The Antidiabetic Drug Metformin Activates the AMP-Activated Protein Kinase Cascade via an Adenine Nucleotide-Independent Mechanism

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Metformin, a drug widely used to treat type 2 diabetes, was recently shown to activate the AMP-activated protein kinase (AMPK) in intact cells and in vivo. In this study we addressed the mechanism for this effect. In intact cells, metformin stimulated phosphorylation of the key regulatory site (Thr-172) on the catalytic (α) subunit of AMPK. It did not affect phosphorylation of this site by either of two upstream kinases in cell-free assays, although we were able to detect an increase in upstream kinase activity in extracts of metformin-treated cells. Metformin has been reported to be an inhibitor of complex 1 of the respiratory chain, but we present evidence that activation of AMPK in two different cell types is not a consequence of depletion of cellular energy charge via this mechanism. Whereas we have not established the definitive mechanism by which metformin activates AMPK, our results show that the mechanism is different from that of the existing AMPK-activating agent, 5-aminoimidazole-4-carboxamide (AICA) riboside. Metformin therefore represents a useful new tool to study the consequences of AMPK activation in intact cells and in vivo. Our results also show that AMPK can be activated by mechanisms other than changes in the cellular AMP-to-ATP ratio. *Diabetes* 51:2420–2425, 2002

Metformin and phenformin are derivatives of guanidine, the active ingredient of French lilac, used to treat diabetes in medieval Europe (1). They were introduced as oral antihyperglycemic agents in the late 1950s, although phenformin was subsequently withdrawn due to problems of lactic acidosis. Metformin, the form used in Europe, was introduced into the U.S. in 1994 and is now very widely used to treat type 2 diabetes. Although its molecular target has been unclear, it promotes insulin-stimulated glucose uptake in muscle (2) and lowers hepatic glucose output (3). It also affects lipid metabolism, lowering plasma triglycerides (4), and free fatty acids (5), the latter possibly due to inhibition of catecholamine-stimulated lipolysis (6).

AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts as a sensor of cellular energy charge (7,8). Once activated by ATP depletion, it switches on ATP-producing catabolic pathways and switches off ATP-consuming anabolic pathways, via direct phosphorylation of metabolic enzymes and via effects on gene expression. The AMPK cascade is activated by exercise (9), and studies with transgenic mice expressing a dominant-negative mutant in skeletal muscle suggest that it is wholly responsible for the effects of hypoxia and partly responsible for the effects of contraction on muscle glucose uptake (10). Winder and Hardie (7) proposed that AMPK represented a target for the development of drugs aimed at type 2 diabetes, and this prediction appears to have been fulfilled by a recent report that metformin activates AMPK in intact cells and in vivo (11). Whereas it is not yet certain that AMPK is the sole target, the metabolic effects of metformin are very similar to those of the AMPK activator, 5-aminoimidazole-4-carboxamide (AICA) riboside. The latter is a nucleoside that is converted inside cells to a nucleotide, ZMP, which mimics the effects of AMP on the AMPK system (12,13). In muscle, AICA riboside stimulates glucose uptake via increased translocation (14) and expression (15) of GLUT4 and promotes insulin-stimulated glucose uptake (16). It inhibits expression of gluconeogenic enzymes in hepatoma cells (17) and endogenous glucose output in vivo (18). Like metformin, it also decreases plasma triglycerides and free fatty acids in vivo (18) and inhibits catecholamine-stimulated lipolysis in rat adipocytes (12,19).

Zhou et al. (11) did not establish the mechanism by which metformin activates AMPK. They proposed that it might act by stimulating phosphorylation of AMPK by upstream kinase(s) or by inhibiting dephosphorylation by protein phosphatases. A third mechanism was suggested by recent findings that metformin can act as an inhibitor of complex 1 of the respiratory chain (20). This suggested that it might activate AMPK by decreasing cellular energy charge. In this article, these various possibilities have been addressed.

RESEARCH DESIGN AND METHODS

Materials. AMPK (21), AMPKK1 (21), protein phosphatase-2C (PP2C) (22), anti-α1 and -α2 antibodies (23), and phosphospecific antibody against Thr-172 (anti-pT172) (24) were purified as previously described. Phosphospecific acetyl-CoA carboxylase (ACC) antibody was raised against the peptide

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ACC, acetyl-CoA carboxylase; AICA, 5-aminoimidazole-4-carboxamide; AMPK, AMP-activated protein kinase; anti-pT172, phosphospecific antibody against Thr-172; DTT, dithiothreitol; GST-KD, glutathione-S-transferase kinase domain; HPLC, high-performance liquid chromatography; PP2C, protein phosphatase-2C.

TMRPSMSpGLHLVK (residues 221–233 of human ACC2; Sp = phosphoserine) as described for anti-pT172 (24). This antibody appears to recognize the homologous AMPK sites on ACC1 (Ser-79) and ACC2 (Ser-221). ExtrAvidin peroxidase conjugate, metformin, and antimycin A were from Sigma, and oligomycin was from Calbiochem.

Cell culture and analyses. CHO cells were cultured in Dulbecco's modified Eagle's medium and H4IIE cells in minimal essential medium, and both media also contained 5% fetal bovine serum and 1% nonessential amino acids (all from Gibco). Immunoprecipitate kinase assays of AMPK were performed using anti-α1 or -α2 antibodies either together or, where specified, individually (25), and results are expressed as the means ± SD of four replicate precipitations. Cellular nucleotides were analyzed by high-performance liquid chromatography (HPLC) (12).

Kinase-kinase assays in H4IIE cell lysates. A fusion protein between the kinase domain of the α1 subunit of AMPK and glutathione-S-transferase (GST-KD) was expressed in *Escherichia coli* (26). The *E. coli* lysate expressing GST-KD was absorbed onto glutathione-Sepharose beads (Amersham-Pharmacia), such that the final concentration of kinase after maximal activation using MgATP and AMPKK in the assay described below was 1 unit in the standard AMPK assay per 5 μl of beads. The slurry was washed with 4 × 1 ml IP buffer (50 mmol/l Tris-HCl, pH 7.4 at 4°C, 50 mmol/l NaF, 5 mmol/l Na pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 1 mmol/l dithiothreitol [DTT], 1 mmol/l benzamidine, 0.1 mmol/l phenylmethane sulfonyl fluoride, and 1 mol/l NaCl) to remove unbound proteins. It was then washed in 3 × 1 ml assay buffer (50 mmol/l Na Hepes, pH 7.4, 1 mmol/l DTT, and 0.02% Brij-35). For the kinase-kinase assay, 25 μg protein from a lysate of H4IIE cells (cells treated with or without metformin) was incubated with 10 μl of a 50% slurry of the glutathione-Sepharose beads with bound GST-KD and 100 μmol/l AMP, 200 μmol/l ATP, 5 mmol/l $MgCl_2$, and assay buffer in a final volume of 25 μl. After incubation for 20 min at 30°C on a rotary shaker, the beads were washed with 4 × 1 ml IP buffer and 3 × 1 ml assay buffer before a standard AMPK assay.

RESULTS

Metformin does not affect regulation of AMPK by upstream kinases and phosphatases. As previously reported (11), metformin does not activate AMPK when added to cell-free assays (not shown). Recently, we have resolved from rat liver extracts two upstream kinases, termed AMPKK1 and AMPKK2, which activate AMPK by phosphorylating Thr-172 (S.A.H., D.G.H., manuscript in preparation). AMPKK1 corresponds to the "AMPKK" we originally described (21), whereas AMPKK2 is a novel activity. Figure 1*A* shows the activation of AMPK by AMPKK1 and AMPKK2 in the presence of AMP under conditions where the degree of activation was relatively modest (15% of maximal activation) and was proportional to the amount of upstream kinase added. Metformin (0.5 or 5 mmol/l) had no effect on activation by either upstream kinase in the presence of AMP. To ensure that the presence of the natural activator, AMP, did not obscure an effect of metformin, we also repeated the experiment in its absence (Fig. 1*B*). Because AMP promotes phosphorylation by the upstream kinases (27), the extent of activation was much smaller (note the different scale and higher amount of upstream kinase used), but metformin was still without effect.

As well as promoting phosphorylation and activation, the binding of AMP to AMPK also inhibits its dephosphorylation and inactivation (22). Figure 1*C* shows that, whereas AMP provided substantial protection against inactivation by PP2C, metformin at concentrations from 0.2 to 20 mmol/l had no effect.

Metformin activates AMPK in CHO cells without affecting the ADP-to-ATP ratio. To examine the effect of metformin in intact cells, we initially used Chinese hamster ovary (CHO) fibroblasts. Incubation with metformin concentrations from 0.25 to 2.5 mmol/l for 18 h

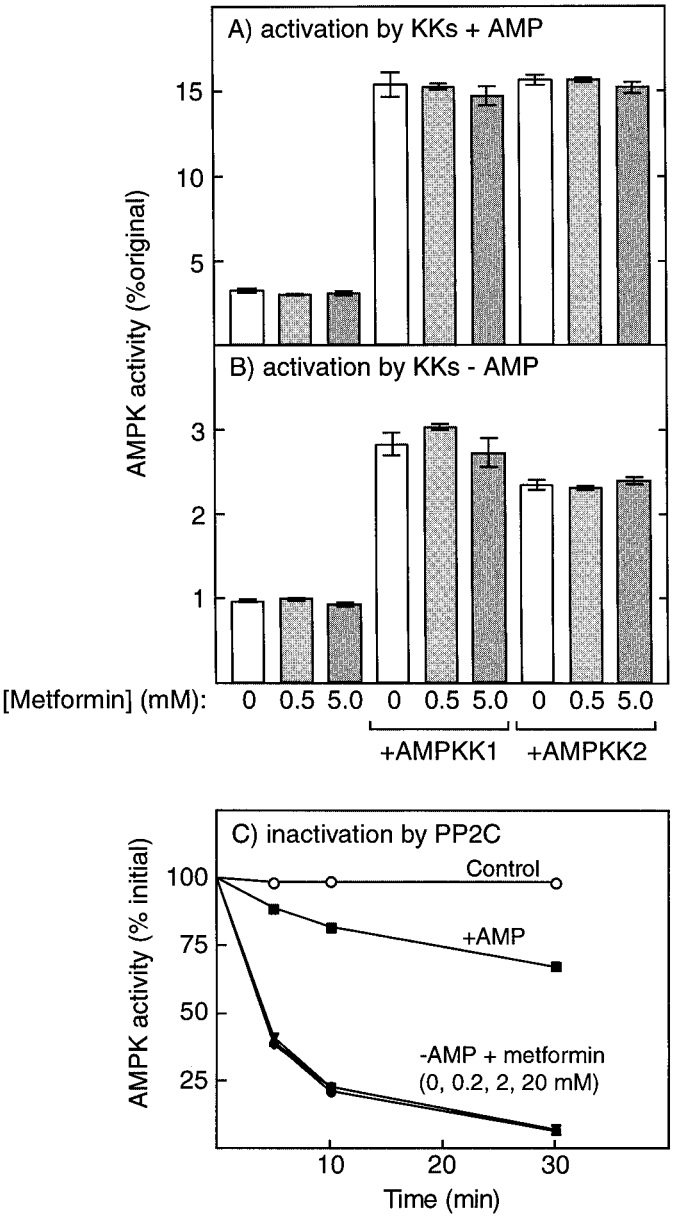

FIG. 1. Effect of metformin on reactivation of purified AMPK by AMPKK1 and AMPKK2 in the presence (*A*) and absence (*B*) of 200 μmol/l AMP. *C*: effect of metformin on inactivation by PP2C. Reactivation by AMPKKs was measured as in ref. 21 and is expressed as the percent of the activity before dephosphorylation (mean ± standard deviation of duplicate assays). Amount of AMPKK1/2 added was 0.4 units/ml (*A*) or 1.6 units/ml (*B*). Inactivation by PP2C was measured as in ref. 20, with single determinations at each time point. The results for 0, 0.2, 2, and 20 mmol/l metformin (filled circles, triangles, diamonds, and inverted triangles, respectively) were indistinguishable.

produced increasing activation of AMPK (Fig. 2*A*). Activation of AMPK was associated with phosphorylation of the α subunits on Thr-172 assessed using phosphospecific antibodies (Fig. 2*B*), with no change in protein level detected using anti-α1/-α2 antibodies (not shown). Using a phosphospecific antibody, we showed that the activation of AMPK was also associated with a large increase in phosphorylation of the target protein, ACC (Fig. 2*B*), with no change in expression detected by probing blots with ExtrAvidin (not shown).

The threefold increase in AMPK activity induced by 2.5 mmol/l metformin occurred without any detectable

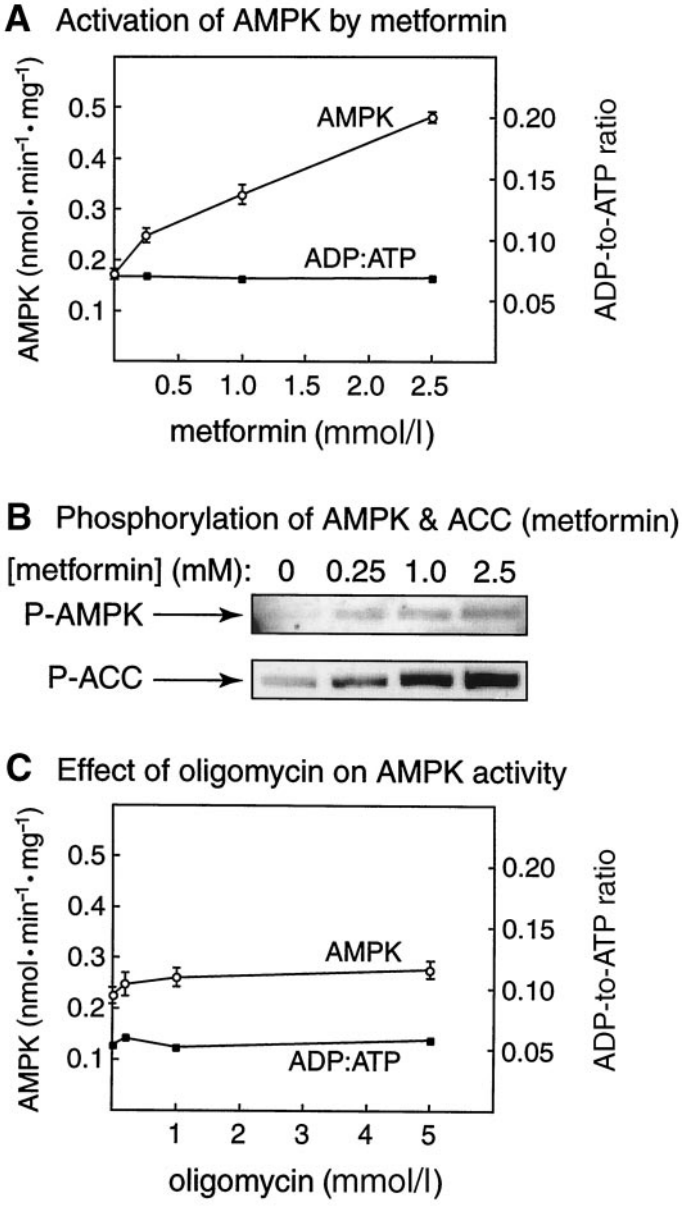

FIG. 2. *A*: **Effect of metformin on total AMPK activity (measured using an immunoprecipitate kinase assay with anti-α1 + -α2 antibodies) and ADP-to-ATP ratio in CHO cells.** *B*: **Phosphorylation of Thr-172 on AMPK-α and the AMPK site on ACC assessed using phosphospecific antibodies.** *C*: **Effect of oligomycin on AMPK activity and ADP-to-ATP ratio in CHO cells.**

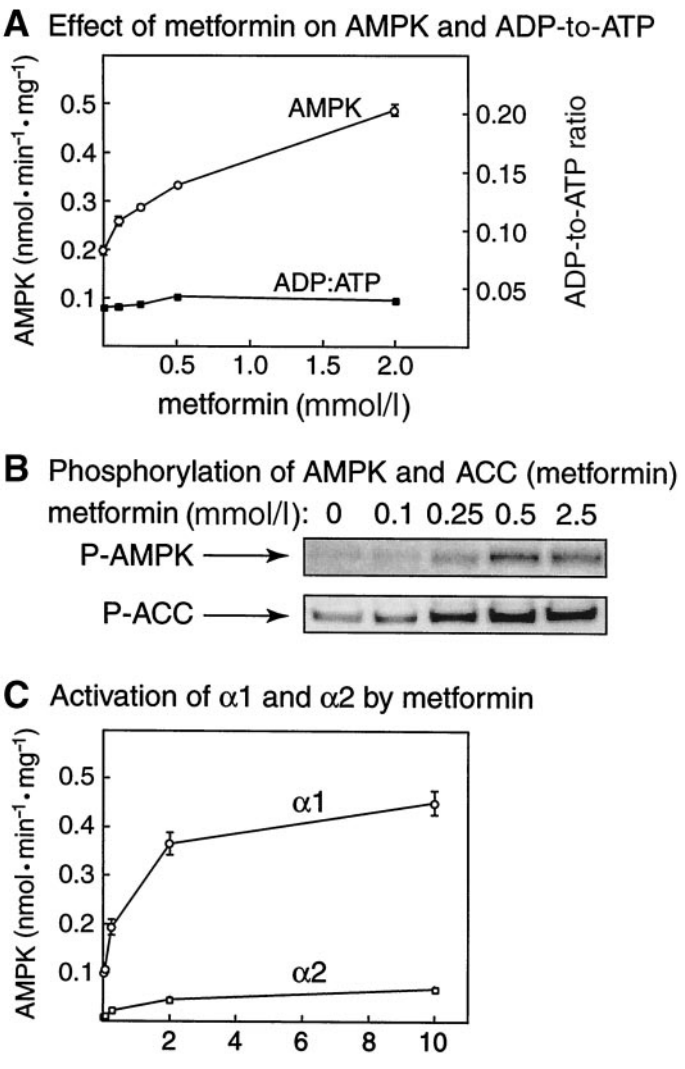

FIG. 3. *A*: **Effect of metformin on total AMPK activity and ADP-to-ATP ratio in H4IIE cells.** *B*: **Effect of metformin on phosphorylation of Thr-172 on AMPK-α and the AMPK site on ACC in H4IIE cells assessed using phosphospecific antibodies.** *C*: **Effect of metformin on the activity of the α1 and α2 complexes in H4IIE cells.**

change in cellular ADP-to-ATP ratio (Fig. 2*A*). To test whether agents that inhibit mitochondrial ATP synthesis would activate AMPK in these cells, we used oligomycin and antimycin A. Oligomycin produced only a very modest activation of AMPK (1.2-fold), even at concentrations as high as 5 mmol/l, and there were no significant changes in cellular ADP-to-ATP ratio (Fig. 2*C*). Similarly, antimycin A at concentrations of up to 25 μmol/l did not produce any significant activation of AMPK (not shown). These results indicate that this cell line does not utilize mitochondrial respiration significantly for ATP synthesis, and we therefore switched to a different cell line.

Metformin activates AMPK in H4IIE cells without affecting the ADP-to-ATP ratio. In the rat hepatoma cell line, H4IIE, incubation for 18 h with metformin from 100 μmol/l to 2 mmol/l produced increasing activation of AMPK without a significant change in cellular ADP-to-ATP ratio (Fig. 3*A*). Activation of AMPK was associated with increased phosphorylation of Thr-172 and a marked phosphorylation of ACC, assessed using phosphospecific antibodies (Fig. 3*B*). Both α1 and α2 isoforms were activated similarly by metformin, although α1 accounts for ~90% of the activity in these cells (Fig. 3*C*).

We also examined the time course of activation of AMPK at low concentrations of metformin (Fig. 4). Even with the lowest concentration used (50 μmol/l), activation was significant after 6 h and continued to slowly increase up to 72 h. Activation was more rapid using 250 μmol/l metformin (Fig. 4*A*). By Western blotting, using antibodies against α1 or α2, there was no change in the expression of AMPK at either concentration of metformin, and the effect was not blocked by an inhibitor of protein synthesis, cycloheximide (data not shown). Figure 4*B* shows that the activation of AMPK was associated with phosphorylation of the AMPK site on ACC, with effects becoming evident at

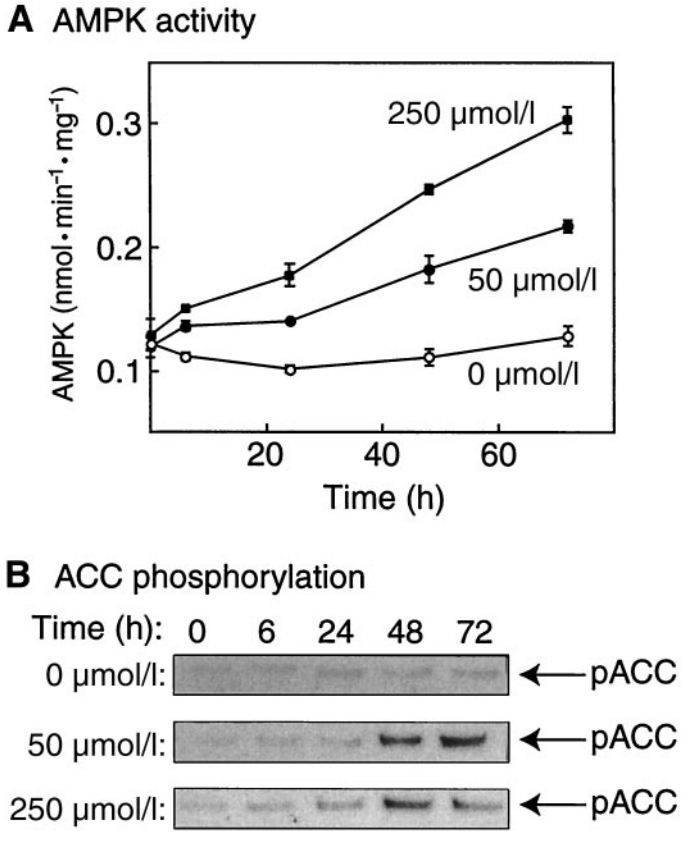

FIG. 4. Time course of activation of total AMPK activity (*A*) and phosphorylation of ACC (*B*) by 50 and 250 μmol/l metformin in H4IIE cells.

later time points, where the activation of AMPK was significant.

Although the effects of metformin were very similar in H4IIE and CHO cells, the effects of oligomycin were quite different. Even at very low concentrations (0.1 to 10 nmol/l, note logarithmic scale), this agent caused an increase in the cellular ADP-to-ATP ratio, and this ratio continued to increase up to 200 nmol/l (Fig. 5*A*). Concentrations higher than this were toxic to the cells. AMPK was also activated by oligomycin, but there was a marked threshold effect in which activation was not evident until oligomycin was >1 nmol/l but had reached a peak by 10 nmol/l. This was particularly striking when the AMPK activity was plotted against the ADP-to-ATP ratio (Fig. 5*B*). As the ADP-to-ATP ratio increased from 0.02 to 0.05 there was no activation, but as it increased from 0.05 to 0.1 there was a three- to fourfold increase in activity. Activation of AMPK was associated with phosphorylation of Thr-172 on AMPK and of the AMPK site on ACC (Fig. 5*C*). Threshold effects were also evident here, with the biggest changes in both parameters occurring between 0.5 and 10 nmol/l oligomycin. In H4IIE cells, AMPK was also activated by antimycin A, with activation reaching twofold at 50 nmol/l and threefold at 200 nmol/l (not shown). Higher concentrations of antimycin A (1, 5, and 25 μmol/l), to which the CHO cells were resistant, appeared to be toxic to H4IIE cells.

Metformin activates upstream kinase(s) in H4IIE cells. Measurement of the activity of upstream kinases using our existing assays is problematic in crude cell

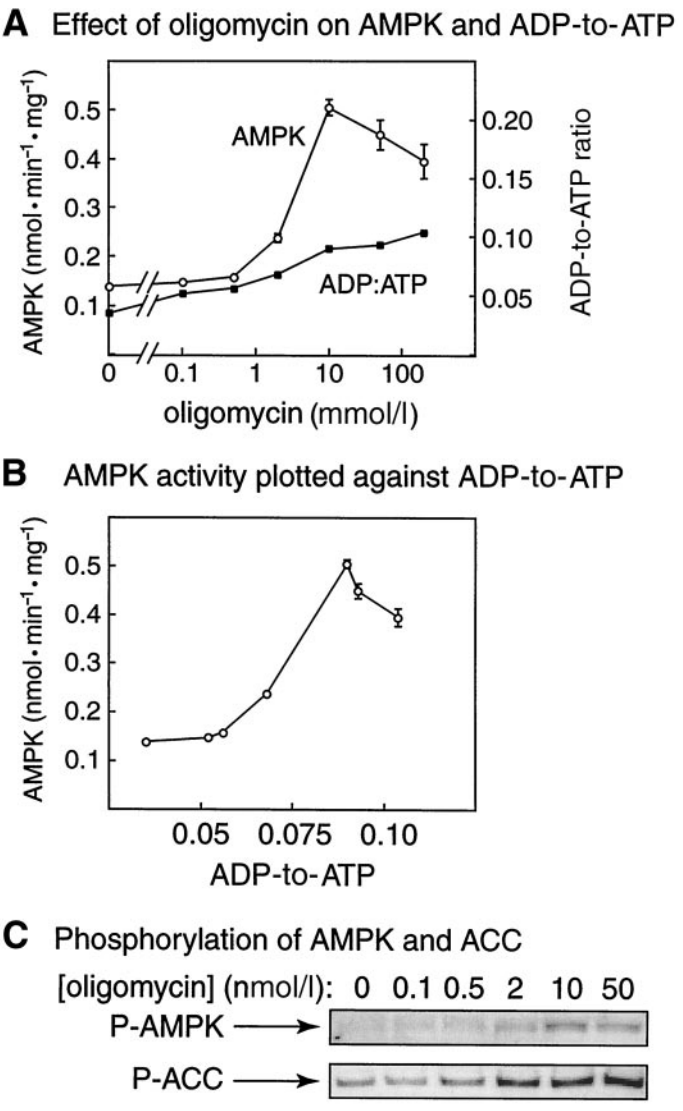

FIG. 5. *A*: Effect of oligomycin on total AMPK activity and ADP-to-ATP ratio in H4IIE cells. *B*: Same data as in *A*, but AMPK activity plotted against ADP-to-ATP ratio. *C*: Effect of oligomycin on phosphorylation of Thr-172 on AMPK-α and the AMPK site on ACC in H4IIE cells assessed using phosphospecific antibodies.

extracts due to the presence of endogenous AMPK. To overcome this problem, we developed a novel assay using a bacterially expressed fusion between glutathione-S-transferase and the kinase domain of the rat α1 subunit (26). The fusion protein was bound to glutathione-Sepharose beads, which were incubated in the presence of AMP and MgATP with lysates of H4IIE cells that had been treated with or without metformin (10 mmol/l for 16 h). The beads were then washed to remove endogenous AMPK and the activity of the fusion protein determined. Metformin caused a 1.6-fold increase in kinase-kinase activity, from 0.10 ± 0.01 to 0.16 ± 0.01 units/mg (mean ± SE) ($n = 4$ for separate batches of cells; $P < 0.001$ by t test).

DISCUSSION

Whereas we have not completely established the mechanism by which metformin activates AMPK, we have ruled out some of the possibilities suggested by Zhou et al. (11)

and Owen et al. (20). Metformin stimulates phosphorylation of AMPK at Thr-172 in intact cells (Figs. 2*B* and 3*B*) and appeared to cause an increase in the activity of upstream kinase(s) that could be detected in a lysate of H4IIE cells by their ability to activate a bacterially expressed α1 kinase domain. However, metformin did not stimulate activation of AMPK in cell-free assays by either of the two upstream kinases (AMPKK1 or AMPKK2) that we have found to phosphorylate Thr-172, either in the presence or absence of AMP. Similarly, it had no effect on inactivation of AMPK by PP2C. There are at least two explanations for our failure to find activation of upstream kinases in cell-free assays. Firstly, metformin might act on an upstream kinase different from AMPKK1 or AMPKK2. If that explanation is correct, it acts in a different manner from AMP, which binds to AMPK and stimulates activation by both AMPKK1 and AMPKK2 (compare Figs. 1*A* and 1*B*) and also inhibits inactivation by PP2C (Fig. 1*C*). Secondly, metformin may be a pro-drug that is modified inside the intact cell to an active form—this would explain why the activation by low concentrations of metformin is rather slow (Fig. 4*A*). At present, we favor this latter explanation, although the active metabolite must be a minor species because the drug is largely excreted in urine in unmodified form (28).

Owen et al. (20) reported that metformin was an inhibitor of complex 1 of the respiratory chain and suggested that this might be important in its mechanism of action. Because AMPK is known to be activated by falling energy charge, this was an attractive idea to explain how metformin activates the kinase. However, our results exclude this possibility for the following reasons:

1) Our CHO cell line was resistant to both oligomycin or antimycin A, with little or no effect of the drugs at concentrations that killed the H4IIE cells. Oligomycin inhibits the mitochondrial F1 ATP synthase, whereas antimycin A inhibits the repiratory chain at the bc_1 complex. The fact that the CHO cells were resistant to two inhibitors that inhibit mitochondrial ATP synthesis via completely different mechanisms shows that these cells must be capable of generating their ATP by glycolysis alone. Lagarde and Siminovitch (29) have previously studied lines of CHO cells that were cross-resistant to oligomycin and antimycin and have shown that they had increased lactate production and increased specific activities of glycolytic enzymes compared with nonresistant cells. Despite the resistance of our CHO cells to oligomycin and antimycin A, AMPK was activated in these cells by metformin over the same concentration range as in H4IIE cells (250 μmol/l to 2.5 mmol/l), with no effect on the cellular ADP-to-ATP ratio. The effect of metformin to activate AMPK in CHO cells therefore cannot be an effect on the respiratory chain.

2) In H4IIE cells, where both oligomycin and metformin activated AMPK to similar extents (c.f. Figs. 3*A* and 5*A*), only oligomycin caused a significant change in the ADP-to-ATP ratio.

In both of these cell lines, the AMP levels were too low to be reliably measured using the HPLC analysis system available to us. However, because the cellular AMP-to-ATP ratio varies as the square of the ADP-to-ATP ratio (8), we could use the ADP-to-ATP ratio as a surrogate measurement. We cannot completely rule out the possibility that metformin could cause a local change in AMP concentration that was not reflected in a change in total cellular ADP-to-ATP. However, we think this is unlikely because in the case of oligomycin, which causes an activation of AMPK in H4IIE cells of similar magnitude to that produced by metformin, changes in ADP-to-ATP ratio were easily measurable (compare Figs. 3 and 5).

The plasma concentrations of metformin in patients treated with the drug are estimated to be 10–20 μmol/l, although higher concentrations may be reached in the portal vein (20). In our cell lines, these concentrations are clearly submaximal in terms of AMPK activation, although we did observe a significant activation after treatment with 50 μmol/l metformin for 6 h in H4IIE cells, and the activation reached twofold if the incubation was continued up to 72 h (Fig. 4). An important point is that an increase in phosphorylation of ACC was evident, even when the degree of phosphorylation and activation of AMPK was very small. To obtain beneficial therapeutic effects of metformin, maximal activation of AMPK may therefore not be necessary. Higher doses of metformin are not used in humans due to gastrointestinal side effects and also because of the risk of lactic acidosis, both of which might be caused by the ability of the compound to act as an inhibitor of the respiratory chain at high concentrations (20). However, it should now be possible to develop a new generation of therapeutic agents more precisely targeted at AMPK, which activate the system at lower concentrations and may have fewer unwanted side effects.

A further interesting finding in this study was the marked threshold effect observed when AMPK was activated using oligomycin in H4IIE cells. Although this agent caused a progressive increase in the ADP-to-ATP ratio over the range from 0.1 to 100 nmol/l, AMPK was only activated over a very narrow range of concentrations from 1 to 10 nmol/l, when the ADP-to-ATP ratio had exceeded a threshold of 0.05. The switch-like nature of this response was evident when the AMPK activity was plotted against the ADP-to-ATP ratio (Fig. 5*B*). These results emphasize the ultrasensitive nature of the response of the AMPK system to a fall in energy charge (30) and indicate that it only becomes significantly activated when the fall in cellular energy status exceeds a critical threshold.

Although the mechanism by which metformin activates AMPK remains unclear, it must be different from that of AICA riboside, which acts by being converted to the AMP mimetic agent, ZMP (12,13). Metformin will therefore be a useful reagent to test for novel cellular processes regulated by AMPK. Whereas the specificity of metformin for AMPK remains uncertain and AICA riboside is not completely specific [e.g., ZMP activates other AMP-regulated enzymes, such as fructose-1,6-bisphosphatase (31) and glycogen phosphorylase (32)], if the same effects are observed using both agents, one can be more confident that they are indeed mediated by AMPK.

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