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Metformin improves atypical protein kinase C activation by insulin and phosphatidylinositol-3,4,5-(PO₄)₃ in muscle of diabetic subjects

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Abstract *Aims/hypothesis:* Metformin is widely used for treating type 2 diabetes mellitus, but its actions are poorly understood. In addition to diminishing hepatic glucose output, metformin, in muscle, activates 5'-AMP-activated protein kinase (AMPK), which alone increases glucose uptake and glycolysis, diminishes lipid synthesis, and increases oxidation of fatty acids. Moreover, such lipid effects may improve insulin sensitivity and insulin-stimulated glucose uptake. Nevertheless, the effects of metformin on insulin-sensitive signalling factors in human muscle have only been partly characterised to date. Interestingly, other substances that activate AMPK, e.g., aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR), simultaneously activate atypical protein kinase C (aPKC), which appears to be required for the glucose transport effects of AICAR and insulin. *Methods:* Since aPKC activation is defective in type 2 diabetes, we evaluated effects of metformin therapy on aPKC activity in muscles of diabetic subjects during hyperinsulinaemic-euglycaemic clamp studies. *Results:* After metformin therapy for 1 month, basal aPKC activity increased in muscle, with little or no change in insulin-stimulated aPKC activity. Metformin therapy for 8 to 12 months improved insulin-stimulated, as well as basal aPKC activity in muscle. In contrast, IRS-1-dependent phosphatidylinositol (PI) 3-kinase activity and Ser473 phosphorylation of protein

kinase B were not altered by metformin therapy, whereas the responsiveness of muscle aPKC to PI-3,4,5-(PO₄)₃, the lipid product of PI 3-kinase, was improved. *Conclusions/interpretation:* These findings suggest that the activation of AMPK by metformin is accompanied by increases in aPKC activity and responsiveness in skeletal muscle, which may contribute to the therapeutic effects of metformin.

Keywords Akt · Atypical protein kinase C · Diabetes · Insulin · Insulin resistance · Metformin · Muscle · Protein kinase B · Protein kinase C-zeta

Abbreviations AICAR: aminoimidazole-4-carboxamide-1- β -D-ribose · AMPK: 5'-AMP-activated protein kinase · aPKC: atypical protein kinase C · DNP: dinitrophenol · PA: phosphatidic acid · PDK1: 3-phosphoinositide-dependent protein kinase-1 · PI: phosphatidylinositol · PIP₃: phosphatidylinositol-3,4,5-(PO₄)₃ · PKB: protein kinase B · PMSF: phenylmethylsulphonylfluoride

Introduction

The mechanism of action of metformin, a widely used agent for treating type 2 diabetes mellitus, is uncertain, but recent studies suggest involvement of 5'-AMP-activated protein kinase (AMPK) [1–3], which is activated by metformin in muscle and liver, and which, in serving as a metabolic energy sensor of 5'-AMP/ATP levels, increases energy (ATP) production by: (1) promoting glucose uptake into muscle; and (2) diminishing lipid synthesis and increasing the oxidation of fatty acids in both muscle and liver.

Improvement in glucose homeostasis in type 2 diabetes during metformin therapy is thought to be largely mediated through diminished hepatic glucose output [4, 5]. However, particularly with long-term therapy, metformin provokes increases in insulin-stimulated glucose disposal [6–10], presumably at least partly by increasing glucose utilisation in muscle. Moreover, in vivo treatment of rats with metformin provokes increases in insulin-stimulated glucose transport, as measured in isolated skeletal muscle

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preparations [11, 12], and metformin itself increases 2-deoxyglucose uptake in cultured myocytes [13, 14]. Thus, metformin-induced increases in basal and/or insulin-stimulated glucose transport may contribute to increases in glucose disposal following metformin treatment of diabetic subjects. On the other hand, it is unclear if the effects of metformin on glucose utilisation are mediated by muscle AMPK, or by a general improvement in insulin signalling in response to better glycaemic control, or by alterations in circulating lipids or cytokines.

Effects of metformin on insulin-sensitive and other signalling factors in human muscle are largely unknown. However, metformin increases the phosphorylation/activity of AMPK in muscles of diabetic subjects [2]. In muscle preparations of rodents and cultured L6 myotubes, substances that activate AMPK – e.g., aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR), which enters cells and mimics 5'-AMP, and dinitrophenol (DNP), which uncouples mitochondrial oxidative phosphorylation, thereby increasing 5'-AMP levels – also activate atypical protein kinase C (aPKC) isoforms, PKC- ζ and PKC- λ . These are required for increases in GLUT4 translocation to the plasma membrane and glucose transport during AICAR and DNP action in L6 myotubes [15]. Importantly, this activation of aPKC by AMPK occurs in the absence of activation of phosphatidylinositol (PI) 3-kinase, which mediates increases in aPKC and protein kinase B (PKB/Akt) activity during insulin-stimulated GLUT4 translocation and glucose transport. Instead, increases in aPKC activity that accompany AMPK activation during AICAR and DNP action appear to depend upon the activation of proline-rich tyrosine kinase-2, extracellular receptor-activated kinase and phospholipase D, which generates the acidic phospholipid, phosphatidic acid (PA), a direct activator of aPKCs [15].

As mentioned, insulin stimulates glucose transport in muscle by activating IRS-1/2-dependent PI 3-kinase, which, via increases in PI-3,4,5-(PO₄)₃ (PIP₃) and action of 3-phosphoinositide-dependent protein kinase-1 (PDK1), activates both aPKCs and PKB/Akt, which together mediate increases in glucose transport. In this regard, it should be noted that: (1) aPKC activation is defective in muscles of type 2 diabetic [16, 17], obese glucose-intolerant [16, 18] and obese glucose-tolerant [17, 19] humans; and (2) the activities of IRS-1-dependent PI 3-kinase and PKB/Akt are unchanged in muscles of diabetic subjects following metformin treatment [20]. On the other hand, it is not known if basal or insulin-stimulated aPKC activity in muscle is altered by metformin treatment.

Here, in hyperinsulinaemic–euglycaemic clamp studies of type 2 diabetic human subjects, we evaluated whether metformin alters basal and insulin-stimulated aPKC activity in skeletal muscle. Interestingly, we found that metformin initially provoked increases in basal aPKC activity, and, later, after 8 to 12 months, provoked increases in basal and insulin-stimulated aPKC activity in diabetic muscle. In contrast, basal and insulin-stimulated activation of both IRS-1-dependent PI 3-kinase and PKB were unchanged. Of

further note, the metformin-induced increase in aPKC activity appeared to be at least partly due to improved ability of aPKCs to respond to the lipid product of PI 3-kinase, i.e., PIP₃.

Subjects and methods

Patients

We studied six type 2 diabetic subjects, two women and four men. As previously reported [16], results of aPKC activation and other parameters in men and women are indistinguishable and were therefore considered together. The subjects were free of cardiovascular, renal, neuropathic and other medical problems. Five of six subjects had reasonable glycaemic control, with HbA_{1c} levels between 5 and 7.5%; one subject had an HbA_{1c} of 11.6% (Table 1). Antidiabetic medications (sulfonylurea or sulfonylurea plus metformin) were discontinued 2 to 4 weeks prior to the basal (i.e., no metformin treatment) clamp/biopsy study, which was also performed after 1 month ('short-term') or 8 to 12 months ('long-term') of treatment with metformin, 1,000 mg twice daily. In addition to the diabetic subjects, five age-matched non-diabetic men were simultaneously studied in the clamp/biopsy procedure to provide control samples for side-by-side analyses with samples from diabetic subjects (see below). Note that responses to

Table 1 Clinical characteristics of the non-diabetic and diabetic subjects

	Non-diabetic	Diabetic, pre-metformin	Diabetic, post-long-term metformin
Age	47±2	50±3	51±3
BMI	28±2	36±2 ^a	36±2
Fasting blood glucose (mmol/l)	5.4±0.2	9.5±1.1 ^a	7.2±0.6 ^c
HbA _{1c} (%)	5.4±0.1	7.3±0.9	6.1±0.3
Serum insulin (μU/ml)	14.1±4.2	20.7±8.6	20.7±3.2
Serum C-peptide (ng/ml)	2.9±0.5	3.6±0.6	3.5±0.6
Serum triglycerides (mg/dl)	134±20	473±302	414±234
Glucose disposal rate (mg kg ⁻¹ min ⁻¹)	13.9±1.6	2.9±0.5 ^b	6.2±1 ^c

Measurements in diabetic subjects were done while off antidiabetic medications (at study entry), and after long-term metformin treatment. Measurements in non-diabetic subjects were at study entry

p values reflect comparisons of indicated groups. Unless indicated, group differences did not attain statistical significance

^a*p*<0.025 vs non-diabetic

^b*p*<0.001 vs non-diabetic

^c*p*<0.025 vs diabetic, pre-metformin

metformin treatment in the diabetic subject who had the decidedly higher HbA_{1c} level were similar to those in patients with lower HbA_{1c} levels, and results of all diabetic subjects were therefore considered together.

Informed consent was obtained prior to entry into the study. All procedures were reviewed, approved and monitored by the Institutional Review Board of the University of South Florida College of Medicine, and the Research and Development Committee of the James A. Haley Veterans Hospital. Studies were performed in accordance with the Declaration of Helsinki and Good Clinical Practice.

Clamp/biopsy studies

Hyperinsulinaemic–euglycaemic clamp/muscle biopsy studies were conducted in the morning after an overnight fast, as described [16, 19]. Subjects received physiological saline i.v., and after anaesthetisation of the biopsy site by s.c. injection of 0.5% lidocaine (without epinephrine), a small incision was made in the skin and a percutaneous biopsy of the vastus lateralis muscle was performed with a 14-gauge biopsy needle. Insulin was then given i.v. in a priming dose over 10 min, and then at a constant rate proportional to body weight (42 pmol kg body weight⁻¹ min⁻¹) to rapidly raise (within 15 min) and steadily maintain serum insulin concentrations at approximately 3 nmol/l, which provides maximal responses for aPKC and PKB activation [16]. Plasma glucose levels were clamped at 5 to 6.1 mmol/l (measured every 5 min) by varying the rate of a 20% glucose infusion. Forty minutes after starting insulin, a second biopsy was obtained. The clamp was continued for another 90 min to estimate steady-state insulin-stimulated glucose disposal rates during the final 30 min. Muscle samples were rapidly frozen in liquid N₂ and stored at -70°C.

Muscle kinase analyses

Samples were homogenised (Polytron) in buffer containing 20 mmol/l Tris–HCl (pH 7.5), 50 mmol/l sucrose, 2 mmol/l EDTA, 2 mmol/l EGTA, 2 mmol/l Na₃VO₄, 2 mmol/l NaF, 2 mmol/l Na₄P₂O₇, 1 mmol/l phenylmethylsulphonyl-fluoride (PMSF), 20 µg/ml leupeptin, 10 µg/ml aprotinin and 1 µmol/l LR-microcystin, as described [15, 16, 19]. Muscle homogenates were supplemented with 150 mmol/l NaCl, 1% Triton X-100 and 0.5% Nonidet, and 500 µg of lysate were protein-immunoprecipitated at 4°C with anti-serum for determination of total aPKC or IRS-1-dependent PI 3-kinase enzyme activity. Precipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and assayed as described below. In each assay, replicate immunoprecipitates prepared from muscles of several non-diabetic and diabetic patients, studied without and with metformin treatment, and studied without (basal) and with insulin stimulation, were assayed simultaneously to provide intra-assay side-by-side comparisons.

Atypical PKC activation

Atypical PKC activity was measured as described previously [15, 16, 19]. In brief, aPKCs were immunoprecipitated with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognises a common epitope in the C-termini of PKC-ζ and PKC-λ/ι, collected on Sepharose-AG beads, and incubated for 8 min at 30°C in 100 µl buffer containing 50 mmol/l Tris–HCl (pH 7.5), 100 µmol/l Na₃VO₄, 100 µmol/l Na₄P₂O₇, 1 mmol/l NaF, 100 µmol/l PMSF, 4 µg phosphatidylserine (Sigma Chemical Co., St Louis, MO, USA), 50 µmol/l [γ-³²P]ATP (NEN/Life Science Products, Boston, MA, USA), 5 mmol/l MgCl₂ and, as substrate, 40 µmol/l serine analogue of the PKC-ε pseudosubstrate (BioSource, Camarillo, CA, USA). After incubation, ³²P-labelled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

In some assays, 10 µmol/l PIP₃ (Matreya, State College, PA, USA), a maximally effective concentration [see 16, 19, 21], was added to directly activate aPKCs in immunoprecipitates prepared from basal unstimulated muscles. Note that aPKC immunoprecipitates contain coprecipitated PDK1, which promotes the phosphorylation of the activation loop of aPKCs [21, 22].

PKB activation

PKB activation was assessed by Western blot analysis and immunoblotting for Ser473 phosphorylation (rabbit polyclonal antiserum from Cell Signaling Technology, Beverly, MA, USA) as described [16, 19, 22].

IRS-1-dependent PI 3-kinase activation

IRS-1-dependent PI 3-kinase was precipitated with rabbit polyclonal antiserum (Upstate Cell Signaling, Lake Placid, NY, USA) and assayed in the presence of PI, MgCl₂ and [γ-³²P]ATP as described [16, 19, 22]. ³²P-labelled PI-3-PO₄ was isolated by thin layer chromatography and quantified with a PhosphorImager (BioRad, Hercules, CA, USA).

AMPK activation

Immunoprecipitable AMPK (combined α1 and α2) activity was measured in muscle lysates by the method of Wojtaszewski et al. [23] using rabbit polyclonal antiserum (Cell Signaling Technologies) and SAMS peptide (Upstate Cell Signaling) as substrate.

Western blot analyses

As described [15, 16, 19, 21, 22], muscle lysates were immunoblotted for PKC-ζ/λ (C-terminal rabbit polyclonal

antiserum from Santa Cruz Biotechnologies that recognises aPKCs ζ and λ) and phospho-Ser473-PKB (rabbit polyclonal antiserum from Cell Signaling Technology). Samples from untreated and metformin-treated diabetic groups were simultaneously blotted, and resulting chemiluminescence was quantified with a PhosphoImager equipped with a chemiluminescence molecular bioanalysis program (Bio-Rad) or by laser scanning densitometry (LKB, Bromma, Sweden).

Blood/plasma analyses

Levels of HbA_{1c}, fasting glucose, immunoreactive insulin, C-peptide and triglycerides were determined as described [16, 22].

Statistical methods

Data are reported as actual counts per minute per immunoprecipitate observed in aPKC and AMPK assays, and in relative values when comparing results of immunoblots. Data are expressed as means $\pm$ SE. Statistical differences between two means, and relative differences between two samples, were determined by Student's unpaired and paired *t* tests, respectively. Statistical differences between three or more groups were determined by one-way ANOVA and the least significant multiple comparison method. Differences were considered to be significant at $p < 0.05$.

Results

Clinical characteristics

Table 1 shows some of the clinical characteristics of the non-diabetic and diabetic subjects upon entry into the study. Characteristics of diabetic subjects were measured while off antidiabetic medications, and after long-term metformin treatment. Of further note, fasting plasma glucose levels diminished from a pretreatment mean level of 9.5 ± 1.1 ($n=6$) to 6.8 ± 0.7 ($n=4$) and 7.2 ± 0.6 ($n=6$) mmol/l during short- and long-term metformin treatment, respectively, (mean maximal decrement, 49 ± 14 ; $p < 0.025$). HbA_{1c} levels also showed a downward trend with short-term and long-term metformin therapy from 7.25 ± 0.94 to 6.88 ± 0.65 to $6.05 \pm 0.32\%$ ($n=4-6$), respectively, but these changes were not significant. Similarly, glucose disposal rates increased from 2.90 ± 0.53 ($n=6$) in the absence of metformin treatment to 6.50 ± 2.42 ($n=4$) and 6.19 ± 0.95 ($n=6$; $p < 0.025$) mg kg body weight⁻¹ min⁻¹ following short-term and long-term metformin treatment, respectively. There were no significant changes in serum levels of insulin, C-peptide and triglycerides, or in body weight with either short-term (not shown) or long-term metformin treatment (Table 1).

Atypical PKC activity

In non-diabetic subjects, as reported previously [16, 19], insulin provoked approximately twofold increases in muscle aPKC activity (Fig. 1). Levels of both basal and, more importantly, insulin-stimulated aPKC activity were diminished in diabetic subjects (Fig. 1). Interestingly, short-term metformin treatment provoked increases in basal aPKC activity, but had little or no effect on insulin-stimulated aPKC activity (Fig. 1). However, with long-term metformin treatment, insulin-stimulated, as well as basal, aPKC activity was increased (Fig. 1). Neither short-term nor long-term metformin treatment altered muscle immunoreactive aPKC levels (data not shown). Interestingly, similar metformin-induced alterations of aPKC activity were observed in each diabetic subject, regardless of the initial HbA_{1c} level.

Although not portrayed, in four diabetic subjects we performed additional clamp studies 3 to 4 weeks after stopping long-term metformin treatment, and levels of basal and insulin-stimulated aPKC activity had reverted back to the original low levels observed prior to starting metformin therapy (i.e., similar to findings to those shown in Fig. 1). It therefore seems clear that: (1) metformin-induced improvements in aPKC activation require continuous administration of metformin; and (2) improvements seen after long-term metformin treatment are unlikely to have been due to long-term improvement in factors not directly influenced by metformin, e.g., diet, weight, psychological factors, etc.

PKB activation

In contrast to increases in aPKC activity, insulin-stimulated phosphorylation (presumably reflecting activation) of

Fig. 1 Effects of short-term (S-T) and long-term (L-T) metformin (MET) treatment on basal and insulin-stimulated aPKC activity in muscles of diabetic subjects. Muscle aPKC activity was measured prior to and following insulin administration in the hyperinsulinaemic-euglycaemic clamp procedure in the presence and absence of metformin therapy. For comparison, basal and insulin-stimulated aPKC activities observed in control non-diabetic subjects are depicted. Values are means $\pm$ SE of the number of determinations shown in parentheses. # $p < 0.025$; *** $p < 0.001$

Ser473 in PKB was mildly, but not significantly, altered by long-term metformin treatment ($122 \pm 39\%$; $n=6$ comparisons) (Fig. 2), expressed as the percent of the insulin-stimulated value observed in the absence of metformin treatment, set as 100%. However, it should be noted that the enzymatic activation and phosphorylation of muscle PKB following comparable insulin stimulation during the clamp procedure was not found to be significantly diminished in previous studies of type 2 diabetic subjects [16, 24]. Accordingly, it is not surprising that we did not observe here a significant change in PKB activation/phosphorylation during the present clamp studies in response to short-term or long-term metformin therapy.

IRS-1-dependent PI 3-kinase activity

Unlike PKB activation, the activation of IRS-1-dependent PI 3-kinase by insulin has been found to be significantly diminished in muscles of type 2 diabetic subjects during comparable clamp studies [16, 24]. However, like PKB, it has been reported that 3 to 4 months of metformin therapy did not improve the activation of IRS-1-dependent PI 3-kinase in diabetic muscle [20]. We, too, did not find any significant change in basal or insulin-stimulated IRS-1-dependent PI 3-kinase activity following short-term or long-term metformin treatment (data not shown).

Effects of PIP₃ on aPKC activity

Previous studies have shown that the ability of the lipid product of PI 3-kinase, PIP₃, to directly activate immunoprecipitated aPKC is impaired in muscles of type 2 diabetic or obese humans [16, 19] and monkeys [22]. Here, we found that long-term metformin treatment led to significant improvement in the ability of PIP₃ to directly activate

Fig. 3 Effects of metformin treatment on PIP₃-dependent activation of aPKC. Atypical PKC was precipitated from basal (i.e., not insulin-stimulated) muscles of diabetic subjects who were on or off long-term metformin treatment, and incubated in the presence or absence of 10 $\mu\text{mol/l}$ PIP₃. Prior to metformin treatment, there was little or no response to PIP₃, i.e., an increase of only approximately 30% above the basal level. Values are means $\pm$ SE of four subjects. * $p < 0.05$ from t test

aPKC in immunoprecipitates prepared from muscles of diabetic subjects, i.e., from 1.30 ± 0.24 to 3.96 ± 1.15 ($n=4$ determinations; $p < 0.05$, t test; fold-increases in aPKC activity upon addition of PIP₃ to the aPKC assay) (Fig. 3). Accordingly, this improved responsiveness of aPKCs to PIP₃ may play an important role in improving insulin-stimulated aPKC activation following long-term metformin treatment.

AMPK activity

In line with work by Musi et al. [2], we found that enzyme activity of AMPK, measured in samples taken prior to insulin administration in the clamp study, was increased in muscles of patients receiving long-term metformin treat-

Fig. 2 Effects of long-term metformin treatment on phosphorylation/activation of PKB in diabetic muscle. Muscle PKB activation by insulin in the presence and absence of metformin therapy was determined in conjunction with studies of aPKC activation described in Fig. 1. **a** Phospho-Ser473-PKB in percent of insulin-stimulated value without metformin treatment. Values are means $\pm$ SE of six subjects. **b** Immunoblots of phospho-Ser473-PKB α/β in each subject

ment (295 ± 77 vs 599 ± 64 cpm per immunoprecipitate; $n=5$; $p < 0.025$, t test).

Discussion

It was particularly interesting to find that metformin, in the short-term provoked increases in basal aPKC activity, and in the long-term provoked increases in both basal and insulin-stimulated aPKC activity. The increases in basal aPKC activity following metformin treatment most probably resulted from increases in AMPK activity, since: (1) other substances that activate AMPK in muscle, such as AICAR and DNP, also provoke increases in aPKC activity in isolated extensor digitorum longus muscles and L6 myotubes [15]; and (2), as discussed further below, metformin provokes increases in aPKC activity in rat skeletal muscle and L6 myotubes by a mechanism dependent on AMPK (Sajan MP, Farese RV, unpublished observations). The increases in insulin-stimulated aPKC activity observed with more prolonged metformin treatment, on the other hand, may have been partly due to increases in the ability of aPKCs to respond to PIP_3 , as well as long-term improvement in glycaemic control and general but undefined insulin-signalling mechanisms.

Increases in aPKC activity that occur with AMPK activation during AICAR and DNP treatment, and with PI 3-kinase activation during insulin treatment, appear to be required for the activation of GLUT4 translocation/glucose transport during actions of AICAR and DNP [15], as well as insulin [25–30]. However, we can only speculate on whether the increases observed in our study in muscle aPKC activity following metformin treatment were effective in stimulating glucose transport into muscles of metformin-treated diabetic subjects, either without or with concurrent acute insulin administration. In this regard, we did not directly measure glucose transport or GLUT4 translocation, and we only examined effects of insulin plus metformin, but not metformin alone, on whole-body glucose disposal. However, we and others (see above) have observed increases in glucose disposal rates following metformin treatment during clamp studies, but as hepatic glucose output was not measured by isotopic dilution methods, it can only be speculated that disposal into muscle was increased by metformin. On the other hand, at the relatively high levels of insulin used in the present clamp studies, hepatic insulin resistance is likely to have been overcome or minimised. In addition, metformin treatment of rats *in vivo* provokes increases in insulin-stimulated glucose transport, as measured *in vitro* in isolated muscle strips [11, 12], and metformin treatment of L6 myotubes [13] and cultured human myocytes [14] increases glucose transport, in the absence of concurrent insulin treatment. Moreover, we have recently found that metformin, via AMPK, provokes increases in aPKC activity and thereby increases glucose transport even more effectively than insulin in L6 myotubes (Sajan MP, Farese RV, unpublished observations). Accordingly, it is reasonable to believe that metformin treatment, particularly when prolonged, pro-

voked increases in basal and insulin-stimulated glucose transport in the present clamp studies.

In assessing whether metformin increased basal or insulin-stimulated glucose transport, either here or in previous studies, several additional issues need to be considered. First, in human diabetic muscle, responsiveness of aPKC to PIP_3 and other acidic lipids such as PA is diminished (Sajan MP, Farese RV, unpublished observations), even with seemingly good glycaemic control [16], and this poor responsiveness may limit aPKC and aPKC-dependent responses to either metformin or insulin, particularly in initial phases of metformin therapy. Second, a simple increase in aPKC activity may not result in the activation of glucose transport unless the activated aPKC is present in specific cellular locations that participate in GLUT4 translocation, and, unless other co-required activators are simultaneously present. Unfortunately, there is little or no information on factors that are responsible for correctly localising aPKCs, or serving as coactivators, during the actions of either AMPK or insulin in muscle. Nevertheless, as mentioned above, since metformin increases insulin-stimulated whole-body glucose disposal, it is conceivable that at least some of the activated aPKC in metformin-treated muscles is correctly localised for GLUT4 translocation, particularly with concurrent insulin administration. In any event, further clamp studies, particularly following long-term metformin therapy, and perhaps with measurement of GLUT4 translocation to plasma membranes or uptake of labelled glucose, will probably be needed to more definitively answer this question. Along these lines, it should be noted that direct intramuscular injection of adenovirus encoding wild-type aPKC leads to increases in insulin-stimulated glucose transport in rat muscles [31], and plasmid- and adenoviral-mediated increases in wild-type or constitutively active aPKC lead to increases in basal and insulin-stimulated GLUT4 translocation and glucose transport in preparations of adipocytes and myocytes [29, 30]. Thus, there is experimental evidence suggesting that increases in the availability of functionally active aPKC can enhance insulin effects on glucose transport in muscle.

Diminished ability of immunoprecipitated aPKCs to respond to the direct addition of PIP_3 has been observed not only in muscles of type 2 diabetic humans [16] and monkeys [22], but also in muscles of high-fat-fed rats [32] and mice (Sajan MP, Farese RV, unpublished observations), muscles of obese humans [19] and monkeys (Sajan MP, Farese RV, unpublished observations), and cultured adipocytes and myocytes of obese humans [33]. Accordingly, this defect in aPKC responsiveness to the lipid product of PI 3-kinase, PIP_3 , along with diminished ability of insulin to activate IRS-1-dependent PI 3-kinase and generate PIP_3 , could reasonably account for the diminished ability of insulin to activate aPKCs in muscles and adipocytes in each of the above described situations. It was therefore interesting to find here that metformin improved the ability of muscle aPKC to respond to PIP_3 . The mechanism whereby metformin improved aPKC responsiveness to PIP_3 is not certain, but conceivably

may involve AMPK activation, which in turn would be expected to diminish fatty acid synthesis and increase fatty acid oxidation, and thereby diminish the availability of intracellular lipids that may directly or indirectly diminish aPKC activity. It is also possible that effects of metformin in liver (possibly on lipid metabolism) may be more primary, and may secondarily alter aPKC activity in muscle, e.g., by improving circulating NEFA, triglycerides and/or other inhibitory lipids, or by acting through other pathways involving cytokines or other modulators of insulin action, or the central nervous system. Further studies are required to examine these and other possibilities.

Interestingly, in rodent studies metformin activates aPKC in muscle, but not in liver, despite the fact that AMPK is activated in both tissues (Sajan MP, Farese RV, unpublished observations). The reason for this tissue specificity is uncertain, but it is probably fortuitous, since activation of aPKC in the liver is attended by increases in expression of sterol regulatory element binding protein-1c, which transactivates genes responsible for lipid synthesis and secretion of triglyceride-rich VLDL-lipoproteins [34].

Finally, it was particularly interesting to find that basal and insulin-stimulated PKC- ζ activity/activation in diabetic muscle following long-term metformin therapy had improved to levels that were comparable with those existing in muscles of non-diabetic subjects. This remarkable improvement in aPKC activity/activation is noteworthy, as it provides evidence for: (1) the improvement or reversibility of at least certain aspects of the aPKC signalling defect in diabetic muscle; and (2) the efficacy of metformin to serve as an insulin-sensitising agent in insulin-resistant states, at least with regard to aPKC activation in muscle. On the other hand, it should be noted that there is a residual defect in insulin-induced activation of IRS-1-dependent PI 3-kinase that persists in diabetic muscle, and glucose disposal rates were only partially improved, even after long-term metformin treatment. If we can extrapolate from findings in studies in rodents [35, 36], there most probably is, in addition to defects in insulin signalling to IRS-1 and aPKC in diabetic muscle [16, 17], a concomitant insulin-signalling defect to IRS-1-dependent PI 3-kinase and PKB, but not to aPKC, in diabetic liver [37]. At this point, we have no information on whether these hepatic insulin-signalling defects to IRS-1-dependent PI 3-kinase and PKB can be improved by metformin therapy, or whether metformin improves hepatic glucose output by metabolic effects that are independent of changes in insulin signalling in the liver. In view of these considerations, further studies are needed to see if it is more advantageous to use metformin, and/or other insulin sensitisers, in the very earliest phases of IGT and type 2 diabetes, or, for that matter, in insulin-resistant pre-diabetic states [37], when insulin signalling in the liver is better maintained, and insulin-signalling defects are limited more to muscle.

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