Metabolic actions of metformin in the heart can occur by AMPK-independent mechanisms

Ramesh Saeedi, 1 Hannah L. Parsons, 1 Richard B. Wambolt, 1 Kim Paulson, 1 Vijay Sharma, 1 Jason R. B. Dyck, 2 Roger W. Brownsey, 2 and Michael F. Allard 1

1 Department of Pathology and Laboratory Medicine, James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, University of British Columbia-Saint Paul’s Hospital; and 2 Department of Biochemistry and Molecular Biology, Diabetes Research Group, Life Sciences Institute, University of British Columbia, Vancouver, British Columbia; and 3 Department of Pediatrics, Cardiovascular Research Group, University of Alberta, Edmonton, Alberta, Canada

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AMP-activated protein kinase (AMPK) is considered to be a metabolic fuel gauge activated by decreases in cellular energy state with the activation occurring by allosteric mechanisms, the phosphorylation of the α-catalytic subunit by one or more upstream kinas (AMPK), and/or the inhibition of phosphatase action (21, 22, 31). More recently, it has been shown that AMPK can be activated in cells without changes in energy state by a calcium-dependent pathway involving calcium/calmodulin-dependent protein kinase kinases as upstream AMPK kinases (24, 29, 31, 61) and by long-chain fatty acids (8, 14). Once activated, AMPK inhibits energy-using anabolic pathways and stimulates energy-producing catabolic pathways. In the heart, AMPK is involved in the control of fatty acid oxidation, glucose uptake, and glycolysis (64). AMPK activation in the heart leads to the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) (13, 46), resulting in a reduction in malonyl-CoA levels (36). The reduction in malonyl-CoA, which inhibits carnitine palmitoyltransferase-1, a key enzymatic step that controls the entry of long-chain fatty acids into the mitochondria, is responsible for the stimulation of fatty acid oxidation (36, 52). The oxidation of fatty acids is facilitated by AMPK-induced increases in vascular lumen lipoprotein lipase (4) and the translocation of the fatty acid transport protein FAT/CD36 to the sarcolemma to increase the cellular uptake of fatty acids (41). The activation of AMPK in the heart is also accompanied by the translocation of glucose transporters to the sarcolemma and enhanced rates of glucose uptake (51, 52, 62) as well as the stimulation of glycolysis due to the phosphorylation and activation of phosphofructokinase-2 (43).

Metformin, an insulin-sensitizing biguanide widely used to treat Type 2 diabetes mellitus (59), the metabolic actions of which are believed to be mediated by the AMPK activation in the liver and skeletal muscle (16, 67), has also been shown to activate AMPK in isolated cardiac myocytes (6, 35, 63) and in intact hearts (66). As a consequence, metformin is now widely used as a means to activate AMPK in experimental model systems. The concentration of metformin used with isolated cardiac myocytes (35) and intact hearts (66) was sufficiently high, however, to cause a reduction in high-energy phosphates leading to an elevation of AMP (15, 66), presumably because metformin inhibits complex I of the respiratory chain (20). Such findings indicate that metformin at these concentrations not surprisingly caused the activation of myocardial AMPK via an adenosine nucleotide-dependent mechanism. However, an adenine nucleotide-independent activation of AMPK by metformin has been proposed in other cell types (23), including the liver, where metformin may act to prevent alcohol-induced injury via an AMPK-independent pathway (5).

Of additional importance is the fact that clinically relevant and metabolically active concentrations of metformin are substantially lower than those typically used experimentally (27), raising the distinct possibility that the metabolic effects of metformin occur without alterations in cellular energy status and in the absence of AMPK activation. Although the concentrations of metformin that change energy state activate AMPK...
in the heart, it is not known whether lower concentrations that do not alter AMPK activity have metabolic effects. Moreover, although metformin has been shown to alter metabolism in tissues such as the liver and skeletal muscle (28, 56, 67) and cardiac myocytes (6, 63), the metabolic effects of metformin in the intact working heart remain to be determined.

Thus we set out to test the hypothesis that metformin has AMPK-independent effects on energy metabolism in the heart muscle using isolated working rat hearts and cultured heart-derived H9c2 cells as experimental model systems. Furthermore, we also investigated selected signal transduction pathways potentially responsible for the metabolic actions of metformin.

MATERIALS AND METHODS

Materials

H9c2 (2-1) cells (passage number 12) were obtained from American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada). Metformin, all-trans retinoic acid, SP-600125, SB-203580, and PD-98059 were obtained from Sigma (St. Louis, MO). 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C) was obtained from Merck (San Diego, CA). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzoypyran-4-one (LY-294002) was obtained from Cell Signaling Technology (Danvers, MA).

Animals

Male Sprague-Dawley rats weighing 350–450 g were housed in a temperature-controlled (22 ± 1°C) and light-controlled (12-h:12-h light-dark cycle) room. The rats had free unlimited access to food and water. The care of and the experiments performed on the animals were approved by the Institutional Animal Care Committee and were in accordance with the guidelines set forth by the Canadian Council on Animal Care and with the American Physiological Society “Guiding Principles for Research Involving Animals and Human Beings.”

Isolated Heart Preparation and Perfusion Protocol

Hearts from halothane (3% to 4%)-anesthetized rats were isolated and perfused as working preparations with Krebs-Henseleit (KH) solution under normoxic nonischemic conditions at a left atrial preload of 11.5 mmHg and an aortic afterload of 80 mmHg, as described (3). The KH solution contained 1.2 mM [1-14C]palmitate prebound to 3% bovine serum albumin, 5.5 mM [5-3H]glucose, and 100 mM Na+ K+ ATPase, and sealed with a rubber stopper fitted with a scintillation vial containing 95% O2-5% CO2 at 37°C in a humidified atmosphere of 95% O2-5% CO2 (7). The differentiation toward a cardiac phenotype was induced by culture in DMEM containing 1% horse serum and 0.1 µM all-trans retinoic acid for 4 days (45). Retinoic acid was prepared in the dark in DMSO and stored at −20°C until use. The concentration of DMSO in the culture media was <0.2%. The media was changed every day.

For metformin treatment, H9c2 cells were treated with or without 2 mM metformin in KH solution containing 0.4 mM [1-14C]palmitate prebound to 3% bovine serum albumin, 5.5 mM [5-3H]glucose, and 10−5 M insulin for 8 h. We found that 8 h was required for metformin to achieve maximal metabolic effects (data not shown), in keeping with view that metformin is poorly taken up by cells and prolonged treatment is needed for maximal effects (27). In selected experiments, AMPK was inhibited by either Compound C (67) or a dominant-negative form of AMPK (DN-AMPK) by means of adenosine gene transfer. The recombinant, replication-deficient adenoviral containing mutant DN-AMPK mutant was constructed, as described (30). Briefly, cDNA containing a dominant-negative catalytic α2-subunit with a mutation in which aspartic acid 157 was altered to alanine (55, 60) was subcloned into a pAdTrack shuttle vector, linearized with SfiI and inserted into adenovirus using the pAdEasy-1 system for homologous recombination in Escherichia coli. The recombinant adenovirus vector contains a reporter gene of green fluorescent protein (GFP), which serves as a marker of successful viral infection and protein expression.

Compound C (40 µM) was preincubated with differentiated H9c2 cells for 30 min before the addition of 2 mM metformin (67). For the viral-mediated transfer of DN-AMPK, differentiated H9c2 cells were infected with adenoviruses containing either GFP or DN-AMPK at a multiplicity of infection (MOI) of 150 virus particles/cell. The cells were allowed to express protein for 24 h before the addition of metformin.

Myocardial Fatty Acid Oxidation, Glucose Oxidation, Glycolysis, and Glucose Uptake

In hearts, palmitate oxidation was determined by the quantitative collection of 14CO2 released from [1-14C]palmitate as a gas and dissolved in the perfusate as 14C bicarbonate (3). Rates of glycolysis were determined by quantitatively measuring the rate of 3H2O production released into the perfusate from [5-3H]glucose (3). During its catabolism in the glycolytic pathway, [5-3H]glucose is completely detrified at the enolase and triose phosphate isomerase steps of the pathway (47). Perfusion and gas samples were taken every 10 min of perfusion. Rates of glucose uptake by the heart were determined using a previously described method (11). Metabolic rates are expressed as nanomoles per minute per gram dry weight.

In cultured H9c2 cells, radiolabeled palmitate or glucose was added to the media 7 h after exposure to metformin or vehicle, and rates of fatty acid oxidation and glycolysis were measured over a subsequent 1-h period, as described (3, 26). Briefly, preoxygenated KH solution containing [U-14C]palmitate (0.4 µCi/µmol) complexed to BSA was added to the cells in a final volume of 2 ml. The flasks were then sealed with a rubber stopper fitted with a scintillation vial containing cellulose filter paper soaked with 0.3 ml of 1 M hyamine hydroxide. Rates of fatty acid oxidation were measured by the quantitative
collection of $^{14}$CO$_2$ released as a gas and dissolved in the cell media as $^{[14]}$C bicarbonate. After incubation for 1 h at 37°C, the reaction was stopped by the injection of 5 M H$_2$SO$_4$ (0.5 ml), which also liberates $^{14}$CO$_2$ dissolved in the KH solution. The filter papers were taken for scintillation counting after 2 h of gentle shaking at room temperature (26). As in the intact heart, rates of glycolysis were determined by quantitatively measuring the rate of $^3$H$_2$O production released into the perfusate from $[5$-$^3$H]glucose (3). A trace amount of $[5$-$^3$H]glucose (1.0 μCi/ml) was added to the media 7 h later to determine rates of glycolysis over a subsequent 1-h period. Glucose uptake by H9c2 cells was measured as described (65). Metabolic rates are expressed as nanomoles substrate per hour per milligram protein.

Activity of AMPK and Other Kinases

The measurement of isoform-specific AMPK activity was determined on myocardial homogenates or H9c2 cell lysate, as described with minor modifications (2, 8). Briefly, H9c2 cells or samples of frozen myocardium were homogenized in buffer containing (in mM) 50 Tris, 1 EDTA, 1 EGTA, 50 NaF, 5 Na$_4$P$_2$O$_7$, and 1 DTT and 0.25 M mannitol. After centrifugation, the supernatant containing ~500 μg myocardial protein or 150 μg H9c2 cell protein was incubated with isoform-specific anti-α1 or anti-α2 AMPK antibodies (Upstate, Charlottesville, VA) bound to protein A-Sepharose. The immunoprecipitate was washed and recentrifuged three times at 4°C with AMPK resuspension buffer containing (in mM) 100 Tris, 1 EDTA, 1 EGTA, 50 NaF, 5 Na$_4$P$_2$O$_7$, and 1 DTT and 10% glycerol and 0.12% Triton. The activity of AMPK in the immunoprecipitate was measured by determining the incorporation of $^{32}$P into the synthetic SAMS peptide (in the case of intact hearts) (40) or the AMARA peptide (in the case of H9c2 cells) (9). Additionally, the total AMPK activity was measured from homogenized H9c2 cells by saturated ammonium sulfate precipitation assay using the AMARA peptide (12). The extent of the phosphorylation can be used as a marker of AMPK activity and that of other kinases, using a previously described method (2, 40). ACC is a downstream target of AMPK, and we also determined by immunoblot analysis as a further means to assess AMPK activity and that of other kinases, using a previously described method (65). Metabolic rates are expressed as nanomoles substrate per hour per milligram protein.

Statistical Analysis

Results are expressed as means ± SE. Differences among groups were compared by means of ANOVA using number-crunching statistical software, version 2000 (Statistical Solutions, Saugus, MA). Newman-Keuls was used as a post hoc test to identify the location of significant differences when the ANOVA yielded a significant F-ratio. A P value of <0.05 was considered significant.

RESULTS

Isolated Rat Heart

Heart function. Cardiac output and hydraulic work of metformin-treated hearts were significantly higher than those in vehicle-treated hearts, whereas heart rate was slightly but significantly lower in hearts exposed to metformin (Table 1).

Table 2. Myocardial glucose and glycogen metabolism in hearts perfused with or without 2 mM metformin

<table>
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<tr>
<th>Metabolites in Hearts and H9c2 Cells</th>
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| Adenine nucleotides, creatine, and creatine phosphate were determined in perchloric acid extracts of frozen ventricular tissue (40). Since HPLC measures the total amount of AMP in tissue extracts with the functionally relevant free AMP representing a small fraction of the total, the concentration of free AMP was determined using the colorimetric method (Roche/Hitachi, Mississauga, ON, Canada), and the total myocardial glycogen content and the amount synthesized were measured, as described (40). Glycogen content was determined after the digestion of cells with 0.4 M KOH, followed by the precipitation of glycogen in 66% ethanol, and acid hydrolysis of glycogen to glucose, as described (32). Glucose was measured using a diagnostic kit (Sigma). The glycogen content of cells was expressed as nanomoles of glucose per milligram protein.

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<table>
<thead>
<tr>
<th>Glucose uptake, mmol·min$^{-1}$·g dry wt$^{-1}$</th>
<th>Control</th>
<th>Metformin, 2 mM</th>
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<tbody>
<tr>
<td>2,129±138</td>
<td>1,535±92*</td>
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<tr>
<th>Glycogen, μmol/g dry wt</th>
<th>Control</th>
<th>Metformin, 2 mM</th>
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<tr>
<td>138.4±5.5</td>
<td>130.0±8.2</td>
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<table>
<thead>
<tr>
<th>Glycogen synthesis, mmol·min$^{-1}$·g dry wt$^{-1}$</th>
<th>Control</th>
<th>Metformin, 2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>120.2±6</td>
<td>276.25±25*</td>
<td></td>
</tr>
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Values are means ± SE; n = 4–8 hearts/group. *P < 0.05, significantly different from control.

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Metformin increased palmitate oxidation (Fig. 1) with that of control (control, 14.09 ± 0.59; and metformin, 11.38 ± 0.75 mg/g dry wt; P < 0.05). This difference in the rates of fatty acid oxidation was not simply due to an increase in cardiac work in response to metformin because the rates of fatty acid oxidation remain elevated in metformin-treated hearts when the rates are normalized to cardiac work (data not shown).

Glycolysis (Fig. 1B) and glucose uptake (Table 2) were significantly reduced in response to metformin in hearts perfused with 1.2 mM palmitate. When compared with that of vehicle-treated hearts, overall glycolysis content was not different, although the synthesis of glycogen was increased by metformin (Table 2).

Activity of AMPK and PKB phosphorylation. The myocardial isoform-specific activity of AMPK was not significantly increased by metformin (Table 3). Metformin also failed to increase total AMPK activity measured in myocardial homogenates (data not shown). Neither the phosphorylation of Thr172 of the α-catalytic subunit of AMPK (Fig. 2A) nor the phosphorylation of ACC differed from that in vehicle-treated control hearts (Fig. 2B). The phosphorylation state of PKB in hearts exposed to metformin was determined because metformin has been shown to enhance the insulin-induced phosphorylation of PKB in cardiac myocytes (6). Metformin did not significantly alter PKB phosphorylation (Fig. 2C).

Myocardial high-energy phosphates. Metformin treatment did not significantly alter the content of adenine nucleotides (Table 4). The myocardial content of phosphocreatine was significantly elevated in metformin-treated compared with control hearts (Table 4). Free AMP was not elevated in metformin-treated hearts; in fact, there were no significant differences among groups [control, 0.38 ± 0.08 vs. metformin, 0.20 ± 0.09 μmol/l; n = 5; P = not significant (NS)]. Taken together, these results indicate that, if anything, energy state was improved in the heart by this concentration of metformin.

H9c2 Cells

Effects of metformin on metabolism. Metformin (2 mM) significantly increased rates of glycolysis compared with control values (control, 18.7 ± 1.3 vs. metformin, 35.0 ± 2.2 mmol·h⁻¹·mg protein⁻¹; P < 0.05). Of note, we found that 10

![Figure 2](http://ajpheart.physiology.org/)
Metformin, a clinically relevant concentration, also significantly increased glycolysis above the control rates of glycolysis (control, 18.7 ± 1.3 vs. metformin, 27.4 ± 2.6 nmol·h⁻¹·mg protein⁻¹; P < 0.05). The rates of glucose uptake were also increased significantly by 2 mM metformin in H9c2 cells (control, 24.5 ± 2.4 vs. metformin, 34.9 ± 1.2 nmol·h⁻¹·mg protein⁻¹; n = 6/group, P < 0.05). Fatty acid oxidation rates were significantly lower in H9c2 cells treated with 2 mM metformin compared with those in control (control, 4.6 ± 0.14 vs. metformin, 3.7 ± 0.15 nmol·h⁻¹·mg protein⁻¹; P < 0.05). Metformin did not have any significant effect on total glycogen content (control, 412.6 ± 26.8 vs. metformin, 442.1 ± 44 nmol/mg protein; P = NS).

**AMPK activity.** The activity of AMPK did not differ between control and metformin-treated H9c2 cells (control, 205.87 ± 22.14 vs. metformin, 244.24 ± 34.71 pmol·min⁻¹·mg protein⁻¹; P = NS). Similarly, the phosphorylation state of both AMPK and ACC was not altered by metformin (Fig. 3). Collectively, these data indicate that AMPK is not activated by 2 mM metformin in heart-derived H9c2 cells, in keeping with data obtained in the heart.

**Inhibition of AMPK fails to alter the metabolic actions of metformin in H9c2 cells.** To more clearly determine the role of AMPK in metformin-induced changes in metabolism in H9c2 cells, AMPK was inhibited by molecular and pharmacological means, before treatment with 2 mM metformin. Twenty-four hours after the infection of cells with adenovirus containing DN-AMPK-α2 (MOI, 150), nearly 100% of cells showed green fluorescence (Fig. 4A), indicative of successful viral-mediated gene transfer and protein expression. Importantly, AMPK activity was significantly reduced in H9c2 cells infected with DN-AMPK-α2 compared with that of control cells or cells exposed to adenovirus containing GFP alone (Fig. 4B).

The rates of glycolysis were stimulated by metformin to the same degree, regardless of the presence or absence of DN-AMPK (Fig. 5). The inhibition of AMPK by 40 μM Compound C, a pharmacological inhibitor of AMPK, also significantly reduced AMPK activity (Fig. 6A) but failed to reduce the acceleration of glycolysis induced by metformin (Fig. 6B). Taken together, these findings indicate that the acceleration of glycolysis by metformin at a concentration of 2 mM and under these experimental conditions occurs independently of AMPK activation.

**Metabolic actions of metformin in H9c2 cells occur by p38 MAPK- and PKC-dependent pathways.** The modulation of other signaling pathways, including MAPK, PKC, and phosphatidylinositol 3-kinase (PI3K)-PKB pathways, has been im-
complicated in the cellular effects of metformin (6, 33, 37, 42). As such, we evaluated the role of these pathways in mediating the metabolic response of H9c2 cells to metformin by the use of pharmacological inhibitors. We found that metformin elevated the measured activity of p38 MAPK (control, 12.4 ± 1.6 vs. metformin, 20.0 ± 2.7 pmol·min⁻¹·mg protein⁻¹; n = 9–12; P < 0.05) and increased the phosphorylation of PKC (Fig. 7) in H9c2 cells. The treatment of cells with 10 μM SB-203580, reportedly a selective p38 MAPK inhibitor, significantly reduced but did not abolish the metabolic effects of metformin (Fig. 8A). In contrast, the pretreatment of cells with SP-600125 and PD-98059, specific inhibitors of JNK and ERK pathways, respectively, had no effect on the metformin-induced acceleration of glycolysis in H9c2 cells (data not shown). The pretreatment of cells with 10 nM calphostin C, a potent and specific inhibitor of conventional and novel isoforms of PKC (34, 38), completely abolished the metabolic actions of metformin (Fig. 8B), whereas the PI3K inhibitor LY-294002 (10 μM) did not significantly alter metformin-induced changes in glycolysis (Fig. 8C). Interestingly, insulin did not increase the rates of glycolysis in H9c2 cells (insulin, 19.05 ± 0.75 vs. no insulin, 20.56 ± 0.91 nmol·h⁻¹·mg protein⁻¹; n = 9–19; P = NS).

**DISCUSSION**

In the current study, we found that metformin altered fatty acid oxidation and glucose utilization in the intact heart and isolated heart muscle cells. The metabolic changes induced by metformin in the heart occurred in the absence of a reduction in energy status and without the measurable activation of AMPK. We confirmed that the metabolic effects of metformin occurred independently of AMPK by showing that the inhibition of AMPK activity by either the overexpression of DN-AMPK or by the administration of the AMPK inhibitor Compound C failed to prevent the metabolic effects observed. These findings were extended by demonstrating that the metabolic effect of metformin is abrogated by the pharmacological inhibition of p38 MAPK and PKC.

Metformin has been reported to reduce (10), stimulate (67), or not significantly alter (18) rates of fatty acid oxidation in a variety of tissues and cells. This variability likely reflects the underlying cell- or tissue-specific characteristics that determine the nature of the metabolic response to metformin. In the current study, we show that metformin stimulates myocardial fatty acid oxidation (Fig. 1A) in the intact heart. This finding serves to emphasize that cell- or tissue-specific characteristics are key determinants of the metabolic response produced by metformin. The exact mechanisms responsible for the cell- and tissue-specific metabolic response are not yet known.

Metformin led to net degradation of endogenous triglycerides in isolated perfused hearts. Endogenous triglycerides have been shown to contribute substantially to the energy production in the heart and to be simultaneously synthesized and degraded (53). With the data available, it is not possible to determine whether the metformin-induced changes in total myocardial triglyceride content are due to reduced synthesis or the enhanced degradation of triglycerides.

The suppression of glucose use by metformin in intact hearts is not surprising with the suppression of glucose catabolism likely being a reflection of the well-recognized glucose-fatty acid cycle in which elevated fatty acid oxidation rates lead to

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**Fig. 5. Rates of glycolysis in Ad.GFP- or Ad.DN-AMPK-treated H9c2 cells in the presence or absence of 2 mM metformin for 8 h. Values are means ± SE. *P < 0.05 vs. corresponding untreated control H9c2 cells; n = 22–24 dishes/group.**

**Fig. 6. AMPK activity (A) and rates of glycolysis (B) in H9c2 cells incubated with or without Compound C. H9c2 cells were preincubated with or without 40 μM Compound C for 30 min before 8-h study in the presence or absence of 2 mM metformin. White bar, control H9c2 cells; light gray bar, Compound C-treated H9c2 cells; black bar, metformin-treated H9c2 cells; and dark gray bar, Compound C plus metformin-treated H9c2 cells. Values are means ± SE. *P < 0.05 vs. control; $P < 0.05 vs. corresponding untreated control H9c2 cells; n = 6 dishes/group.**
a reduction in glucose uptake, glycolysis, and glucose oxidation (49). Metformin reportedly enhances both glucose and fatty acid catabolism in noncardiac tissues (18, 57, 67). Our data indicate that the stimulatory effect of metformin on fatty acid oxidation (Fig. 1A), when present, is the predominant effect in the heart, accompanied by a compensatory reduction in glucose use (Fig. 1B and Table 2).

In contrast to the intact heart, metformin decreased fatty oxidation rates in cultured H9c2 cells, presumably as a consequence of the increased rates of glucose use and the well-recognized reciprocal relationship between fatty acid and glucose use (49). The discrepancy between the intact heart and cultured H9c2 cells may possibly be due to the fact that cultured cells are quiescent with substantially lower energy requirements and that the fate of fatty acid taken up by the cell is directed predominantly toward storage rather than oxidation (48). An additional factor that may have contributed to the discrepant responses of H9c2 cells and hearts to metformin is the finding that H9c2 cells were resistant to the effects of insulin. The exposure of cardiac myocytes to insulin for prolonged periods is known to cause insulin resistance (6).

Several studies on tissue and cells provide support for the concept that metformin activates AMPK (6, 16, 23, 54, 63, 66, 67). However, we found that the activity and phosphorylation state of AMPK, measured in intact rat hearts and heart-derived H9c2 cells, were not significantly altered by metformin, even though the concentration used yielded metabolic effects. It is important to recognize that the failure to detect measurable changes in AMPK activity or the phosphorylation in tissue extracts does not rule out the allosteric activation of AMPK (40), an effect that can be assessed by measuring the phosphorylation of ACC, a downstream target of AMPK. In the current study, the phosphorylation of ACC did not differ between metformin-treated and -untreated hearts and H9c2 cells, in keeping with the measured activity and phosphorylation of AMPK. Thus these findings indicate that AMPK is not activated in the intact heart and H9c2 cells by metformin at the concentration used and under these experimental conditions.

The absence of effects of DN-AMPK and Compound C on the metformin-induced changes in metabolism (Figs. 5 and 6B) confirms that the activation of AMPK is not necessary for the metabolic effects of metformin to occur in the heart muscle, at

Fig. 7. Representative immunoblots and corresponding densitometric analysis of phosphorylation state of PKC protein in H9c2 cells treated with or without 2 mM metformin for 8 h (n = 3 dishes/group). Values are means ± SE and are expressed as arbitrary density units. *P < 0.05 vs. control.

Fig. 8. Role of p38 MAPK, PKC, and PKB in the metabolic actions of metformin in H9c2 cells. Cells were preincubated with 10 μM SB-203580 (p38 MAPK inhibitor; A), 10 nM calphostin C (PKC inhibitor; B), 10 μM LY-294002 (phosphatidylinositol 3-kinase inhibitor; C), or vehicle for 30 min before study in the presence or absence of 2 mM metformin. White bar, control-treated H9c2 cells; light gray bar, inhibitor-treated H9c2 cells; black bar, metformin-treated H9c2 cells; and dark gray bar, inhibitor plus metformin-treated H9c2 cells. Values are means ± SE. *P < 0.05 vs. control; $P < 0.05 vs. inhibitor; @P < 0.05 vs. metformin; n = 6–15 dishes/group.
least without changes in cellular energy status. This result is consistent with the observation that the metabolic effect of metformin in the liver occurs independently of AMPK (19). The finding that the exposure of H9c2 cells to Compound C caused a reduction in measurable AMPK activity is interesting because Compound C is considered an allosteric inhibitor of AMPK (67), the effects of which should disappear during the purification procedure (17). The detection of a measurable reduction in AMPK activity by Compound C, which has also been observed by others in neural tissue (44), indicates that the phosphorylation state of AMPK has been decreased and suggests that Compound C has other as yet uncharacterized actions on AMPK.

Cellular energy status, including the absence of elevation in free AMP concentration, was not impaired by 2 mM metformin in the current experiments (Table 4). This finding contrasts with those of others in which higher concentrations of metformin (5–10 mM) were found to cause a reduction in cellular energy status in cardiac myocytes and intact hearts (15, 66) accompanied by an increase in AMPK activity in the intact heart (66). The reduction in cellular energy status likely occurred because metformin inhibits complex 1 of the respiratory chain when used at high concentrations (20). The fact that cellular energy status was not impaired by metformin in the current experiments (Table 4) indicates that no significant inhibition of complex 1 of the respiratory chain occurred and provides a good explanation as to why AMPK was not activated.

A relationship between metformin-induced AMPK activation and a metabolic effect has been reported previously in adult cardiac myocytes (6, 63). In one study, a lower concentration of metformin (1 mM) activated AMPK and increased glucose uptake in cardiac myocytes, but the duration of the exposure to metformin was very prolonged (18 h) (63). A 4-h duration of exposure to the same concentration of metformin increased glucose uptake but did not activate AMPK significantly (6), whereas the exposure to substantially higher concentrations of metformin (5 and 10 mM) for 4 h caused both the activation of AMPK and the stimulation of glucose uptake (6). The results of these experiments and our own highlight the fact that the metabolic actions of metformin are concentration and time dependent.

The absence of a role for AMPK indicates that other signaling pathways are responsible for the metabolic effects of lower concentrations of metformin in the heart muscle. Investigations in other tissues suggest PKC (39) and p38 MAPK (37) as potential mediators of the effects of metformin in the heart muscle. By the use of selective inhibitors of p38 MAPK and PKC pathways in H9c2 cells, we show that the metformin-induced acceleration of glycolysis in H9c2 cells is partially abrogated by the inhibition of p38 MAPK and completely abrogated by the inhibition of PKC, suggesting that the metabolic effects of metformin are related to p38 MAPK and PKC pathways. It is important to recognize that SB-203580, the p38 MAPK inhibitor used in these experiments, has been shown to influence glucose use independently of p38 MAPK (50), indicating that an alternative means of inhibiting p38 MAPK is required to fully characterize its role.

H9c2 cells were used as a model system in the current experiments. These cells, which were originally derived from rat embryonic ventricle, have morphological characteristics similar to those of immature embryonic cardiomyocytes and biochemical and electrophysiological properties similar to those of adult cardiac cells (25). Such cells, however, cannot be considered completely representative of cardiac myocytes.

Several important corollaries arise from the consideration of the results of our study and those of others. First, the metabolic actions of metformin are dependent on the duration of exposure to metformin and the concentration of metformin used. Second, the activation of AMPK by metformin in the heart muscle requires a reduction in cellular energy status, leading to an elevation of AMP.

GRANTS

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REFERENCES


