Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR

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Rat ventricular papillary muscle incubations with 5-aminoimidazole-4-carboxamide-1-β-D-arabinofuranoside (AICAR), a competitive inhibitor of AMPK, decreased myocardial basal glucose uptake. However, AMPK activity is also increased by myocardial ischemia, and we examined whether AMPK stimulates glucose uptake and GLUT-4 translocation in isolated rat papillary muscles. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an activator of AMPK, as well as cyanide-induced chemical hypoxia and insulin, increased 2-[3H]deoxyglucose uptake two- to threefold. Wortmannin, a PI3K inhibitor, did not affect either AICAR- or the cyanide-stimulated increase in deoxyglucose uptake but eliminated the insulin-stimulated increase in deoxyglucose uptake. Immunofluorescence studies demonstrated translocation of GLUT-4 to the myocyte sarcolemma in response to stimulation with AICAR, cyanide, or insulin. Preincubation of papillary muscles with the kinase inhibitor Iodotubercidin or adenosine 9-β-o-arabinofuranoside (araA), a precursor of ara-ATP (a competitive inhibitor of AMPK), decreased AICAR- and cyanide-stimulated glucose uptake but did not affect basal or insulin-stimulated glucose uptake. In vivo infusion of AICAR caused myocardial AMPK activation and GLUT-4 translocation in the rat. We conclude that AMPK activation increases cardiac muscle glucose uptake through translocation of GLUT-4 via a pathway that is independent of PI3K. These findings suggest that AMPK activation may be important in ischemia-induced translocation of GLUT-4 in the heart.

GLUT-4; ischemia; adenosine 5'-monophosphate-activated protein kinase; 5-aminoimidazole-4-carboxamide-1-β-o-ribofuranoside

Both insulin stimulation and ischemia have been shown to cause the translocation of GLUT-4 to the sarcolemma of heart muscle, where it is biologically active (29, 31, 41). Previous studies in heart and skeletal muscle have demonstrated that insulin-mediated translocation of GLUT-4 requires phosphatidylinositol 3-kinase (PI3K) activation and is inhibited by the PI3K inhibitor wortmannin (4, 8, 33). In contrast, ischemia causes translocation of GLUT-4 in the heart through a pathway that is independent of PI3K activation (8). In addition, both hypoxia and contraction also cause GLUT-4 translocation in skeletal muscle through a PI3K-independent pathway (22, 23, 33, 40), suggesting that a common pathway based on metabolic stress is shared between ischemia- and contraction-stimulated translocation of GLUT-4. However, the mechanisms responsible for the translocation of GLUT-4 to the cell surface in response to ischemia-contraction remain to be elucidated.

One potential regulator of the translocation of GLUT-4 in response to ischemia-contraction is AMP-activated protein kinase (AMPK). AMPK activity increases in the setting of increased intracellular AMP and decreased creatine phosphate and is thought to act as a metabolic stress protein (12, 13, 26). Previous studies have suggested the possibility that AMPK may play a role in the PI3K-independent signaling pathway for GLUT-4 translocation in the ischemic heart or contracting muscle.

First, both myocardial ischemia (19, 20) and skeletal muscle contraction (17, 34, 38) have been shown to cause the activation of AMPK. Second, studies have shown that activation of AMPK with the nucleoside 5-aminoimidazole-4-carboxamidine-1-β-o-ribofuranoside (AICAR) increases glucose uptake in skeletal muscle, which is not inhibited by wortmannin (1, 14, 24).

Although AMPK activation leads to increased myocardial fatty acid utilization in the heart (20), the role of AMPK activation in regulating myocardial glucose uptake is not known. In addition, the effects of AMPK activation on GLUT-4 translocation have not been assessed in either heart or skeletal muscle. The present studies were therefore performed to determine whether activation of myocardial AMPK by AICAR causes the translocation of heart GLUT-4 to the sarcolemma, resulting in increased myocardial glucose uptake in vitro. Further studies examined whether inhibition of AMPK affects the activation of myocardial glucose utilization by AICAR or chemical hypoxia. Finally, we also investigated whether AICAR infusion increases AMPK activity and stimulates GLUT-4 translocation in the intact heart in vivo.

EXPERIMENTAL PROCEDURES

Rat ventricular papillary muscle incubations. The effects of various stimulators and inhibitors of myocardial glucose uptake and phosphorylation were studied in quiescent papillary muscles. Hearts were isolated from fasted (5 h) male Sprague-Dawley rats (300–350 g) and immediately placed in ice-cold Dulbecco's PBS. After the atria were removed, the left
ventricle was cut open, and the anterior and posterior papillary muscles (~3–6 mg) were dissected free. The isolated papillary muscles were initially incubated for 30 min at 37°C in PBS containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose, and 1% BSA with or without wortmannin (0.5 µM). Incubations were then continued either with no other additions or with insulin (10 µM), potassium cyanide (15 mM), or AICAR (1 mM) for 90 min. 2-[1-^3H]deoxyglucose (1 µCi/ml) was present for the final 60 min to estimate rates of glucose uptake and phosphorylation. [U-^14C]mannitol (0.1 µCi/ml) was also added to incubations to correct the heart muscle 2-deoxyglucose content for 2-deoxyglucose present in the extracellular fluid space.

After the incubations, papillary muscles were washed three times with ice-cold saline, blotted dry, and weighed. Muscles were then solubilized at 60°C with Soluene-350 (Packard Instrument, Meriden, CT) and subjected to liquid scintillation counting. In experiments determining the effects of AICAR stimulation on myocardial glycogen content, tissue glycogen content was measured in freeze-dried papillary muscles as previously described (28).

To further elucidate the mechanism of AICAR-stimulated glucose uptake, inhibitors of different pathways were added to papillary muscles before incubation with AICAR. The potential roles of protein kinase C (PKC) activation or adenosine-receptor stimulation in mediating AICAR stimulation of glucose uptake were studied in papillary muscles incubated with 0.5 µM calphostin C (a PKC inhibitor) or 10 µM 8-(sulfophenyl)-theophylline (an adenosine-receptor blocker). The effect of inhibiting AMPK on AICAR- and potassium cyanide-stimulated 2-deoxyglucose uptake was determined by preincubating papillary muscles for 60 min with the protein kinase inhibitor iodothubercidin (50 µM, kindly provided by Dr. Gary Lopaschuk, University of Alberta) or with adenine-β-D-arabinofuranoside (araA, 500 µM), a precursor of araATP (a competitive inhibitor of AMPK; see Ref. 16). Muscles were then incubated with media alone or with the addition of potassium cyanide, AICAR, or insulin, and glucose uptake was estimated as described above.

Immunofluorescence studies. To determine the subcellular localization of GLUT-4, immunofluorescence studies were performed on papillary muscles incubated as described above and then fixed in 0.5% paraformaldehyde at 4°C. Tissue sections were processed and incubated with rabbit anti-GLUT-4 primary antibody and rhodamine-labeled goat anti-rabbit IgG secondary antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) as described previously (41). The slides were then examined using a Zeiss LSM410 confocal microscope. Contrast and brightness settings were chosen so that all pixels were within the linear range. All images are the product of eightfold line averaging.

In vivo AICAR infusion. To confirm the effect of AICAR on AMPK activity and GLUT-4 translocation in vivo, awake, unrestrained chronically catheterized rats (18) received intravenous infusions of either saline or AICAR (40 mg/kg bolus over 2 min followed by a constant infusion of 7.5 mg·kg$^{-1}$·min$^{-1}$ for 80 min). The plasma AICAR concentration was determined spectrophotometrically (9). Glucose was infused intravenously at a variable rate to maintain the arterial plasma glucose concentration at euglycemic levels during AICAR infusion because AICAR infusion can cause hypoglycemia (36).

After the infusion, hearts were removed, and the ventricular muscle was either used for the isolation of intracellular and sarcosomal membranes for GLUT-4 immunoblot analysis or used to determine AMPK activity. Sarcosomal and intracellular membrane fractions were isolated by differen-
nide, or insulin. Wortmannin inhibited insulin-mediated myocardial 2-deoxyglucose uptake but did not attenuate the increase in 2-deoxyglucose uptake mediated by either AICAR or cyanide.

Figure 2 demonstrates the subcellular distribution of GLUT-4 determined by immunofluorescence confocal microscopy. Although there is a relatively homogeneous distribution of GLUT-4 throughout the individual cardiac myocytes in papillary muscles incubated with glucose alone, the addition of AICAR, cyanide, or insulin resulted in a greater amount of GLUT-4 localizing to the sarcolemma of the myocytes. These findings indicate that AICAR causes translocation of GLUT-4 to the cardiac myocyte cell surface to a similar degree as known stimulators of translocation (i.e., insulin and ischemia; see Refs. 29 and 41).

Both activation of PKC and adenosine-receptor activation have been suggested to play a role in the translocation of GLUT-4 and increased muscle glucose uptake in response to increased work or ischemia (15). However, incubation of papillary muscles with the PKC inhibitor calphostin C did not affect the increase in 2-deoxyglucose uptake mediated by AICAR (Fig. 1). In addition, blocking the adenosine receptor with 8-(sulfo-phenyl)-theophylline did not decrease the AICAR-stimulated 2-deoxyglucose uptake in ventricular papillary muscles.

AICAR stimulation results in an increase in skeletal muscle utilization of both glucose and fatty acids (24). To determine whether AICAR stimulation of cardiac muscle AMPK would result in an increase in glucose uptake in the presence of fatty acids, papillary muscles were incubated as described above with glucose (5 mM) in the presence or in the absence of palmitate (0.5 mM) bound to 5% BSA. Under unstimulated conditions, the rate of glucose uptake was reduced by ~30% by the addition of palmitate (Table 1). AICAR (1 mM) increased glucose uptake in papillary muscles almost twofold in both the absence and presence of free fatty acid. In addition, incubation of papillary muscles with AICAR had no significant effect on the tissue content of glycogen (Table 1), which is in keeping with our recent findings of no change in skeletal muscle glycogen content in rats infused with AICAR in vivo (1).

To determine if inhibition of AMPK decreases glucose uptake under conditions of chemical hypoxia, AICAR, or insulin stimulation, papillary muscles were preincubated with a precursor of araATP, a competitive inhibitor of AMPK (16). Preincubation of papillary muscles with araA (500 µM) had no effect on glucose uptake under control conditions (Fig. 3). Similarly, araA had no effect on insulin-stimulated glucose uptake. However, preincubation with araA significantly decreased the uptake of glucose in papillary muscles subjected to
cytide-induced chemical hypoxia and tended to decrease AICAR-stimulated glucose uptake. Furthermore, preincubation of papillary muscles with varying concentrations of araA resulted in a significant dose-dependent decrease in AICAR-stimulated glucose uptake (Fig. 3).

Previous studies have also demonstrated that iodotubercidin can inhibit AMPK activation (16). To determine if iodotubercidin can inhibit AMPK-mediated increases in glucose uptake, papillary muscles were preincubated with iodotubercidin before stimulation with AICAR, cyanide, or insulin. Iodotubercidin (50 µM) decreased cyanide- and AICAR-stimulated glucose uptake to basal levels. In contrast, iodotubercidin had no effect on either basal glucose utilization or insulin-stimulated utilization (Fig. 4). Therefore, two different inhibitors of AMPK, araA and iodotubercidin, can inhibit both AICAR- and cyanide-stimulated glucose uptake.

AICAR was infused in vivo in awake, unrestrained rats to confirm that AICAR activates AMPK and translocates GLUT-4 in the intact heart. The intravenous infusion of AICAR in vivo resulted in an arterial plasma AICAR concentration (0.89 ± 0.07 mM) that was similar to that used for the in vitro incubations (1 mM). The mean arterial blood pressure (115 ± 3 vs. 111 ± 17 mmHg) and heart rate (409 ± 13 vs. 431 ± 19 min⁻¹) were similar for rats infused with AICAR and rats infused with saline. AICAR stimulated whole body glucose uptake, and a steady-state glucose infusion rate of 134 ± 11 µmol·kg⁻¹·min⁻¹ was required to maintain euglycemia. The myocardial AMPK activity in AICAR-infused rats was approximately threefold higher than that for saline-infused animals (Fig. 5; P < 0.0005). In addition, AICAR infusion caused an increase in the sarcomemmal content of GLUT-4 with a concomitant decrease in the intracellular content of GLUT-4 as determined by membrane fractionation, resulting in a 57% increase in the sarcomemmal-to-intracellular content ratio for GLUT-4 (Fig. 6; P < 0.05).

**DISCUSSION**

The present study demonstrates that AICAR, a specific activator of AMPK (6), causes translocation of GLUT-4 to the surface of cardiac myocytes and increases heart muscle glucose uptake. In addition, like hypoxia-ischemia, AICAR causes GLUT-4 translocation through a pathway that does not require activation of PI3K. In addition, both AICAR- and hypoxia-mediated glucose uptake, but not insulin-stimulated glucose uptake, were inhibited by the addition of either araA or iodotubercidin, inhibitors of AMPK. Given the fact that both AICAR and ischemia cause activation of AMPK (6, 19, 20) and translocation of GLUT-4 (31, 41), these results support a role for AMPK activation in the PI3K-independent translocation of GLUT-4 in response to metabolic stressors.

AICAR is taken up by cells, phosphorylated, and accumulates as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside monophosphate, mimicking the ability of AMP to activate AMPK (6). Infusion of AICAR in vivo has previously been shown to cause hypoglycemia in mice (36). It was hypothesized that the hypoglycemia was caused by inhibition of gluconeogenesis, a known effect of AMPK activation (37). Subsequent studies in perfused rat hindlimbs demonstrated that AICAR increases skeletal muscle glucose uptake in the presence of maximally stimulating doses of insulin (24), suggesting that the hypoglycemic effect of AICAR is also due to increased cellular glucose uptake. In addition, recent studies have also demonstrated that AICAR-mediated activation of AMPK stimulates skeletal muscle glucose uptake (1, 14). However, these previous studies did not...
investigate whether AICAR increased muscle glucose uptake by stimulating the translocation of GLUT-4. Thus the present study is not only the first to demonstrate that AICAR increases glucose uptake in the heart but also the first to demonstrate that AICAR causes translocation of GLUT-4 to the cell surface in muscle tissue.

AMP activates AMPK through both covalent and noncovalent mechanisms (13). Increased AMP concentrations cause AMPK kinase to phosphorylate AMPK, thereby increasing its activity. In addition, an increased AMP concentration allosterically activates the enzyme. Myocardial ischemia (27) and skeletal muscle contraction (17) increase the intracellular content of AMP with a concomitant stimulation of AMPK activity (17, 19). AMPK kinase activity is presumably increased by ischemia or muscle contraction, although it has not been studied.

Given the responsiveness of AMPK to the energy charge of the cell, the enzyme has been described as a “fuel gauge” for controlling the supply of energy providing substrates to the cell (12). In keeping with this role, AMPK activation results in a cellular shift favoring catabolic metabolic pathways over anabolic pathways. Specifically, AMPK causes phosphorylation and inactivation of acetyl-CoA carboxylase (3, 39), with a resulting decrease in malonyl-CoA and a shift from lipogenesis (30) to fatty acid oxidation (19, 20). Previous studies suggested that AICAR also stimulates glycogen mobilization and phosphorylase activity in skeletal muscle but did not measure glycogen content directly (42). However, in the present study, the glycogen content of papillary muscles was not affected by AICAR. Similarly, we have recently demonstrated that AICAR infusion does not affect the glycogen content of skeletal muscle (1). Our findings suggest that either phosphorylase is not affected in cardiac muscle or more complex interactions between glucose uptake and increased glycogen breakdown occur with AMPK activation. On the basis of our results, AMPK activation would appear to primarily increase glucose transport rather than affect glycogen mobilization.

The current study, as well as studies in skeletal muscle (1, 14), demonstrates that AICAR stimulation of...
glucose uptake is not attenuated by PI3K inhibition. Our findings also confirm previous studies that demonstrate that insulin-mediated glucose uptake is inhibited by the PI3K inhibitor wortmannin but that hypoxia-stimulated glucose uptake does not decrease with PI3K inhibition (8, 33). Although PI3K activation is not required for ischemia-contraction-stimulated or AICAR-stimulated glucose uptake, it remains unclear whether insulin stimulation and ischemia-contraction stimulation share a common distal pathway that is responsible for GLUT-4 translocation. Recent studies have shown that the protein kinase Akt is activated by insulin stimulation via PI3K but is not affected by skeletal muscle contraction (2), suggesting that, if there is a common pathway, it is further downstream than Akt. Insulin and ischemia-contraction may also cause the translocation of separate pools of intracellular GLUT-4-containing vesicles to the cell surface (5) or may trigger translocation of a common intracellular GLUT-4 pool through distinct distal pathways.

Several other PI3K-independent pathways have been proposed to be responsible for ischemia-contraction-stimulated GLUT-4 translocation, including PKC activation, Jun NH2-terminal kinase activation, p38 kinase activation, nitric oxide release, and adenosine-receptor stimulation (for review, see Ref. 15). Stimulation of cells with phorbol esters causes translocation of GLUT-4, which can be blocked by PKC inhibition (11, 25). However, the present studies suggest that PKC activation does not play a role as a downstream mediator of AMPK-mediated GLUT-4 translocation, which is in keeping with previous studies demonstrating that phorbol esters stimulate GLUT-4 translocation by a pathway that is distinct from hypoxia (11). In addition, AICAR can increase adenosine content (10), which has been shown in some studies to increase glucose uptake during insulin stimulation (21, 35), raising the possibility that adenosine-receptor stimulation was responsible for increased glucose uptake. However, adenosine-receptor blockade with 8-(sulfophenyl)-theophylline did not affect AICAR-mediated increases in glucose uptake in the present study.

Our experiments demonstrated inhibition of AICAR- and cyanide-stimulated glucose uptake with the serine/threonine protein kinase inhibitor iodotubercidin as well as a precursor of araATP, a competitive, but nonspecific, inhibitor of AMPK. In contrast, neither inhibitor affected insulin-stimulated glucose uptake. These findings provide further support for the role of AMPK activation in both AICAR- and cyanide-stimulated glucose uptake. Our results are also in keeping with preliminary studies demonstrating that iodotubercidin decreases postischemic myocardial AMPK activation (32). We cannot exclude the possibility that mechanisms other than AMPK activation may contribute to the cyanide-stimulated translocation of GLUT-4 and increase in glucose uptake in the present study. However, our experiments excluded the possible influences of adenosine receptor stimulation and PKC or PI3K activation. Further evaluation of the role of AMPK in ischemia-contraction-mediated translocation of GLUT-4 will require the development of inhibitors/activators of AMPK with greater specificity or the use of systems in which AMPK is overexpressed or deleted.

Chemical hypoxia appeared to stimulate glucose uptake and phosphorylation to a somewhat greater extent than AICAR (~17% when all of the experiments were combined). Because of the size of isolated papillary muscle, we were unable to determine if AMPK was activated to a greater extent with cyanide than with AICAR. However, AICAR is known to stimulate liver AMPK in vitro (6), and our studies demonstrate that AICAR infusion in vivo activates myocardial AMPK in whole heart. It is possible that other pathways are also activated in cyanide-stimulated heart muscle. Furthermore, although AICAR activation of AMPK stimulates the translocation of GLUT-4, it would not necessarily increase the metabolic demand for glucose, which would conceivably affect the transport and phosphorylation of glucose. This is in contrast to physiological (e.g., exercise) and pathophysiological (e.g., ischemia) states where muscle AMPK activation occurs in response to increased energy needs.

In summary, the present studies support the novel hypothesis that metabolic stressors, such as ischemia, cause the translocation of myocardial GLUT-4 through a pathway that includes AMPK. This pathway is distinct from the insulin-stimulated pathway that requires PI3K activation. The subsequent steps responsible for GLUT-4 translocation in response to changes in the energy charge of the cell require definition.

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