Metformin action in human hepatocytes: coactivation of atypical protein kinase C alters 5'-AMP-activated protein kinase effects on lipogenic and gluconeogenic enzyme expression

Mini P. Sajjan · Robert A. Ivey III · Robert V. Farese

Received: 12 April 2013 / Accepted: 10 July 2013 / Published online: 10 August 2013
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Abstract

Aims/hypothesis Atypical protein kinase C (aPKC) levels and activity are elevated in hepatocytes of individuals with type 2 diabetes and cause excessive increases in the levels of lipogenic and gluconeogenic enzymes; aPKC inhibitors largely correct these aberrations. Metformin improves hepatic gluconeogenesis by activating 5'-AMP-activated protein kinase (AMPK). However, metformin also activates aPKC in certain tissues; in the liver, this activation could amplify diabetic aberrations and offset the positive effects of AMPK. In this study, we examined whether metformin activates aPKC in human hepatocytes and the metabolic consequences of any such activation.

Methods We compared protein kinase activities and alterations in lipogenic and gluconeogenic enzyme levels during activity of the AMPK activators metformin and AICAR, relative to those of an aPKC- ι inhibitor, in hepatocytes from non-diabetic and type 2 diabetic human organ donors.

Results Metformin and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) activated aPKC at concentrations comparable with those required for AMPK activation. Moreover, both agents increased lipogenic enzyme levels by an aPKC-dependent mechanism. Thus, whereas insulin- and diabetes-dependent increases in lipogenic enzyme levels were reversed by aPKC inhibition, such levels were increased in hepatocytes from non-diabetic donors and remained elevated

in hepatocytes from diabetic donors following metformin and AICAR treatment. In addition, whereas aPKC inhibition diminished gluconeogenic enzyme levels in the absence and presence of insulin in hepatocytes from both non-diabetic and diabetic donors, metformin and AICAR increased gluconeogenic enzyme levels in hepatocytes from non-diabetic individuals, but nevertheless diminished gluconeogenic enzyme levels in insulin-treated hepatocytes from diabetic donors.

Conclusions/interpretation Metformin and AICAR activate aPKC together with AMPK in human hepatocytes. Activation of aPKC increases lipogenic enzyme levels and alters gluconeogenic enzyme levels, and therefore appears to offset the positive effects of AMPK.

Keywords AICAR · AMP-activated protein kinase · Hepatocytes · Metformin · Protein kinase C- ι · Type 2 diabetes

Abbreviations

ACC	Acetyl-CoA carboxylase
ACPD	2-Acetyl-1,3-cyclopentanedione
AICAR	5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMPK	5'-AMP-activated protein kinase
aPKC	Atypical protein kinase C
CBP	CREB-binding protein
CREB	cAMP response element-binding protein
ERK	Extracellular signal-regulated kinase
FAS	Fatty acid synthase
ICAP	[1H-imidazole-4-carboxamide,-5-amino]-2,3-dihydroxy-4-hydroxymethyl-cyclopentane-[1R-(1a,2b,3b,4a)]
ICAPP	[1H-imidazole-4-carboxamide-5-amino]-2,3-dihydroxy-4-[(phosphono-oxy)methyl]cyclopentane-[1R-(1a,2b,3b,4a)]

M. P. Sajjan · R. A. Ivey III · R. V. Farese (✉)
Research Service-151, James A. Haley Veterans Medical Center,
13000 Bruce B. Downs Blvd, Tampa, FL 33612, USA
e-mail: rfarese@health.usf.edu

M. P. Sajjan · R. V. Farese
Division of Endocrinology and Metabolism, Department of Internal
Medicine, University of South Florida College of Medicine, Tampa,
FL, USA

PA	Phosphatidic acid
PGC-1 α	Peroxisome proliferator-activated receptor- γ -coactivator-1- α
PI3K	Phosphatidylinositol 3-kinase
PIP ₃	Phosphatidylinositol-3,4,5-(PO ₄) ₃
PKC	Protein kinase C
PLD	Phospholipase D
SREBP-1c	Sterol receptor element binding protein-1c
TORC2	Target of rapamycin-C2

Introduction

Metformin is widely used for treating type 2 diabetes mellitus. Metformin improves hyperglycaemia primarily by diminishing levels of the hepatic gluconeogenic enzymes PEPCCK and glucose-6-phosphatase (G6Pase), thereby reducing hepatic glucose output [1]. Metformin also increases glucose transport in muscle by improving insulin signalling [2] and by direct effects on glucose transport [3].

The actions of metformin in liver and muscle are largely attributed to activation of 5'-AMP-activated protein kinase (AMPK) [3–5]. Although metformin apparently activates AMPK in mouse liver via liver kinase B1 (LKB1) [6], in human hepatocytes, metformin activates AMPK by inhibiting mitochondrial respiratory chain activity and increasing 5'-AMP at the expense of ATP [7].

How AMPK diminishes gluconeogenic enzyme levels is uncertain. It has previously been reported that, in mouse liver, metformin and the AMPK activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) increase Ser-436 phosphorylation of cAMP response element-binding protein (CREB)-binding protein (CBP) and disrupt the formation of a complex between CBP, CREB and target of rapamycin-C2 (TORC2), which is required for the transcription of *Pgc-1 α* , and subsequent *Pepck* and *G6pase* expression [8]. The authors proposed that AMPK increases CBP phosphorylation by activating atypical protein kinase C (aPKC), which directly phosphorylates Ser-436-CBP [8]. Consonant with this idea, AICAR [3, 9] and metformin [3] activate aPKC in rodent muscle independently of phosphatidylinositol 3-kinase (PI3K), but dependent on extracellular signal-regulated kinase (ERK) and phospholipase D (PLD), which generates phosphatidic acid (PA), a direct activator of aPKC- ι , γ and ζ [3, 9].

As in previous reports [3, 10–14], He et al found that insulin activates hepatic aPKC by a PI3K-dependent mechanism, but further noted that this similarly leads to Ser-436-CBP phosphorylation and disruption of the CREB/CBP/TORC2 complex [8]. However, insulin also diminishes PEPCCK and G6Pase levels by PI3K/Akt-dependent phosphorylation of Ser-256-FoxO1, thereby causing nuclear exclusion and inactivation of FoxO1, which is co-required for CREB/

CBP/TORC2/PGC-1 α -induced increases in *Pepck/G6pase* (also known as *G6PC*) expression [15, 16]. The relative contributions of Akt-dependent Ser-256-FoxO in relation to aPKC-dependent phosphorylation of Ser-436-CBP to diminish *Pepck/G6pase* expression during insulin action are currently uncertain.

Militating against the idea that aPKC activation diminishes *Pepck/G6pase* expression during metformin and insulin action is the finding that inhibition of hepatic aPKC by either adenovirally mediated production of kinase-inactive aPKC [13] or small-molecule inhibitors of aPKC [14, 17] leads to decreased levels of *Pepck* and *G6pase*. Moreover, aPKC inhibition, like insulin, increases phosphorylation of Ser-256-FoxO1 [14, 17]. Although the mechanism underlying increases in FoxO1 phosphorylation during aPKC inhibition is uncertain, aPKC binds to and phosphorylates, and thus may inhibit, Akt [18]; in addition, aPKC: (1) increases levels of TRB3, a pseudokinase that inhibits hepatic Akt [19]; and (2) phosphorylates and inhibits IRS-1 [20], which is required for insulin activation of Akt, but not aPKC, in the liver [21, 22].

Another problem that may ensue from hepatic aPKC activation during metformin treatment arises from the fact that aPKC participates in mediating insulin-induced increases in the expression of hepatic lipogenic genes [12–14, 17]. Thus, metformin-induced increases in hepatic aPKC activity may increase the levels of sterol receptor element binding protein-1c (*Srebp-1c*), which transactivates the production of multiple lipogenic enzymes, including fatty acid synthase (*Fas*).

Here, we questioned whether metformin and AICAR activate aPKC in human hepatocytes, and whether increases in hepatic aPKC activity can offset the positive effects that simple AMPK activation would otherwise have on hepatic gene expression. We compared the effects of two AMPK activators, metformin and AICAR, with those of an inhibitor of aPKC on levels of lipogenic and gluconeogenic factors in hepatocytes of non-diabetic and type 2 diabetic humans. In the latter regard, we recently reported that aPKC activity is elevated, protein and mRNA levels of *aPKC- ι* are increased, and levels of gluconeogenic and lipogenic enzymes are increased in hepatocytes of type 2 diabetic humans [14]; moreover, PKC- ι inhibitors largely reverse aberrant increases in levels of lipogenic and gluconeogenic factors in hepatocytes/livers of type 2 diabetic humans [14] and mice [17].

Methods

Kinase activators and inhibitors Metformin and AICAR were purchased from Sigma (St Louis, MO, USA). PKC- ι inhibitor, [1H-imidazole-4-carboxamide,-5-amino]-2,3-dihydroxy-4-hydroxymethyl-cyclopentane-[1R-(1a,2b,3b,4a)] (ICAP) was custom-synthesised by Southern Research (Birmingham, AL, USA) or United Chemical Resources (Birmingham, AL,

USA) (>95% purity). We used ICAP instead of 1H-imidazole-4-carboxamide, 5-amino-1-[2,3-dihydroxy-4-[(phosphonoxy)methyl]cyclopentyl-1R-(1a,2b,3b,4a)] (ICAPP) [see 14, 17] because ICAP synthesis is easier and much less costly and, although ICAP is itself inactive, it can be converted to the active compound, ICAPP, by adenosine kinase (see below). In some cases, we also used a newly developed inhibitor of both PKC- ι and PKC- ζ -acetyl-1,3-cyclopentanedione (ACPD) (Sigma); as will be reported separately, this inhibitor differs from ICAP in that it inhibits both recombinant PKC- ι and PKC- ζ , but, like ICAPP, does not inhibit conventional or novel PKCs, Akt or AMPK.

Hepatocyte incubation Cryopreserved hepatocytes (70–90% viability; purchased from Zen-Bio Corp., Research Triangle, NC, USA) were harvested from the perfused livers of non-diabetic individuals (two women and six men; ages 43–60 years, mean $\pm$ SEM 51 $\pm$ 3 years; BMI 30 $\pm$ 2 kg/m²) and individuals with type 2 diabetes (two women and four men; ages 46–68 years, 60 $\pm$ 4 years; BMI 27 $\pm$ 2 kg/m²) who were maintained on life support as transplant donors (these hepatocytes were obtained from the same patient groups described previously [14]). Diabetic donors were hyperglycaemic and undergoing insulin treatment, but other pertinent laboratory and clinical data are not available in transplant donors.

As previously described [14], unless otherwise indicated, hepatocytes were incubated (10⁶ cells/100 mm plate) overnight (approximately 16 h) in Dulbecco's minimal essential medium containing 5% FCS, 100 units/ml sodium-penicillin and 100 μ g/ml streptomycin-sulfate, 2 μ mol/l dexamethasone; then for 2 h in William's E medium (Sigma) containing GlutaMAX (Invitrogen, Carlsbad, CA, USA), 100 units/ml sodium-penicillin, 100 μ g/ml streptomycin-sulfate and 100 nmol/l dexamethasone; then for 4 h in similar medium supplemented with 25 mg/ml transferrin and 0.25 μ g/ml sodium selenite. Where indicated, 1 μ mol/l insulin and varying concentrations of ICAP, AICAR and metformin were also present in the media throughout all incubations. Note: (1) this concentration of insulin was needed to maintain a high level of insulin activation of aPKC during prolonged incubation; indeed, 100 nmol/l insulin was considerably less effective than 1 μ mol/l insulin in maintaining increases in aPKC and Akt activity in non-diabetic hepatocytes; and (2) the effects of metformin on AMPK activity develop slowly and reach their maximum at 24 h in rat and human hepatocytes [7].

In some studies, where indicated, we used a protocol described previously [14], whereby after overnight incubation in insulin-containing medium as described above, hepatocytes were incubated for 3 h in similar but insulin-free Williams E medium, followed by 6 h $\pm$ 100 nmol/l insulin, $\pm$ 1 or 10 mmol/l metformin and $\pm$ 100 nmol/l ICAP.

After incubation, cells were sonicated in homogenising buffer for protein studies or placed into TRIzol reagent (Invitrogen) for mRNA studies.

All experimental procedures involving human materials 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.

Tissue preparation As previously described [14], hepatocytes 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 phenylsulfonylfluoride (PMSF), 20 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 mmol/l Na₄P₂O₇, 2 mmol/l Na₃VO₄, 2 mmol/l NaF and 1 μ mol/l microcystin, and then supplemented with 1% TritonX-100, 0.6% Nonidet and 150 mmol/l NaCl and cleared by low-speed centrifugation.

aPKC, Akt and AMPK assays As previously described [11–14, 17], 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 with 98% homology; human and mouse muscle contain primarily PKC- ι/λ and little PKC- ζ ; mouse and human liver contain substantial amounts of both PKC- ι/λ and PKC- ζ [23]). Immunoprecipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies) and incubated for 8 min at 30°C in 100 μ l buffer containing 50 mmol/l Tris/HCl (pH 7.5), 100 μ mol/l Na₃VO₄, 100 μ mol/l Na₄P₂O₄, 1 mmol/l NaF, 100 μ mol/l PMSF, 4 μ g phosphatidylserine (Sigma), 50 μ mol/l [γ -³²P]ATP (NEN Life Science Products, Beverly, MA, USA), 5 mmol/l MgCl₂ and, as substrate, 40 μ mol/l serine analogue of the PKC- ϵ pseudosubstrate (Millipore, Bedford, MA, USA). After incubation, ³²P-labelled substrate was trapped on P-81 filter paper and counted. aPKC activation was also assessed by immunoblotting for phosphorylation of the auto(trans)phosphorylation site Thr-555/560 in PKC- ι/ζ , which is required for, and reflective of, activation [23].

As previously described [14], for assays of recombinant PKC- ι and PKC- ζ (5–10 ng/assay; BioVision, Mountain, CA, USA), 10 fmol/l phosphatidylinositol-3,4,5-(PO₄)₃ (PIP₃; Matreya, Pleasant Gap, PA, USA) was added to activate and define aPKC activity (activity unattributable to PIP₃ was subtracted).

Activation of AMPK was assessed by measurement of immunoprecipitable AMPK activity as previously described [3, 14], and by immunoblotting for phosphorylation of both threonine-172-AMPK and the AMPK substrate serine-79-acetyl-CoA carboxylase (ACC).

Western analyses Western analyses were conducted as previously described [11–14, 17], using: anti-phospho-serine-473-Akt and GAPDH antisera (Santa Cruz Biotechnologies); anti-phospho-threonine-560/555-PKC- ζ /PKC- ι / λ antiserum (Invitrogen); mouse monoclonal anti-PKC- ι / λ antibodies (Transduction Laboratories, Bedford, MA, USA); and anti-phospho-threonine-172-AMPK and anti-phosphoserine-79-ACC antisera (Millipore). Samples from experimental groups were compared on the same blots and corrected for recovery as needed by measurement of GAPDH immunoreactivity.

mRNA measurements As previously described [11–14, 17], 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}) and checked for purity by electrophoresis on 1.2% agarose gels. mRNA was quantified by quantitative real-time RT-PCR using TaqMan reverse transcription reagent (Applied Biosystems, Carlsbad, CA, USA) and SYBR Green (kit from Applied Biosystems) with human nucleotide primers [see 13, 14, 17], and hypoxanthine phosphoribosyl-transferase mRNA for recovery correction. Values are expressed relative to the mean control unstimulated non-diabetic value.

Statistical evaluations Data are expressed as mean $\pm$ SEM, and p values were determined by one-way ANOVA and least-significant multiple comparison methods. Values exceeding 2 SDs from the mean value were discarded.

Results

Activation of AMPK by metformin and AICAR in human hepatocytes Treatment of human hepatocytes for 24 h with either metformin or AICAR increased AMPK activity, as evidenced by increases in: (1) phosphorylation of Thr-172-AMPK and the AMPK substrate Ser-79-ACC (Fig. 1); and (2) immunoprecipitable AMPK enzyme activity (Fig. 2). Maximal activation of AMPK was seen at 1 mmol/l metformin and 100 nmol/l AICAR (Fig. 2). Note that treatment with 10 mmol/l metformin had variable effects on AMPK activity and, in some cases, diminished energy-dependent processes (e.g. aPKC activation; see below); this probably reflects varying degrees of limitation in ATP availability [7].

Although we did not determine whether metformin and AICAR activate aPKC through activation of ERK and PLD-mediated increases in PA in human hepatocytes, we found that, like PIP_3 , which mediates insulin effects on aPKC [23], PA activated recombinant aPKC, with potency comparable with that of PIP_3 (Fig. 3b).

Activation of aPKC by metformin and AICAR in human hepatocytes As in mouse liver [8], treatment of human hepatocytes with maximally effective concentrations of metformin or AICAR for 24 h increased phosphorylation of Thr-555/560-PKC- λ/ζ , the autophosphorylation site, reflective of and required for aPKC activation (Fig. 1). Dose-dependent increases in immunoprecipitable aPKC enzyme activity were also seen following 24 h treatments, with maximal increases seen at 1–3 mmol/l metformin and 100 nmol/l AICAR (Fig. 4). In these comparisons, metformin- and AICAR-induced increases in aPKC activity were approximately 50–60% of those elicited by combined treatment with metformin or AICAR plus insulin; however, in individual comparisons, 1 mmol/l metformin and 100 nmol/l AICAR provoked increases in aPKC activity comparable in magnitude with those elicited by insulin (see below). We also noted that treatment with 10 mmol/l metformin in overnight incubations produced variable alterations, which, on average, failed to increase basal aPKC activity and, moreover, partially diminished insulin-stimulated aPKC activity (Fig. 4). Indeed, even more marked inhibition of insulin-stimulated aPKC was seen in 6 h incubations with 10 mmol/l metformin, perhaps reflecting greater availability of metformin in shorter incubations (not shown).

Inhibition of aPKC activity by ICAP in human hepatocytes ICAP diminished insulin-stimulated aPKC activity by approximately 50% in human hepatocytes (Figs 1 and 4), with maximal inhibition seen at 100 nmol/l (Fig. 4). However, ICAP itself did not directly inhibit recombinant PKC- ι (Fig. 3c), indicating that ICAP must be converted intracellularly to the active inhibitory compound ICAPP, which contains a phosphate group linked to the 4-methyl-hydroxy group and which binds to and specifically inhibits PKC- ι (Fig. 3a) and 98% homologous PKC- λ (not shown), but no other PKCs, including aPKC- ζ (72% homology) and PKC- α , β , δ , ϵ and θ [14]. Consonant with this idea: (1) AICAR is itself inactive but is phosphorylated intracellularly by adenosine kinase to the active compound AICAR- PO_4 (ZMP), which acts as an analogue of 5'-AMP; (2) ICAP is structurally identical to AICAR, except that ICAP has a cyclopentyl ring in place of the ribose ring in AICAR; (3) addition of adenosine kinase along with ICAP to the incubation of recombinant PKC- ι led to an inhibitory effect comparable with that of ICAPP (compare with Fig. 3a, d); and (4) incubation of ICAP with adenosine kinase and γ - ^{32}P PO_4 -ATP yielded ^{32}P PO_4 -labelled ICAPP, as determined by purification with thin-layer chromatography (50% inhibition at approximately 1 μ mol/l). It can also be noted (Fig. 4) that: (1) insulin-stimulated aPKC activity resistant to ICAP probably reflects PKC- ζ , which is also present in human hepatocytes; and (2) the resistance of basal in relation to insulin-stimulated aPKC activity to inhibition by ICAP may reflect that insulin-activated aPKC would be expected to have

Fig. 1 Effects of ICAP, AICAR and metformin (MET) on phosphorylation of AMPK, ACC and aPKC in human hepatocytes. Hepatocytes from non-diabetic humans were treated for 24 h with 100 nmol/l ICAP, 100 nmol/l AICAR or 1 mmol/l metformin, and then examined for relative levels of (a) phospho-threonine (p-Thr)-172-AMPK, (b)

phospho-serine (p-Ser)-79-ACC and (c) p-Thr-555/560-PKC- α/ζ . Representative immunoblots are shown above the bar charts. Bar charts show values relative to untreated mean values and are mean $\pm$ SEM of five determinations; * p <0.05; ** p <0.01; *** p <0.001

an open substrate-binding site that may be more sensitive to inhibitors than inactive closed aPKC, and/or a substantial amount of insulin-insensitive non-aPKC kinase(s) co-immunoprecipitates with aPKC.

Effects of ICAP on AMPK activity in human hepatocytes

Despite structural similarities to AICAR, ICAP, at concentrations that maximally inhibited aPKC (Fig. 4), did not increase the phosphorylation of AMPK or ACC (Fig. 1) or immunoprecipitable AMPK enzyme activity (Fig. 2). In addition, despite structural similarities to ICAP, AICAR, at concentrations that maximally activated AMPK (Fig. 2), not only failed to inhibit but instead increased aPKC phosphorylation at Thr-555/560 (Fig. 1) and aPKC enzyme activity (Fig. 4). Further, although not shown, effects of 10 μ mol/l AICAR on both AMPK and aPKC activity were comparable with those elicited by 0.1–1 μ mol/l AICAR, indicating that increases in both activities had plateaued.

Fig. 2 Dose-dependent effects of ICAP, metformin and AICAR on AMPK activity in human hepatocytes. Hepatocytes of non-diabetic humans were treated for 24 h with indicated concentrations of (a) ICAP, (b) metformin or (c) AICAR and then examined for immunoprecipitable AMPK activity. Values are mean $\pm$ SEM of five determinations; * p <0.05; *** p <0.001

Effects of metformin and AICAR vs ICAP on lipogenic and gluconeogenic enzyme levels in hepatocytes of non-diabetic and type 2 diabetic humans As in previous ICAPP studies [14]: (1) insulin provoked increases in the expression of the lipogenic factors *SREBP-1c* and *FAS*, and decreases in the expression of genes for the gluconeogenic enzymes *PEPCK* and *G6Pase*, in non-diabetic hepatocytes; (2) the expression of these lipogenic and gluconeogenic factors was increased basally and insulin had no further effect on these factors in hepatocytes of type 2 diabetic humans; and (3) 100 nmol/l ICAP largely diminished both insulin-induced increases in the expression of the lipogenic factors *SREBP-1c* and *FAS* in non-diabetic hepatocytes and diabetes-induced increases in both lipogenic and gluconeogenic factors in diabetic hepatocytes (Fig. 5).

In contrast to ICAP treatment: (1) basal expression of *SREBP-1c* and *FAS* increased following the treatment of non-diabetic hepatocytes with 1 mmol/l metformin and

Fig. 3 Effects of ICAPP and ICAP±adenosine (Ado) kinase on aPKC activity. Recombinant PKC- ι was incubated with 10 fmol/l PIP₃ [which maximally activated recombinant aPKC – see (b)] and indicated doses of (a) ICAPP, (c) ICAP or (d) ICAP+100 ng Ado-kinase, along with other components of the aPKC assay system. (b) PIP₃ and PA had similar dose-dependent effects on recombinant aPKC activity

100 nmol/l AICAR (Fig. 6b, d), and concomitant insulin treatment did not provoke further increases in *SREBP-1c*/*FAS* expression (Fig. 5); and (2) diabetes-dependent increases in the expression of *SREBP-1c* and *FAS* were not improved by either 1–3 mmol/l metformin or 100 nmol/l AICAR treatment in diabetic hepatocytes (Fig. 5).

As in ICAPP studies [14], treatment with 100 nmol/l ICAP was attended by decreases in the expression of *PEPCK* and *G6Pase* in hepatocytes of both non-diabetic and type 2 diabetic humans incubated in the absence of insulin; moreover, insulin did not elicit further decreases in *PEPCK*/*G6Pase* expression (Fig. 5). In contrast to ICAP, basal expression of *PEPCK* and *G6Pase* trended higher following the treatment of non-diabetic hepatocytes with 1–3 mmol/l metformin and 100 nmol/l AICAR, and concomitant insulin treatment failed to significantly improve *PEPCK*/*G6Pase* expression in non-diabetic hepatocytes (Fig. 5). In addition, 100 nmol/l AICAR and 1 mmol/l metformin did not diminish the basal expression of *PEPCK* and *G6Pase* in diabetic hepatocytes (Fig. 5). On

the other hand, in type 2 diabetes mellitus hepatocytes, 1 and 3 mmol/l metformin and 100 nmol/l AICAR improved the insulin effects on *PEPCK*/*G6Pase* expression (Fig. 5).

To determine whether the stimulatory effects of metformin and AICAR on *SREBP-1c* and *FAS* expression are dependent on aPKC, we used a newly developed inhibitor of PKC- ι and PKC- ζ , ACPD, instead of ICAP, as metformin and AICAR activate both aPKCs [3], and to avoid competition between ICAP and AICAR, which are probably similarly transported and phosphorylated by adenosine kinase (see above). Indeed, in hepatocytes of non-diabetic humans, 1 μ mol/l ACPD markedly inhibited the increases in aPKC activity elicited by metformin, AICAR and insulin (Fig. 6a; note that metformin- and AICAR-induced increases in aPKC were equal to that of insulin). In contrast, ACPD did not diminish AMPK activation by AICAR and metformin (Fig. 6c). Most importantly, ACPD largely inhibited AICAR- and metformin-induced increases in the expression of both *SREBP-1c* (Fig. 6b) and *FAS* (Fig. 6d).

Fig. 4 Effects of ICAP, metformin and AICAR on aPKC activity in human hepatocytes. Hepatocytes from five non-diabetic humans were treated for 24 h with the indicated concentrations of (a) ICAP, (b) metformin or (c) AICAR, in the presence and absence of 1 μ mol/l insulin, and then examined for immunoprecipitable aPKC activity. Values are mean $\pm$ SEM of five determinations; * p <0.05; ** p <0.01; *** p <0.001

Fig. 5 Effects of ICAP, AICAR and metformin on the expression of lipogenic and gluconeogenic factors in basal and insulin-stimulated hepatocytes from non-diabetic and type 2 diabetic (T2DM) humans. Hepatocytes from non-diabetic and T2DM humans were treated for 24 h without (O) or with 100 nmol/l ICAP (I), 100 nmol/l AICAR (A) or 1 or 3 mmol/l metformin (1 M and 3 M, respectively), in the presence (solid bars) and absence (open bars) of 1 μ mol/l insulin, and then examined for mRNA levels of (a) *SREBP-1c*, (b) *PEPCK*, (c) *FAS* and (d) *G6Pase*.

Relative values are mean $\pm$ SEM of five determinations normalised to the control after standardising to hypoxanthine phosphoribosyl-transferase mRNA for recovery correction. * p <0.05, agent-treated basal or agent-treated insulin-stimulated value vs corresponding basal or insulin-stimulated value of the O group; † p <0.05, insulin-treated value of the O group vs basal value of the O group; and ‡ p <0.05, basal diabetic value of the O group vs basal non-diabetic value of the O group

Effects of ICAP on phosphorylation of FoxO1 in human hepatocytes As in ICAPP studies in human hepatocytes [14] and mouse liver [17], ICAP provoked increases in phosphothreonine-256-FoxO1 in non-diabetic human hepatocytes that were comparable with or greater than those elicited by insulin

(Fig. 7). These effects of ICAP and insulin on FoxO1 phosphorylation were seen in 6 h but not 24 h incubations, perhaps reflecting an autoregulatory limitation consequent to prolonged suppression of gluconeogenic enzyme levels. As also shown in Fig. 7, 100 nmol/l insulin increased aPKC and

Fig. 6 AICAR and metformin increase the expression of lipogenic factors by an aPKC-dependent mechanism in human hepatocytes. Hepatocytes from four non-diabetic humans were treated for 24 h without or with 1 μ mol/l ACPD and 100 nmol/l AICAR, 1 mmol/l metformin and 1 μ mol/l insulin, and then examined for activities of (a) aPKC and (c) AMPK and mRNA levels of (b) *SREBP-1c* and (d) *FAS*. Values are

mean $\pm$ SEM of four determinations. *SREBP-1c* and *FAS* values are expressed relative to mean values of samples untreated with ACPD, AICAR and metformin immunoprecipitate. *** p <0.001, agonist-treated value vs the basal untreated value; ††† p <0.001, ACPD-inhibited value vs the adjacent uninhibited value

Fig. 7 Effects of ICAP on phosphorylation of FoxO1 in human hepatocytes. As described in the Methods, hepatocytes from non-diabetic humans were incubated first for 16 h with 1 μ mol/l insulin and then for 3 h in insulin-free medium, and then treated for 6 h $\pm$ 100 nmol/l ICAP and $\pm$ 100 nmol/l insulin, as indicated. Tissue extracts were examined by western analysis for phospho-serine (p-Ser)-256-FoxO1. Bar chart values (expressed relative to the mean value of samples untreated with insulin or ICAP) are mean $\pm$ SEM of four determinations. For comparison, the effects of insulin and ICAP on the activation of aPKC (phospho-threonine [p-Thr]-555/560-PKC- α/ζ) and Akt (p-Ser-473-Akt1/2) in these conditions are shown. ** p < 0.01; *** p < 0.001

Akt phosphorylation/activation, and 100 nmol/l ICAP diminished aPKC but not Akt phosphorylation in these 6 h experiments.

Discussion

Metformin and AICAR activated aPKC in concentrations that activate AMPK in human hepatocytes. This is important, as hepatic aPKC inhibition by expression of kinase-inactive aPKC [12, 13] short-hairpin RNA for knockdown of hepatic IRS-2, which controls hepatic aPKC [12], or small-molecule aPKC inhibitors [14, 17], has salutary (inhibitory) effects on the expression of hepatic lipogenic and gluconeogenic factors that contribute importantly to lipid and carbohydrate abnormalities in insulin-resistant syndromes. Accordingly, except in states of maximal aPKC activation, hepatic aPKC activation by metformin and AICAR would diminish the positive effects of these agents on lipogenic and gluconeogenic factors by simple AMPK activation.

Activation of aPKC in human hepatocytes by metformin and AICAR apparently derives from AMPK activation, as activation profiles of aPKC and AMPK followed similar dose–response relationships. In addition, it should be noted that, in muscle, aPKC activation by metformin and AICAR is dependent on AMPK, and AMPK activation is independent of aPKC [3, 9]. Similarly, with a specific aPKC inhibitor, we found that AMPK activation is independent of aPKC in

human hepatocytes (we were unable to use the AMPK inhibitor compound C as it unexpectedly inhibited aPKC).

In support of the idea that hepatic aPKC activation may diminish the therapeutically desirable effects of simple AMPK activation, both metformin and AICAR were less effective than the aPKC inhibitor ICAP in diminishing insulin-dependent and diabetes-dependent increases in expression of the lipogenic factors *SREBP-1c* and *FAS* in hepatocytes of non-diabetic and type 2 diabetic humans. Indeed, the expression of lipogenic factors increased following metformin and AICAR treatment in non-diabetic hepatocytes, and diabetes-dependent increases in their expression were not significantly improved by metformin and AICAR in hepatocytes of type 2 diabetic humans. In contrast, ICAP largely reversed both insulin-induced and diabetes-induced increases in lipogenic factors. Of course, we cannot rule out the possibility that the failure of metformin and AICAR to improve *SREBP-1c* and *FAS* expression in diabetic hepatocytes resulted from an aPKC-independent mechanism.

Failure to find more significant salutary effects of metformin and AICAR on hepatic lipogenic factors in diabetic hepatocytes may explain why metformin has limited effects on weight loss and hyperlipidaemia. This failure to improve the expression of lipogenic factors further suggests that the positive effects of metformin on lipid metabolism in vivo may reflect alterations in processes other than direct improvements of hepatic *SREBP-1c* and *FAS* expression; for example, metformin-induced anorectic tendencies and decreases in hyperinsulinaemia (and thus decreases in hepatic aPKC activation) owing to improvements in hepatic and/or muscle glucose metabolism. In addition, AMPK directly phosphorylates/inhibits ACC, and this may increase fatty acid oxidation and diminish fatty acid synthesis.

It was also important to find that, as with ICAPP [14, 17], ICAP diminished expression of *PEPCK* and *G6Pase* basally (i.e. in the absence of insulin treatment) in hepatocytes of both non-diabetic and type 2 diabetic humans. In contrast, metformin and AICAR did not diminish basal expression of these gluconeogenic enzymes in non-diabetic hepatocytes, and seemed to provoke upward trends in the expression of these factors that were not reversed by concomitant insulin treatment. On the other hand, metformin and AICAR did improve insulin-induced decreases in *PEPCK* and *G6Pase* expression in diabetic hepatocytes, and this sensitising mechanism may be important for metformin-induced improvements in hepatic gluconeogenesis. That this salutary action required the presence of insulin correlates with the fact that metformin is most useful for treating earlier, but not later, phases of type 2 diabetes, when insulin secretion diminishes.

The mechanism whereby metformin and AICAR enhanced the effects of insulin on gluconeogenic enzymes in type 2 diabetic hepatocytes is uncertain. One possibility is that metformin and AICAR increased the phosphorylation and nuclear

exclusion of TORC2 [6] independently of aPKC, and thereby restored the ability of insulin to disrupt the CREB/CBP/TORC2 complex needed for *PEPCK/G6Pase* expression. Another possibility is that metformin and AICAR may have enhanced the effects of insulin on gluconeogenic enzymes by increasing aPKC-dependent phosphorylation and nuclear exclusion of CBP in accordance with the mechanism advanced by He et al [8]. This possibility seems remote, as: (1) aPKC activity is increased basally in the hepatocytes of rodent models of type 2 diabetes [11–13, 17] and humans [14 and present results]; and (2) as seen presently and previously [12–14, 17], aPKC inhibition diminishes basal hepatic gluconeogenic enzyme expression. On the other hand, He et al reported that, whereas insulin had little ability to phosphorylate CBP in high-fat-fed mice, metformin was fully effective and, moreover, acutely lowered blood glucose levels [8]. In this scenario, however, since overall hepatic aPKC activity is increased in hyperinsulinaemic high-fat-fed mice [13], an important role for aPKC in mediating metformin effects in this model would require a remarkable degree of compartmentalisation (i.e. an aPKC subset downregulated and unresponsive to hyperinsulinaemia, but responsive to metformin). Needless to say, other mechanisms may be operative in metformin-induced sensitisation to insulin.

It was surprising to find that, despite structural similarity between ICAP and AICAR, ICAP did not increase AMPK activity and AICAR did not diminish aPKC activity. This suggests that the one structural difference, that is, the oxygen atom in the ribose ring of AICAR-PO₄, is not only important for AMPK activation, but also serves to prevent aPKC inhibition. On the other hand, the possibility that aPKC inhibition may occur when supra-optimal concentrations of metformin are used must be kept in mind, as aPKC inhibition rather than simple AMPK activation might underlie or contribute to positive effects.

The ability of ICAP to maximally inhibit PKC- ι in intact human hepatocytes at a concentration only one order of magnitude greater than that of ICAPP [4, 17] most likely reflects efficient cellular uptake of ICAP and subsequent conversion to the active phosphorylated compound, ICAPP, probably by the same adenosine transporter and kinase used by AICAR. In this regard, it should be noted that ICAP, in doses slightly greater than those used in ICAPP studies: (1) specifically inhibited hepatic (but not muscle) PKC- λ with no effects on hepatic Akt or AMPK in studies of intact mice; and (2) effectively inhibited aPKC-dependent production of lipogenic and gluconeogenic factors in the livers of diabetic mice (MPS, RVF, unpublished data).

To summarise, in human hepatocytes, metformin and AICAR activated aPKC in concentrations comparable with those required for maximal AMPK activation. Since aPKC inhibition has positive effects on (i.e. diminishes levels of) lipogenic and gluconeogenic factors in human hepatocytes, it

was not surprising to find that the activation of aPKC during optimal metformin and AICAR action on AMPK was accompanied by changes in the levels of lipogenic and gluconeogenic factors that were less salutary than those elicited by ICAP, a specific inhibitor of PKC- ι . The activation of aPKC by metformin and AICAR appeared to explain why metformin and AICAR failed to reverse insulin- and type 2 diabetes-induced increases in the lipogenic factors *SREBP-1c* and *FAS*. The activation of aPKC by metformin and AICAR may also explain why these agents blunted insulin effects on *PEPCK* and *G6Pase* expression in hepatocytes from non-diabetic donors; accordingly, metformin usage in states such as impaired glucose tolerance may be problematic. On the other hand, metformin and AICAR improved insulin effects on *PEPCK* and *G6Pase* in hepatocytes of type 2 diabetic humans, regardless of concomitant aPKC activation. Our findings may explain why metformin has only modest effects on lipid metabolism, and requires insulin for improvements in glucose metabolism.

Funding Supported by funds from the Department of Veterans Affairs Merit Review Program and the National Institutes of Health Grants (DK 065969 to RVF).

Contribution statement RVF conceived, designed and directed the studies, analysed data and wrote the paper. MPS conducted studies and assays, assembled and assisted in interpretation of data, and contributed to writing and revising the paper. RAI conducted assays, assisted in interpretation of data and contributed to the revision of the paper. All authors have approved the final version of the paper.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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