AMPK activation restores the stimulation of glucose uptake in an in vitro model of insulin-resistant cardiomyocytes via the activation of protein kinase B

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Introduction

Insulin resistance is the hallmark of Type 2 diabetes and is a known risk factor for the development of cardiovascular diseases (1, 14, 18, 34, 46, 51). Ischemic heart disease is responsible for one-half of the deaths in the diabetic population. Moreover, in postischemic survival diabetic patients, the rate of detrimental consequences is significantly higher. In muscular tissues, such as the heart or skeletal muscles, insulin resistance impairs glucose uptake despite the presence of higher circulating glucose levels. This defect in glucose uptake stimulation is associated, during ischemia and reperfusion, with a poor recovery of left ventricular function (52). The impairment of insulin-stimulated glucose uptake is explained, at least partially, by perturbations of the insulin signaling pathway that lead to a reduction in the translocation of the glucose transporter GLUT4 to the plasma membrane (37, 49). Even if the mechanisms underlying insulin resistance are not yet fully understood, alteration in the insulin-induced activation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) signaling pathway undoubtedly plays a critical role (8, 36, 38).

Over the past four decades, several pharmacological approaches that improve insulin sensitivity have been developed (1, 15, 56a). Among these, biguanides, which have been shown to positively impact on glucose homeostasis, have become a cornerstone in the treatment of patients with Type 2 diabetes (2, 56a). Although the glucose-lowering effects of biguanides are known to be due to an increased glucose uptake by peripheral tissues (30) and a decreased hepatic glucose production (31), the precise molecular mechanisms underlying their effects have not yet been fully elucidated. For instance, metformin, a dimethylbiguanide widely used to treat Type 2 diabetic patients and that markedly improves insulin-stimulated glucose uptake, does not seem to directly interfere with the insulin signaling pathway (35). It was nonetheless shown to restore insulin-induced PI3K activation in insulin-resistant C2C12 muscle cell line (40).

It has been recently suggested that metformin activates the AMP-activated protein kinase (AMPK) and that this contributes to its therapeutic action (21, 26, 64). Primarily considered as a cellular fuel gauge, AMPK is involved in the regulation of glucose and lipid metabolism in the liver, the skeletal muscle, the heart, and adipose tissues (3, 11, 23, 29, 48). AMPK is a heterotrimeric enzyme that consists of one catalytic (α) and two regulatory (β and γ) subunits. The control of AMPK activity is complex and involves allosteric stimulation by AMP, as well as AMP-dependent phosphorylation of Thr172, a residue located in the activation loop of the α-subunit. Several protein kinases responsible for this phosphorylation have been identified. They include LKB1 (25, 50, 62) and the Ca2+/calmodulin-dependent protein kinase kinase (27, 32, 61). In ischemic-reperfused myocardium, AMPK phosphorylates

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several downstream targets, resulting in the switching on of several ATP-generating pathways and the switching off of ATP-consuming biosynthetic pathways, thereby mollifying the negative effects of ischemia on the heart’s energy balance (29, 63). During myocardial ischemia, AMPK promotes glycolysis by a double mechanism. First, it increases glucose uptake via the stimulation of the GLUT4 transporter translocation to the plasma membrane (47). Second, it indirectly stimulates phosphofructo-1-kinase (PFK-1) activity by phosphorylating and activating the heart isoform of 6-phosphofructo-2-kinase, the enzyme that synthesizes fructose 2,6-bisphosphate, a potent PFK-1 stimulator (44). Finally, during early reperfusion, AMPK helps fatty acid oxidation to predominate over glucose oxidation. Indeed, the AMPK-mediated phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) leads to a decrease in the concentration of malonyl-CoA, the inhibitor of fatty acid transport to the mitochondria, and to the subsequent increase in fatty acid oxidation (12).

Because 1) insulin stimulates glucose uptake via the PI3K/PKB pathway, 2) this pathway is impaired in insulin-resistant cells, 3) AMPK activation also stimulates glucose utilization, 4) metformin improves insulin sensitivity, and 5) AMPK can be activated by metformin, we hypothesize that AMPK activation could counteract insulin resistance in cardiomyocytes.

We therefore studied the relationship between the glucose uptake stimulation and PKB and AMPK signaling pathways in insulin-sensitive and insulin-resistant cardiomyocytes in the presence or absence of insulin and of known activators of the AMPK, including oligomycin (44) and the biguanide molecules, metformin and phenformin.

MATERIALS AND METHODS

Metformin and phenformin were obtained from Sigma. The adenoviral constructions expressing the mouse myc-tagged and constitutively active form of AMPK (CA-AMPK) and/or the green fluorescent protein (GFP) were kindly provided by F. Foufelle and P. Ferré (Paris). Unless otherwise stated, the source of all other materials is given in Refs. 4 and 5.

Preparation, incubation, and treatment of adult rat cardiomyocytes in culture. This study was approved by the Animal Research Committee at the Université Catholique de Louvain.

Cultured adult cardiomyocytes were prepared from male Wistar rats with a method adapted from Ref. 42. Briefly, two hearts were perfused in parallel with a Ca\(^{2+}\)-free Krebs-Henseleit buffer containing 5 mM glucose, 2 mM pyruvate, and 10 mM HEPES (pH 7.4). Cardiomyocyte isolation was obtained by adding 0.2 mM Ca\(^{2+}\), 1 mg/ml collagenase ( Worthington), and 0.4% (wt/vol) BSA to the perfusate. Thirty minutes after the start of the collagenase perfusion, the hearts were removed from the perfusion apparatus and chopped into small fragments. Ca\(^{2+}\) was progressively reintroduced in the medium to reach a final concentration of 1 mM. The pellets containing the cardiomyocytes from the two hearts were then filtered, lumped together, and resuspended in medium 199 (Invitrogen, 5.5 mM glucose) containing 100 mg/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The cardiomyocytes (about 20–30 mg wet weight of cells/ml) were equally distributed in 20 dishes (60-mm diameter) coated with laminin (Sigma) and incubated at 37°C for 1 h. The cultured dishes were then washed with PBS, after which they were randomly assigned to receive either insulin-free medium 199, to maintain insulin sensitivity, or medium 199 containing 10\(^{-7}\) M insulin during 24 h, to induce insulin resistance. The dishes were then washed several times with PBS, reincubated for 1 h in minimal medium (medium 199 with glutamine, BSA, penicillin, and streptomycin) and treated as described in the legends. After treatment, the medium was removed. The cells were then lysed in a cold buffer containing 50 mM HEPES (pH 7.5), 50 mM KF, 1 mM KPi, 5 mM EDTA, 5 mM EGTA, 15 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 mM benzamid, 1 mM PMSF, 1 mM vanadate, and 0.2% (vol/vol) triton. The lysates were then centrifuged (10,000 g, 15 min, 4°C), and the supernatants were stored at -80°C.

Adenoviral infections. Cardiomyocytes were infected (multiplicity of infection 40), as described in the legend for Fig. 8, with two different adenoviral constructions, one expressing both CA-AMPK and GFP, the other expressing GFP alone (60). We verified that, after 2 days, all the cardiomyocytes were infected and expressed CA-AMPK (by immunoblot with the anti-c-myc antibody from Cell Signaling) and/or GFP (by fluorescence microscopy).

Glucose uptake measurement. In muscle, glucose uptake (i.e., glucose transport and phosphorylation) approximates the rate of glucose transport because the rate of phosphorylation by hexokinase exceeds that of glucose transport. We measured glucose uptake by the detritiation rate of [2-\(^{3}\)H]glucose (10), which occurs after glucose phosphorylation during the rapid isomerization of hexose-6-phosphates catalyzed by phosphoglucose isomerase. Because the rate of this reaction is ~100 times faster than that of hexokinase, the rate of detritiation adequately estimates glucose uptake as defined above. However, it should be noted that this method underestimates the true rate of glucose uptake. This underestimation results mainly from incomplete detritiation and to a lesser extent from glycogen synthesis and may amount to 20–25% (for a review, see Ref. 16). To measure glucose detritiation, tracer amounts (0.2 µCi/ml, Amersham) of tritiated glucose were added to the medium (including 5.5 mM glucose) 30 min before the end of the different treatments. Medium was then taken to separate tritiated water from tritiated glucose by column chromatography (10).

Enzyme assays and phosphorylation state measurements. AMPK activity was assayed in the presence of 0.2 mM AMP in a 10% (wt/vol) polyethylene glycol 6000 fraction (44). PKB activity was measured in immunoprecipitates (anti-PKB antibody from Upstate) as previously described (7). One unit of AMPK or PKB activity corresponds to 1 nmol of product formed per minute under the assay conditions. The phosphorylation state of AMPK, PKB, and ACC was measured on immunoblots by use of anti-phospho-Thr172 AMPK (Cell Signaling), anti-phospho-Ser473 PKB (Cell Signaling), and anti-phospho-Ser212 ACC2 (Cell Signaling) antibodies, respectively. The total amount of AMPK, PKB, and ACC was also measured by immunoblotting using anti-AMPK (Cell Signaling), anti-PKB (Upstate), and anti-biotin (Sigma), respectively. Band intensities were quantified by scanning and processing with the program ImageJ (1.33 for Mac OS X).

Protein measurements and statistics. Protein content was estimated by the method of Bradford with BSA as a standard. The results are expressed as means ± SE. The statistical significance was calculated using a one-way ANOVA by using the Bonferroni for post hoc test.

RESULTS

Insulin resistance in cultured cardiomyocytes. The basal level of glucose uptake varied, among the different experiments, between 75 and 150 nmol·mg protein\(^{-1}\)·h\(^{-1}\) but was always similar in insulin-sensitive and insulin-resistant cardiomyocytes. Short-term (30 min) exposure of cardiomyocytes to increasing concentrations of insulin (from 10\(^{-10}\) to 10\(^{-7}\) M) increased glucose uptake in both insulin-sensitive and insulin-resistant cardiomyocytes (Fig. 1A). As expected, the insulin response of the insulin-resistant cardiomyocytes was signifi-
The different responses of glucose uptake and PKB to insulin in insulin-sensitive and insulin-resistant cardiomyocytes suggest that insulin resistance could not be exclusively attributed to PKB.

Effects of metformin and phenformin on glucose uptake and AMPK activity in insulin-resistant cardiomyocytes. Short-term exposure of insulin-resistant cardiomyocytes to increasing concentrations of biguanides induced an 8- to 10-fold stimulation of glucose uptake and a 3- to 5-fold increase in AMPK activity, the latter being confirmed by the increase in the phosphorylation state of AMPK on Thr172 (Fig. 2). The concentrations required for maximal effect were 0.5 and 10 mM for phenformin and metformin, respectively. With both biguanides, a tight relationship between the activation of AMPK and the stimulation of glucose uptake was observed. This correlation was also found in time-course experiments (data not shown).

Comparison of the effects of insulin and phenformin on glucose uptake, AMPK, ACC, and PKB phosphorylation in insulin-sensitive and insulin-resistant cardiomyocytes. Figure 3, inset, shows the experimental protocol used to compare the effect of insulin and phenformin, alone or in combination, in insulin-sensitive and insulin-resistant cardiomyocytes. In insulin-sensitive cardiomyocytes, saturating concentration of insulin (10^{-7} M) or phenformin (0.5 mM) stimulated glucose uptake to a similar extent (Fig. 3A). Maximal glucose uptake was obtained when cells were incubated in the presence of both insulin and phenformin, the combined effect of insulin and biguanide being larger than the sum of their individual effects, suggesting a synergistic mechanism. In insulin-resistant cells, insulin was able to stimulate glucose uptake as efficiently as in insulin-sensitive cells (as previously seen in Fig. 1), but, surprisingly, phenformin exerted a significantly greater effect than insulin (Fig. 3A). Indeed, this phenformin-induced glucose uptake stimulation was higher than that obtained in insulin-sensitive cells. Finally, the combined exposure of cardiomyocytes to phenformin and insulin led to a glucose uptake stimulation similar to that previously evidenced in insulin-sensitive cells.

Phosphorylation of AMPK by phenformin was similar in insulin-sensitive and insulin-resistant cells (Fig. 3B). This phenformin-induced AMPK phosphorylation was partially inhibited by insulin in insulin-sensitive but not in insulin-resistant cardiomyocytes. A similar pattern was observed on the phosphorylation state of ACC, a downstream target of AMPK, although the inhibition by insulin did not reach statistical significance (Fig. 3C).

The phosphorylation state of PKB has been measured to further dissect the effects of phenformin in insulin-sensitive and insulin-resistant cells. As previously shown in Fig. 1, the phosphorylation of PKB induced by insulin was significantly less in insulin-resistant than in insulin-sensitive cardiomyocytes (Fig. 3D). Although phenformin alone did not modify PKB phosphorylation in insulin-sensitive cells, it induced PKB phosphorylation in insulin-resistant cardiomyocytes. Finally, incubation with insulin and phenformin resulted in a striking and similar stimulation of PKB phosphorylation in both insulin-sensitive and insulin-resistant cardiomyocytes. Measurements of PKB activity confirmed the changes in PKB phosphorylation state (insulin-sensitive cells: control 27 ± 10, insulin 158 ± 20, phenformin 38 ± 19, insulin + phenformin 303 ± 49 mU/mg protein; insulin-resistant...
cells: control 87 ± 25, insulin 133 ± 19, phenformin 181 ± 32, insulin + phenformin 349 ± 71 mU/mg protein; n = 3).

To ensure that the synergistic effects of phenformin and insulin were still present at physiological insulin concentrations, we repeated the experiment with 3 × 10^{-9} M insulin. Even at this concentration, phenformin and insulin synergistically stimulated glucose uptake and PKB phosphorylation (Fig. 4). The different concentrations used for the immunoblots in C are 0, 1, 3, 10, and 20 mM for metformin and 5, 15, 50, 150, 500, and 1,500 μM for phenformin. For A and B, values are means ± SE of at least 3 different experiments. *P < 0.05 vs. non-treated cells. Immunoblots in C are representative of 2 different experiments.

Effects of wortmannin on phenformin-induced glucose uptake stimulation, PKB, and AMPK phosphorylation in insulin-sensitive and insulin-resistant cardiomyocytes. To determine the signaling pathway leading to the stimulation of glucose uptake and PKB by phenformin, we used the PI3K inhibitor wortmannin (Fig. 5). Whereas wortmannin totally prevented PKB phosphorylation in all conditions, its effect on the stimulation of glucose uptake varied according to treatment. As expected, wortmannin markedly inhibited the stimulation of glucose uptake by insulin in both insulin-sensitive and insulin-resistant cardiomyocytes. In insulin-sensitive
cells, wortmannin had no effect on the stimulation of glucose uptake by phenformin. However, it prevented the synergistic effect of insulin, decreasing glucose uptake to values similar to those obtained with phenformin alone. In insulin-resistant cardiomyocytes, wortmannin, similarly, attenuated the effects of phenformin alone on glucose uptake to reach values similar to those obtained in insulin-sensitive cells. The effect of wortmannin on insulin-resistant cells incubated with both phenformin and insulin was similar to that obtained in insulin-sensitive cells. In all conditions, the decrease in PKB phosphorylation induced by wortmannin was correlated with a corresponding decrease in PKB activity (data not shown). Similar results were obtained with LY-294002, another PI3K inhibitor (data not shown). Finally, we showed that the effects of wortmannin on glucose uptake were not due to a decrease in the phenformin-induced AMPK phosphorylation (Fig. 5C). In fact, wortmannin induced a slight increase in AMPK phosphorylation.

Effects of metformin on glucose uptake, PKB, and AMPK phosphorylation in insulin-sensitive and insulin-resistant cardiomyocytes. The effects of metformin were similar to those of its analog phenformin (Fig. 6). First, we found that, in insulin-resistant cardiomyocytes, metformin alone was also able to stimulate PKB phosphorylation and glucose uptake more than...
in insulin-sensitive cells. Furthermore, the effects of metformin and insulin on glucose uptake and PKB phosphorylation in both insulin-sensitive and insulin-resistant cells were synergistic. Finally, the effects of wortmannin were also similar. Indeed, in insulin-resistant cardiomyocytes, the stimulation of glucose uptake by metformin was attenuated in the presence of wortmannin and only reached levels similar to those seen in insulin-sensitive cells (control 0.095 ± 0.018, metformin 1.029 ± 0.075, and metformin + wortmannin 0.667 ± 0.081 μmol·mg protein⁻¹·h⁻¹, P < 0.01, in insulin-resistant cells; control 0.068 ± 0.009 and metformin 0.583 ± 0.036 μmol·mg protein⁻¹·h⁻¹ in insulin-sensitive cells, n = 3). As shown in Fig. 7, left, PKB phosphorylation followed a similar pattern as that previously observed with phenformin. Indeed, metformin increased PKB phosphorylation only in insulin-resistant cells, and this was blocked by wortmannin. Finally, as for phenformin, wortmannin induced a slight increase of the metformin-induced AMPK phosphorylation (Fig. 7). Altogether, these results confirm that the effects of metformin and phenformin were grossly similar.

Some differences between the two biguanides were nonetheless apparent. First, in insulin-resistant cells in metformin experiments, PKB phosphorylation state almost returned to basal level in control conditions. Second, in the same experiments, the stimulation of glucose uptake by 10⁻⁷ M insulin alone tended to be lower, albeit nonsignificantly, in insulin-resistant than in insulin-sensitive cells. Third, the stimulation of glucose uptake in insulin-resistant cells by metformin alone was less elevated than by phenformin alone. One possible explanation for these observations is that our in vitro model of insulin resistance is dynamic and, so, probably slightly different 3 h (for phenformin experiments) than 5 h (for metformin experiments) after insulin removal. This could also explain the larger inhibitory effects of insulin on AMPK phosphorylation in metformin compared with phenformin experiments (Fig. 6).

**Effects of oligomycin on glucose uptake, PKB, and AMPK phosphorylation.** We further investigated the putative role of AMPK by using oligomycin, another potent AMPK activator but not related to known antidiabetic effects. Like biguanides, in insulin-sensitive cardiomyocytes, oligomycin increased AMPK phosphorylation and, in parallel, stimulated glucose uptake independently of PKB and in a wortmannin-insensitive manner (Fig. 7 and Table 1). Moreover, in insulin-resistant cells, the similar unexpected combination of glucose uptake overstimulation and PKB phosphorylation was found when AMPK is activated by oligomycin. Indeed, in insulin-resistant cells, oligomycin stimulated glucose uptake twice as much as in insulin-sensitive cells, and this was brought back by wortmannin (Table 1). As shown in Fig. 7, right, this was also accompanied by an increase in PKB phosphorylation that was blocked by wortmannin. As previously shown for biguanides, the oligomycin-induced AMPK phosphorylation seemed to be slightly increased by wortmannin (Fig. 7).

**Effect of CA-AMPK expression on glucose uptake and PKB phosphorylation.** To verify that effects of biguanides and oligomycin on glucose uptake and PKB phosphorylation were due to AMPK activation, we expressed a constitutively active form of AMPK, CA-AMPK, in insulin-resistant cardiomyocytes by adenoviral infection (Fig. 8). This adenoviral-mediated infection of cardiomyocytes was carried out several hours after the beginning of the insulin-resistance induction by exposure to 10⁻⁷ M insulin. Interestingly, CA-AMPK induced a significant stimulation of glucose uptake and potent phosphorylation of both PKB and ACC that mimicked perfectly the oligomycin effects evidenced in these insulin-resistant cells (Fig. 8).

**DISCUSSION**

The aim of the present study was to clarify the complex interplay between AMPK and insulin signaling in insulin-sensitive and insulin-resistant cardiomyocytes (Fig. 9). The results can be summarized as follows: 1) insulin-stimulated glucose uptake and PKB phosphorylation are attenuated in insulin-resistant compared with insulin-sensitive cardiomyocytes; 2) biguanides and oligomycin activate AMPK and increase glucose uptake in both insulin-sensitive and insulin-resistant cardiomyocytes, albeit to a larger extent in insulin-resistant cells; 3) these drugs also increase the phosphorylation state of PKB, albeit solely in insulin-resistant cells; 4) the greater increase in glucose uptake and phosphorylation of PKB seen in the presence of AMPK activators in insulin-resistant cells is blocked by wortmannin, an inhibitor of PI3K, the lipid kinase responsible for the insulin-induced activation of PKB; 5) when combined, insulin and AMPK activators induce a synergistic increase in glucose uptake and PKB phosphorylation in both insulin-sensitive and insulin-resistant cells. This
synergistic effect is completely blocked by wortmannin; and 6) the effects of AMPK activators on glucose uptake and PKB phosphorylation can be mimicked by the expression of CA-AMPK. Taken together, these results show that activation of AMPK can over- or reactivate insulin signaling and increase glucose uptake stimulation via the over- or reactivation of PKB by acting on PI3K (Fig. 9).

Because of the lack of availability of specific AMPK modulators, we had to resort to the use of nonspecific activators. Although biguanides, such as metformin and phenformin, have been shown to activate AMPK, the mechanism by which they act remains uncertain but seems to be independent of the modification of adenine nucleotide concentrations (21, 26). On the other hand, oligomycin requires energy depletion and increase of the AMP-to-ATP ratio to activate AMPK (44). We cannot therefore totally exclude that their effects on glucose uptake and PKB phosphorylation could be unrelated to AMPK activation. However, the fact that very similar observations were made using CA-AMPK indicates the probable involvement of the kinase in these effects.

Synergistic effects of biguanides and insulin on glucose uptake in insulin-sensitive cells. The present experiments show that, in insulin-sensitive cells, insulin and AMPK activators stimulate glucose uptake to a similar extent but act via different signaling pathways. Indeed, insulin action is mediated by a PI3K/PKB signaling pathway that is completely blocked by the PI3K inhibitor wortmannin (57, 59), whereas AMPK activator effects are independent of the PI3K/PKB pathway. Our experiments also demonstrate that when cells are simultaneously exposed to insulin and biguanides, the resultant increase in glucose uptake is larger than the mere sum of the two separate effects, implying a synergistic effect of these drugs on the stimulation of glucose uptake. A similar synergistic effect of insulin, but only at low concentration, and biguanides has been already shown previously in cardiomyocytes (20). Interestingly, our study shows that this synergistic effect involves a
greater PKB activation than that seen with insulin alone. Furthermore, this effect is PI3K dependent because addition of wortmannin completely blocks PKB phosphorylation and brings the level of glucose uptake back to that seen in the presence of the biguanides alone (Fig. 9).

Effects of AMPK activators and insulin on glucose uptake in insulin-resistant cardiomyocytes. One of the salient findings of our study is the observation that the stimulation of glucose uptake by all the AMPK activators used (biguanides as well as oligomycin) in the absence of insulin is more pronounced in insulin-resistant cells than in insulin-sensitive cardiomyocytes and is associated with an unexpected PI3K-dependent increase in PKB phosphorylation (Fig. 9). Indeed, addition of the PI3K inhibitor wortmannin prevents the phosphorylation of PKB and reduces glucose uptake to levels similar to those seen in insulin-sensitive cells. Moreover, CA-AMPK is able to mimic the effects of these AMPK activators, suggesting that this kinase could play a determinant role. In our experiments, insulin-resistance was induced by incubating cardiomyocytes in the presence of glucose and a high concentration of insulin for several hours. This method is similar to that previously established to induce insulin resistance in adipocytes (53) and has been already used to study glucose uptake regulation (54); effects of antidiabetic drugs, including metformin (39, 41); and O-GlcNAc protein modifications (58) in insulin-resistant myocytes. This method induces alterations of the insulin signaling pathway that are very similar to those seen in diabetic models (39).

Several mechanisms have been proposed to account for insulin resistance, the most likely being phosphorylation of the insulin receptor (IR) substrate-1 (IRS-1) on its serine residues (9). This abnormal phosphorylation of IRS-1 is also known to be induced by an incubation with high insulin concentration as in our model (56). This IRS-1 phosphorylation on serine acts as a negative-feedback loop and thus prevents any further normal insulin signaling. In our model, the wortmannin-sensitive phosphorylation of PKB induced by AMPK activation suggests that the possible targets of AMPK could be PI3K or a step located upstream of this lipid kinase, at the level of IRS-1 or the IR. It has been recently shown that AMPK can phosphorylate IRS-1 (33). However, this result remains controversial because this phosphorylation has been reported both to inhibit (45) or to activate (33) the insulin signaling pathway. More recently, Longnus and coworkers (43) have shown that acute in vivo injection of metformin or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), another AMPK activator, enhanced insulin signaling downstream of PKB in rat hearts (43). Even if the cardiac action of metformin and AICAR in these experiments could have been due to their systemic rather than local effects, it was shown that IR, IRS-1, and PI3K did not seem to be involved in the PKB overactivation. By contrast, Tzatsos and Kandror (56) recently reported that, in cultured cardiomyocytes
adipocytes, AMPK could positively regulate the PI3K/PKB signaling pathway by inhibiting the p70 ribosomal protein kinase (p70S6K) and, subsequently, the p70S6K-dependent phosphorylation of IRS-1 on serine residues. This last explanation is not sufficient to explain the PKB overactivation that occurred in our experiments when cardiomyocytes were exposed to both insulin and AMPK activators. Indeed, we found that AMPK activation did not prevent activation of p70S6K by insulin (data not shown). Nevertheless, as AMPK activators are able to induce PKB activation in the absence of insulin in insulin-resistant cells, we can postulate that a key element of the negative-feedback loop involved in the apparition and maintenance of insulin-resistance should be a good target of AMPK action. Indeed, in the absence of insulin, this remaining negative-feedback loop is the sole leftover of the former insulin incubation and is exclusively present in insulin-resistant cardiomyocytes. This precise mechanism of PI3K/PKB regulation by AMPK in insulin-resistant cardiomyocytes remains to be elucidated.

The AMPK-induced PKB phosphorylation seen in our and in other studies (43, 56) is in keeping with previous observations of our group on PKB activity during ischemia in isolated perfused rat hearts. In these previous experiments, we had indeed observed that although prolonged periods of no-flow ischemia blocked insulin signaling via the decrease in intracellular pH (4), shorter periods of ischemia, typically <5 min, were associated with an increase in PKB phosphorylation, a finding that we were not able to explain until now. On the basis of the present observations, this transient increase in the phosphorylation state of PKB during early ischemia could be due to the activation of AMPK that typically occurs within seconds of the onset of ischemia (44). This hypothesis is reinforced by the fact that, in the same study, perfusion of ischemic heart at an extracellular pH of 8.2 completely pre-

Table 1. Effect of wortmannin on oligomycin-induced glucose uptake

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<th>Control</th>
<th>Oligomycin</th>
<th>Oligomycin + Wortmannin</th>
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<tr>
<td>Insulin-sensitive cardiomyocytes</td>
<td>0.09±0.02</td>
<td>0.36±0.06*</td>
<td>0.34±0.04*</td>
</tr>
<tr>
<td>Insulin-resistant cardiomyocytes</td>
<td>0.07±0.01</td>
<td>0.75±0.08*</td>
<td>0.30±0.06*</td>
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Values are means ± SE of glucose uptake in 3 different experiments, in units of μmol·mg protein⁻¹·h⁻¹. The experimental protocol is schematically depicted in Fig. 7. *P < 0.05 vs. control cells; †P < 0.05 vs. insulin-sensitive cells.
Although the present study is mainly focused on the activation probably involved in the protection of the heart against ischemia-induced overactivation of PKB. Once again, this fact can now be explained by the ischemia-induced AMPK activation, which is then no longer masked by the tremendous drop of intracellular pH. This is also in agreement with recent data showing that AMPK can be phosphorylated by PKB and that this prevents the subsequent phosphorylation of AMPK by its upstream kinases (28). Even if this phenomenon probably explains previous results in perfused hearts (5), it is unlikely to account for our present findings. Indeed, in our experiments, when insulin-sensitive cardiomyocytes were incubated with phenformin alone, wortmannin was found to overactivate AMPK while PKB activity was not modified (Fig. 5). Moreover, as explained above, the inhibition of AMPK activation by PKB requires a temporal sequence of events in which PKB must be activated before AMPK (5, 28), which could not be the case in our experiments because insulin was added after AMPK activation. The regulation of AMPK activity in insulin-resistant cardiomyocytes is rather more puzzling. Indeed, AMPK activation in insulin-resistant cardiomyocytes was lower than in insulin-sensitive cells and was completely insensitive to insulin addition (Figs. 3 and 5–7). In conclusion, inhibition of AMPK activation, in our experiments, most probably occurred by a still-unknown mechanism that must involve part of the insulin signaling pathway. It should thus 1) be downstream of PI3K, 2) be sufficiently active under basal conditions to be inhibited by wortmannin in the absence of insulin, and 3) be partially impaired under insulin resistance.

It has to be mentioned that even if AMPK activation is inhibited by insulin, this inhibition is not sufficient to induce significant downstream inhibiting effects. Indeed, AMPK-induced ACC phosphorylation is only slightly, and not significantly, inhibited (Fig. 3). The absence of downstream effect could come from the fact that AMPK inhibition is relatively small and/or, as insulin is added after AMPK activators, the insulin-induced inhibition occurs after AMPK activation.

Study limitations. This study has several limitations that should be acknowledged. First, in our model, cardiomyocytes were insulin resistant for some hours only. It was not therefore possible to evaluate if the long-term effects of lower concentration of biguanides also involve AMPK. In these short-term experiments, the doses of biguanides required to activate AMPK are indeed much larger than those used in humans for the treatment of Type 2 diabetes. Therefore, we cannot exclude that the mechanisms of action of biguanides in clinical practice also involve AMPK-independent pathway(s), for instance in relation to its mitochondrial effect (17, 19, 22). Second, our
experiments were conducted in an in vitro model of insulin resistance. Part of the results obtained here could be related to the method used to induce this insulin-resistance, i.e., incubation with a high concentration of insulin. Moreover, this model does not take into account the probable role played by dyslipidemia in the development of insulin resistance in diabetic patients. The results obtained here, even if they are partially supported by those of other recently published studies (13, 43, 56), should be confirmed in in vivo genetic models of insulin resistance and diabetes. Nonetheless, our study allowed us to demonstrate the importance of AMPK signaling in the restoration of normal insulin sensitivity in cultured cardiomyocytes. This finding reinforces recent data showing that, in liver, improvements in lipid-induced insulin resistance by metformin could also be mediated by the concomitant activation of AMPK and PKB (13).

Summary and conclusions. The present study shows that biguanides and oligomycin are able to stimulate glucose uptake in cardiomyocytes via an AMPK-dependent pathway. Our data also show that AMPK activators can restore sensitivity of glucose uptake to insulin in insulin-resistant cardiomyocytes by stimulating PKB phosphorylation in a PI3K-dependent manner. Future prospects are needed to investigate if AMPK activation in the diabetic heart is a potential therapeutic approach to treat insulin resistance.

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REFERENCES

30. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumanc W, Petersen KF, Landau BR, and...


