinx Division, Eli Lilly and Co., Indianapolis, IN, USA) as described previously [17]. Conventional PKCs (α , β 2) were assayed in the presence of 1 mmol/l CaCl₂ and 100 nmol/l phorbol myristate acetate (PMA); novel PKCs (δ , ϵ) were assayed with 100 nmol/l PMA, as described previously [17].

AMPK was immunoprecipitated from human hepatocytes and assayed with and without PKC- ι inhibitor as described previously [18].

Immunoprecipitable Akt2, IRS-1/PI3K and IRS-2/PI3K activities were measured (kit for Akt2 and anti-IRS-1 serum from Millipore; anti-IRS-2 serum from Santa Cruz Biotechnologies) as described previously [1–3, 10]. For IRS-1/PI3K and IRS-2/PI3K, immunoprecipitates were incubated with phosphatidylinositol and [γ -³²P]ATP, and the end product PI-3'-³²PO₄ was purified by thin layer chromatography.

Western blot analyses Western blot analyses were conducted as described previously [1–3, 10, 11] using: anti-(IRS-2), anti-(PKC- ζ/λ), anti-(p65/RelA-NF κ B subunit) and anti-(Akt1/2) sera (Millipore); anti-(phosphoSer-473-Akt1/2), anti-(phosphoThr-410-PKC- $\zeta/-403$ -PKC- ι/λ) and anti-(glyceraldehyde-3-phosphate dehydrogenase) (GAPDH) sera (Santa Cruz Biotechnologies); anti-(phosphoThr-560-PKC- $\zeta/-555$ -PKC- ι/λ) serum (Invitrogen); anti-(PKC- ζ) serum (gift from T. Sacktor, Downstate Medical Center, New York, NY, USA); and mouse monoclonal anti-(PKC- ι/λ)

Table 3 Effects of ICAPP on activities of conventional PKCs (α and β 2), novel PKCs (δ and ϵ), atypical PKC- ι and AMPK

ICAPP concentration (nmol/l)	PKC- α	PKC- β 2	PKC- δ	PKC- ϵ	PKC- ι	ICAPP concentration (nmol/l)	AMPK
0	5,522±227	6,509±239	4,962±393	7,129±269	5,461±226	0	2,427±83
1	5,866±707	7,286±102	5,279±265	7,068±280	3,980±45	0.1	2,352±342
10	5,088±128	6,757±92	5,293±173	6,694±285	2,239±64	1	2,803±69
100	4,965±137	6,545±118	5,504±729	6,732±332	1,882±246	10	2,815±15
1000	6,187±1,670	6,422±698	5,634±201	6,156±282	1,762±105	100	2,830±15
10,000	4,737±238	6,225±242	4,443±232	5,837±191	1,300±40	1,000	3,173±5

Conventional PKCs were maximally stimulated with 1 mmol/l CaCl₂ and 100 nmol/l PMA. Novel PKCs were maximally stimulated with 100 nmol/l PMA. PKC- ι was maximally stimulated with 10 fmol/l PIP₃. See [Methods](#) for other details. Kinase activity values (cpm) are mean ±range or SEM of duplicates or quadruplicates, respectively; comparable results were seen in a repeat experiment

antibodies (Transduction Labs, Bedford, MA, USA). Samples from experimental groups were compared on the same blots, and corrected for recovery as needed by measurement of GAPDH immunoreactivity.

mRNA measurements As described previously [2, 3, 10], tissues were added to TRIzol reagent (Invitrogen), and RNA was extracted and purified with RNeasy Mini-Kit and RNase-free DNase set (Qiagen, Valencia, CA, USA), quantified (A_{260}/A_{280}), checked for purity by electrophoresis on 1.2% agarose gels and mRNA quantified by quantitative real-time RT-PCR using TaqMan reverse transcription reagent (Applied Biosystems, Carlsbad, CA, USA). mRNAs were measured with a SYBR Green kit (Applied Biosystems)

Fig. 1 Effects of insulin on basal and insulin-stimulated enzyme activities of total aPKC (a), Akt2 (b), IRS-1-dependent PI3K (c) and IRS-2-dependent PI3K (d) in hepatocytes of non-diabetic and type 2 diabetic humans. Hepatocytes of all patients, five non-diabetic and five with type 2 diabetes mellitus (T2DM), were compared simultaneously during a 15 min incubation with or without 100 nmol/l insulin. Shown in (e) are representative immunoblots of levels and autoradiograms portraying PI3K activity of IRS-1 and IRS-2, auto(trans)phosphorylation of PKC-ι/ζ (Thr-555/560), PDK2-dependent phosphorylation of Akt1/2 (Ser-473) and levels of PKC-ι, PKC-ι/ζ (total aPKC) and Akt1/2. Bar graph values are mean±SEM from five patients. Asterisks (* p <0.05; ** p <0.01; *** p <0.001) reflect p values (ANOVA) of adjacent basal and insulin-stimulated groups. Daggers († p <0.05; †† p <0.01; ††† p <0.001) reflect p values of basal versus basal groups, or insulin-stimulated versus insulin-stimulated groups. Immunoppt, immunoprecipitate

and human nucleotide primers as provided in the electronic supplementary material (ESM).

Statistical evaluations Data are expressed as mean±SEM, and p values were determined by one-way ANOVA and the least-significant multiple comparison method.

Fig. 2 Effects of aPKC inhibitors, ICAPP and ATM, on basal and insulin-stimulated total aPKC activity (a), PKC-ι abundance (b), Akt2 activity (c) and PKC-ζ abundance (d) in hepatocytes of non-diabetic and type 2 diabetic (T2DM) humans. Incubation time, 4 or 6 h with or without 1 μmol/l insulin (values at the two times were comparable and therefore pooled). Bar graph values, normalised to unity, are mean±SEM from findings in five to eight patients. Asterisks reflect significant differences (* p <0.05; ** p <0.01; *** p <0.001; ANOVA) between bracketed groups. e Representative immunoblots reflecting changes in basal, insulin-stimulated and inhibitor-treated levels of phosphoThr-555/560-PKC-ι/ζ, PKC-ι, PKC-ζ and PKC-ι/ζ (total aPKC)

Results

Overabundance of PKC- ι and increased activity of PKC- ι and PKC- ι -dependent functions in hepatocytes of type 2 diabetic humans Before culture, resting aPKC activity, PKC- ι protein and mRNA levels, and expression of hepatic lipogenic, proinflammatory and gluconeogenic hepatic enzymes were increased in hepatocytes of type 2 diabetic, compared with non-diabetic, humans (Table 1). Moreover, this pattern of PKC- ι overabundance and hyperactivity, and activation of lipogenic, proinflammatory and gluconeogenic pathways in hepatocytes of type 2 diabetic humans was comparably maintained when hepatocytes were cultured overnight in insulin-containing preincubation medium, but lost if insulin was omitted from the preincubation medium (Table 2). On the other hand, note that a comparable diabetic cellular phenotype was not induced by comparable addition of insulin to the preincubation medium of non-diabetic hepatocytes. Dependence of the diabetic cellular phenotype on the continued presence of insulin is discussed further below.

aPKC inhibitors To elucidate the role of hepatic aPKC in maintaining the phenotype of hepatocytes of type 2 diabetic humans, we used two highly specific aPKC inhibitors

developed by high-throughput screening methods. The first inhibitor, ICAPP, binds to the substrate-binding site of PKC- ι , but not PKC- ζ [16], and had no effect on activities of PKC- α , PKC- β 2, PKC- δ , PKC- ϵ and AMPK (Table 3). The second inhibitor, ATM, binds to Cys-69 in PKC- ι , thereby inhibiting PB1 domain-dependent binding (present in all aPKCs, $\iota/\lambda/\zeta$, but not other PKCs) to factors that regulate downstream processes [12–16]. Note that ATM does not directly inhibit PKC- ι , but, as seen below, diminishes insulin-induced increases in aPKC enzyme activity in intact tissues. Thus inhibitory effects of ATM on downstream processes are mediated by inhibition of both PB1-dependent scaffolding and enzymatic activation of aPKC.

Insulin signalling in hepatocytes of non-diabetic and type 2 diabetic humans Basal and acute insulin-stimulated activities of aPKC and its major, if not sole, activator during insulin action in liver, IRS-2/PI3K, were increased in hepatocytes of type 2 diabetic, relative to non-diabetic, humans (Figs 1 and 2). In contrast, activities of Akt2 and its primary activator, IRS-1/PI3K, were diminished (Figs 1 and 2). Like enzyme activity, aPKC auto(trans)phosphorylation (p-Thr-555/560-PKC- ι/ζ) was increased basally in diabetic hepatocytes and by insulin in non-diabetic hepatocytes (Figs 1

Fig. 3 Dose-related effects of PKC- ι/λ inhibitors, ICAPP (**a** and **d**) and ATM (**c** and **f**) on basal (white bars) and insulin-stimulated (grey bars) activities of total aPKC and Akt2 in hepatocytes of non-diabetic humans. Incubation time 4 h with or without 1 μ mol/l insulin. Also shown are dose-related effects of ICAPP on: (1) activity of 10 ng

recombinant PKC- ι activated by maximally effective 10 fmol/l PIP₃ (**b**); and (2) Ser-256 phosphorylation of FOXO1 in hepatocytes (**e**). Values are mean $\pm$ SEM from four or more determinations. Immunoppt, immunoprecipitate

and 2). Moreover, auto(trans)phosphorylation was diminished by both aPKC inhibitors (Fig. 2).

Like aPKC activity, protein levels of PKC- ι , but not PKC- ζ , were increased twofold in hepatocytes of type 2 diabetic humans (1 ± 0.04 [mean $\pm$ SEM; $n=4$] non-diabetic versus 2.07 ± 0.05 [$n=4$] diabetic, relative units [$p<0.001$]; blots, Figs 1 and 2). Total aPKC levels in diabetic hepatocytes were increased less than PKC- ι , presumably reflecting the presence of sizeable but unchanged amounts of PKC- ζ (Figs 1 and 2). Unlike PKC- ι , Akt levels were comparable in diabetic and non-diabetic hepatocytes (Fig. 1).

Like IRS-1/PI3K activity, IRS-1 levels were diminished by 61% in diabetic hepatocytes (1 ± 0.01 [$n=5$] non-diabetic versus 0.39 ± 0.10 [$n=5$] diabetic, relative units [$p<0.001$]; blot, Fig. 1). In contrast, IRS-2 levels were comparable in diabetic and non-diabetic hepatocytes (blot, Fig. 1).

Production of aPKCs in hepatocytes of non-diabetic and type 2 diabetic humans As in acute signalling studies, activities of aPKC and Akt2 continued to be excessive and diminished, respectively, in hepatocytes of type 2 diabetic humans during more prolonged 4–6 h expression studies (Fig. 2). Along with increases in activity and protein levels, PKC- ι expression (mRNA) was increased in diabetic hepatocytes (Fig. 2). Moreover, PKC- ι expression in non-diabetic hepatocytes was increased modestly but significantly by 4–6 h insulin treatment, and similar upward trends were seen in diabetic hepatocytes. Importantly, diabetes-induced and insulin-stimulated PKC- ι expression was diminished by PKC- ι inhibitors (Fig. 2), suggesting operation of an insulin-driven, feed-forward/positive-feedback mechanism. That protein levels of PKC- ι did not change with inhibitor treatment probably reflects a slower turnover rate relative to that of mRNA.

Fig. 4 Dose-related effects of PKC- ι/λ inhibitor, ATM, on the abundance of lipogenic (SREBP-1c, FAS, ACC), inflammatory (TNF- α , IL-1 β) and glucogenic (PEPCK, G6Pase) enzymes in hepatocytes of

non-diabetic humans. Incubation time 4 h with or without 1 μ mol/l insulin. Values are mean $\pm$ SEM from four or more determinations. Data are from cells used in Fig. 3

Unlike PKC- ι , mRNA expression and protein levels of PKC- ζ were unaltered by insulin, diabetes and aPKC inhibitors (Fig. 2).

Expression of PKC- ι in muscles of non-diabetic and type 2 diabetic humans Different from hepatocytes, but consonant with reports of diminished total aPKC levels in muscles of type 2 diabetic humans [11, 19], muscle PKC- ι mRNA levels were diminished by 57% in diabetic patients (1 ± 0.14 [$n=5$] non-diabetic versus 0.43 ± 0.07 [$n=5$] diabetic, relative units [$p<0.05$]). Muscle PKC- ι protein levels were similarly diminished by 52% (1 ± 0.12 [$n=12$] non-diabetic versus 0.48 ± 0.08 [$n=12$] diabetic, relative units [$p<0.001$]).

Effects of aPKC inhibitors on aPKC activity in human hepatocytes ICAPP potently (IC_{50} 10–100 nmol/l) inhibited both recombinant PKC- ι activated by PIP₃ in vitro (Table 3)

and insulin-stimulated aPKC activity in human hepatocytes (Figs 2 and 3). Note that residual insulin-dependent hepatic aPKC activity that is resistant to ICAPP probably reflects PKC- ζ , which is not inhibited by ICAPP. Also note that basal immunoprecipitable aPKC activity is partially resistant to ICAPP (Figs 2 and 3), and this probably reflects some non-aPKC kinase activity in the immunoprecipitates.

Like ICAPP, ATM diminished insulin-stimulated aPKC activity in intact hepatocytes (Figs 2 and 3), presumably reflecting a requirement for a PB1-dependent scaffolding complex during aPKC activation.

Also note that neither ICAPP nor ATM inhibited insulin-stimulated Akt activation (Figs 2 and 3); ICAPP did not inhibit increases in phosphoinositide-dependent kinase-1 (PDK1)-dependent phosphorylation of PKC- ι/ζ (Thr-403/410) (not shown); and both ICAPP and ATM diminished aPKC auto-(trans)phosphorylation in intact hepatocytes (Fig. 2).

Fig. 5 Dose-related effects of PKC- ι/λ inhibitor, ICAPP, on the abundance of lipogenic (SREBP-1c, FAS, ACC), inflammatory (TNF- α , IL-1 β) and gluconeogenic (PEPCK, G6Pase) enzymes in hepatocytes of

non-diabetic humans. Incubation time 4 h with or without 1 μ mol/l insulin. Values are mean $\pm$ SEM from four or more determinations. Data are from cells used in Fig. 3

Effects of aPKC inhibitors on aPKC-dependent processes in human hepatocytes Accompanying increases in aPKC activity seen basally in hepatocytes of type 2 diabetic humans and after insulin treatment of hepatocytes of non-diabetic and type 2 diabetic humans, there were increases in the abundance of SREBP-1c and SREBP-1c-dependent enzymes, FAS and ACC, and NF κ B-dependent cytokines, TNF- α and IL-1 β (Figs 4–6). Accompanying decreases in aPKC activity induced by ICAPP and ATM, production of SREBP-1c, FAS, ACC, TNF- α and IL-1 β diminished in hepatocytes of non-diabetic and type 2 diabetic humans (Figs 4–6). Note that unaltered abundance of PKC- ζ (see Fig. 2) served as a negative control in abundance studies.

Insulin maintains blood glucose levels largely by decreasing levels of gluconeogenic enzymes, PEPCK and

G6Pase, thereby diminishing hepatic glucose output. Akt at least partly mediates this inhibitory effect of insulin by phosphorylating Ser-256-forkhead box O1 (FOXO1), thus causing nuclear exclusion and diminished ability of FOXO1 to promote gluconeogenic enzyme abundance in response to A-kinase activators. Interestingly, along with decreased Akt activity in hepatocytes of type 2 diabetic humans, there were upward trends in basal abundance of PEPCK/G6Pase and diminished effects of insulin on this production (Fig. 6). Moreover, consonant with previous findings that adenoviral-mediated inhibition of hepatic aPKC elicits insulin-like decreases in fasting-dependent production of PEPCK/G6Pase in rodent liver [2], ICAPP and ATM had insulin-like effects, or enhanced insulin effects, on FOXO1 phosphorylation (Fig. 3 and other unpublished results [M. Sajjan and R. Farese]) and

Fig. 6 Effects of aPKC inhibitors, ICAPP and ATM, on basal and insulin-stimulated abundance of lipogenic (SREBP-1c, FAS, ACC), inflammatory (TNF- α , IL-1 β) and gluconeogenic (PEPCK, G6Pase) enzymes in hepatocytes of non-diabetic and type 2 diabetic (T2DM) humans. Incubation time 4 or 6 h with or without 1 μ mol/l insulin (values at the two times were comparable and therefore pooled). Bar gram values (normalised to unity) are mean $\pm$ SEM from findings in five to eight patients. Asterisks reflect significant differences (* p <0.05; ** p <0.01; *** p <0.001; ANOVA) between bracketed groups. See Fig. 3 for effects of ICAPP and ATM on activities and/or abundance of aPKC and Akt in these hepatocytes

hand, a comparable cycle was not elicited by simple exposure of non-diabetic hepatocytes to overnight insulin treatment, suggesting that the aberrant PKC- ϵ cycle in type 2 diabetes develops more slowly than it dissipates and/or co-requires other factors, such as IRS-2/PI3K hyperactivity, which, for presently uncertain reasons, exists in diabetic hepatocytes. In any case, dissipation of the aberrant PKC- ϵ activation/production cycle by insulin deprivation demonstrates that it is an acquired, reversible abnormality.

That insulin-stimulated aPKC activation was excessive and Akt2 activation was simultaneously diminished probably reflects differential regulation of upstream activators in hepatocytes of type 2 diabetic humans. Thus conserved levels and excessive activity of IRS-2 would account for heightened aPKC activation/production, and diminished levels and diminished activity of IRS-1 would account for diminished Akt activation. These findings in hepatocytes of type 2 diabetic humans agree with: (1) an IRS-2 requirement for hepatic aPKC activation and an IRS-1 requirement for hepatic Akt activation in mouse knockout studies [4–7]; and (2) conserved or hyperactive IRS-2/PI3K/aPKC signalling versus diminished IRS-1/PI3K/Akt signalling in a rodent diabetic liver [1, 3].

Although decreased IRS-1 levels and activity seem to account for diminished Akt activation in hepatocytes of type 2 diabetic humans, it is also possible that overactivity of PKC- α may have contributed to diminished hepatic Akt activity. In support of this idea, hepatic aPKC inhibition by ICAPP provoked increases in Ser-256 phosphorylation of

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Akt substrate FOXO1. On the other hand, aPKC inhibition provoked increases in FOXO1 phosphorylation even in the absence of concurrent insulin stimulation of Akt, and the underlying mechanism is at present uncertain. In any case, increased FOXO1 phosphorylation may explain insulin-like decreases in PEPCK/G6Pase production elicited by PKC- ι inhibition caused in the present study by ICAPP and previously by hepatic levels of kinase-inactive aPKC [2].

That PKC- ι abundance was increased in hepatocytes but suppressed in muscles of type 2 diabetic humans may seem puzzling. However, as increased PKC- ι abundance in diabetic hepatocytes most likely reflects operation of an overactive, insulin-driven, IRS-2/PI3K-dependent, feed-forward, positive-feedback cycle, the fact that abundance of PKC- ι in muscle is diminished could reasonably reflect diminished activation of IRS-1/PI3K and PKC- ι in diabetic muscle [11, 19].

As partial PKC- ι/λ deficiency in muscle induces obesity, features of the metabolic syndrome and glucose intolerance [10], the combination of diminished muscle PKC- ι and excessive hepatic PKC- ι activity in type 2 diabetic humans is particularly problematic. Thus deficient PKC- ι abundance/activation and glucose transport in muscle would exacerbate glucose intolerance, resultant hyperinsulinaemia would increase hepatic PKC- ι abundance/activation and abundance of lipogenic, proinflammatory and gluconeogenic pathways, and these hepatic abnormalities would further impair PKC- ι activation in muscle. This imbalance between underactive muscle and overactive liver PKC- ι is itself a multi-organ vicious cycle that is superimposed on that of the intrahepatic PKC- ι (see Fig. 7). Fortunately, both vicious cycles are likely to be ameliorated by inhibition of hepatic PKC- ι .

With aPKC, Akt is co-required for insulin effects on the abundance of SREBP-1c and lipogenic enzymes in rodent hepatocytes [20, 21]. Indeed, we found that the Akt chemical inhibitor used by Li et al. [21] markedly (>90%) inhibited SREBP-1c activation by insulin in human hepatocytes, while inhibiting Akt2 enzyme activity by 40% (not shown). This lack of correlation suggests that there are multiple Akt pools with notable differences in responsiveness to inhibitors. By analogy, similar heterogeneity in aPKC pools, as well as different biological effects of PKC- ι and PKC- ζ , and unquantifiable scaffolding effects of ATM may explain the lack of strict parallelism between alterations in hepatic aPKC activity and hepatic enzyme production after ATM and ICAPP treatment.

It should be emphasised that, because of limited availability of donor livers, in particular diabetic donors, we were able to study only a relatively small number of human subjects and further studies in various human populations are needed to see if the present findings have widespread application.

To summarise, in contrast with underproduction and underactivation of PKC- ι in muscle, PKC- ι is overproduced

and overactive in hepatocytes of type 2 diabetic humans, most likely largely due to an insulin-driven, IRS-2- and PKC- ι -dependent, feed-forward/positive-feedback cycle. Moreover, this vicious cycle of PKC- ι overproduction/overactivity in liver is accompanied by overproduction of hepatic lipogenic, proinflammatory and glucogenic factors that are presumed to be key players in the pathogenesis of clinical abnormalities in obesity, the metabolic syndrome and type 2 diabetes. Importantly, two newly developed PKC- ι inhibitors specifically inhibit activity and production of PKC- ι and abundance of PKC- ι -dependent pathogenetic factors in isolated human hepatocytes. Further studies are needed to see if these or other PKC- ι inhibitors are useful for treating obesity and type 2 diabetes.

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Contribution statement RVF conceived, designed and directed the studies, analysed data and wrote the paper. MPS conducted studies and assays, and assembled and assisted in interpretation of data. MPS was involved in drafting the article and both authors approved the final version of the paper.

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