Evidence for 5′AMP-Activated Protein Kinase Mediation of the Effect of Muscle Contraction on Glucose Transport

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The intracellular signaling proteins that lead to exercise-stimulated glucose transport in skeletal muscle have not been identified, although it is clear that there are separate signaling mechanisms for exercise- and insulin-stimulated glucose transport. We have hypothesized that the 5′AMP-activated protein kinase (AMPK) functions as a signaling intermediary in exercise-stimulated glucose uptake. This hypothesis was based on recent studies showing the following: *1*) muscle contraction increases AMPK activity and *2*) perfusion of rat hindlimb skeletal muscles with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a compound that results in increased AMPK activity, increased insulin-stimulated glucose uptake. In the current study, isolated rat epitrochlearis muscles were treated to contract in vitro (via electrical stimulation for 10 min) and/or incubated in the absence or presence of AICAR (2 mmol/l), insulin (1 µmol/l), or wortmannin (100 nmol/l). Both contraction and AICAR significantly increased AMPK activity, while the enzyme was not activated by insulin. AICAR, contraction, and insulin all increased 3-*O*-methylglucose (3MG) transport by threefold to fivefold above basal. The phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin completely blocked insulin-stimulated transport, but did not inhibit AICAR- or contraction-stimulated transport. The increase in glucose transport with the combination of maximal AICAR plus maximal insulin treatments was partially additive, suggesting that these stimuli increase glucose transport by different mechanisms. In contrast, there was no additive effect on glucose transport with the combination of AICAR plus contraction. These data suggest that AICAR and contraction stimulate glucose transport by a similar insulin-independent signaling mechanism and are consistent with the hypothesis that AMPK is involved in exercise-stimulated glucose uptake. *Diabetes* 47:1369–1373, 1998

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AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, 5′AMP-activated protein kinase; CPT 1, carnitine palmitoyltransferase; KRBB, Krebs-Ringer bicarbonate buffer; 3MG, 3-*O*-methyl-D-glucose; PI 3-kinase, phosphatidylinositol 3-kinase; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide.

The performance of regular physical exercise results in numerous health benefits including a reduced risk of developing type 2 diabetes (1). Physical exercise has also been widely accepted as a clinically important modality to decrease blood glucose concentrations in patients with diabetes (2). Part of the mechanism through which exercise results in lower blood glucose concentrations involves the acute effects of muscle contraction to increase the rate of glucose uptake into the contracting skeletal muscles and the increase in insulin sensitivity in the period following exercise. Despite the physiological importance of exercise in regulating glucose uptake in skeletal muscle, the molecular mechanisms that mediate this important phenomenon are still not fully understood. However, it is now known that there are distinct intracellular signaling mechanisms that lead to exercise- and insulin-stimulated glucose uptake in skeletal muscle (3).

We have hypothesized that the 5′AMP-activated protein kinase (AMPK) is a key signaling intermediary in the regulation of glucose uptake during exercise. AMPK is a heterotrimeric protein that is activated by an increase in the AMP:ATP ratio and creatine:phosphocreatine ratio, via a complex mechanism that involves allosteric regulation of the subunits, phosphorylation by an AMPK kinase, and decreases in phosphatase activities (4,5). AMPK has been proposed to function as a "fuel gauge" in mammalian cells, switching off ATP-consuming pathways and switching on alternative pathways for ATP regeneration when the cell senses low fuel (decreased ATP) (4). To date, the few studies of AMPK activity in contracting skeletal muscle are consistent with this fuel-gauge hypothesis. AMPK activity is increased in response to both physical exercise (6) and electrical stimulation to produce muscle contractions in situ (7,8). Activation of AMPK results in the phosphorylation and inactivation of acetyl-CoA carboxylase, decreasing malonyl-CoA and thereby relieving inhibition of carnitine palmitoyltransferase (CPT 1) and allowing an increase in fatty acid oxidation during exercise (6–8). A specific role for AMPK in the regulation of glucose uptake was previously proposed when it was discovered that perfusion of the isolated rat hindlimb with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) increased glucose uptake by approximately twofold in the presence of 200 µU/ml insulin (9). AICAR is a compound that is taken up into intact cells, including muscle, and phosphorylated to form 5-aminoimidazole-4-carboxam-

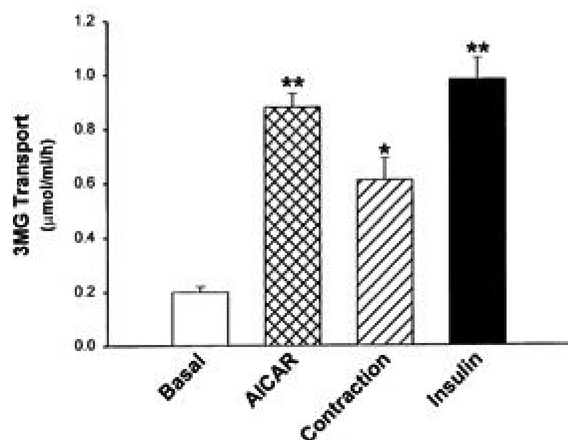

FIG. 1. Effects of AICAR, contraction, and insulin on 3MG transport in isolated rat epitrochlearis muscles. Muscles were preincubated in KRBB followed by treatment with AICAR (2 mmol/l, 30 min), contraction (10 min), or insulin (1 µmol/l, 30 min). After treatment, 3MG transport was measured in the muscles as described in METHODS. *P < 0.001, **P < 0.0001 versus basal. n = 7–22 per group.

ide ribonucleotide (ZMP), the monophosphorylated derivative that mimics the effects of AMP on AMPK (9–11). These intriguing findings together suggest that activation of AMPK is one of the first events in the contraction-induced increase in glucose uptake into skeletal muscle fibers.

In the current study, we have performed experiments to address this hypothesis on the mechanism of contraction-stimulated glucose transport. First, we determined if AICAR, in the absence of insulin, can increase skeletal muscle glucose transport. Next, we determined if, similar to contraction-stimulated glucose transport, AICAR-stimulated glucose transport is wortmannin-insensitive. Finally, we investigated the possibility that AICAR stimulates glucose transport through the same or different pathways as insulin and contraction by determining if the combination of AICAR plus insulin and/or AICAR plus contraction have additive effects on glucose transport. All of our findings are consistent with the hypothesis that AMPK is involved in mediating contraction-stimulated glucose transport in skeletal muscle.

RESEARCH DESIGN AND METHODS

Muscle incubation and contraction. Male Sprague-Dawley rats (Taconic, Germantown, NY) weighing 120–130 g were fasted overnight before the study. Rats were killed and the epitrochlearis muscles were rapidly removed and treated as previously described (12–15) with some modifications. Both ends of each epitrochlearis muscle were tied with suture (silk 4–0) and mounted on an incubation apparatus (Harvard Apparatus, Holliston, MA) with resting tension set to 0.5 g. Muscles were preincubated in 6 ml Krebs-Ringer bicarbonate buffer (KRBB) (117 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 24.6 mmol/l $NaHCO_3$) containing 8 mmol/l D-glucose for 20 min. The muscles were then incubated for 30 min in KRBB containing 8 mmol/l D-glucose in the absence or presence of 2 mmol/l AICAR and/or 1 µmol/l insulin. When contracted, muscles were stimulated during the last 10 min of this incubation period (train rate = 1/min, train duration = 10 s, pulse rate = 100 pulse/s, duration = 0.1 ms, volts = 100 V). When added, wortmannin (at the indicated concentration) was present during the entire experiment. The maximal concentration of vehicle (DMSO) was 0.1%, which did not affect any assay. The buffers were continuously gassed with 95% O_2–5% CO_2, and maintained at 37°C. Muscles were then used to measure AMPK activity or 3-O-methyl-D-glucose (3MG) transport.

AMPK activity. For the measurement of AMPK activity, muscles were immediately frozen in liquid nitrogen at the end of the incubation period. Muscles were weighed and then homogenized in buffer (1:19, wt/vol) containing 10 mmol/l Tris-HCl (pH 7.5), 100 mmol/l mannitol, 50 mmol/l NaF, 1 mmol/l EDTA, 10 mmol/l β-mercaptoethanol, 10 ml/l aprotinin, 10 mg/l leupeptin, 10 mg/l anti-trypsin, and centrifuged at 48,000g for 30 min. AMPK was precipitated from an exact volume of the supernatant by the addition of an equal volume of the homogenate buffer containing 288 mg/ml ammonium sulfate and by incubating tubes for 30 min on ice. The precipitate was collected by centrifugation at 48,000g for 30 min, and the pellet was dissolved in 10% of the original volume of the homogenate buffer. AMPK activity was determined on this extract by the method described previously (6) using the SAMS peptide as substrate (16).

3MG transport. Separate muscles were used for the measurement of 3MG transport. After the incubation period (muscle incubation and contraction) muscles were rinsed in 7.5 ml KRBB containing 8 mmol/l D-mannitol at 30°C for 10 min, and then transport was measured in 2 ml KRBB containing 1 mmol/l 3-O-[^{3}H]-methyl-D-glucose (1.5 µCi/ml) and 7 mmol/l D-[^{14}C]-mannitol (0.3 µCi/ml) (New England Nuclear, Boston, MA) at 30°C for 10 min. AICAR, insulin, and/or wortmannin were added to each buffer if present during the previous incubation period. Transport was terminated by dipping muscle in KRBB containing 80 µmol/l cytochalasin B at 4°C, and muscles were frozen in liquid nitrogen. Muscles were weighed and processed by incubating in 300 µl of 1 mol/l NaOH at 80°C for 10 min. Digestates were neutralized with 300 µl of 1 mol/l HCl, and particulates were precipitated by centrifuging at 13,000g for 2 min. Radioactivity in aliquots of the digested protein was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated.

Statistical analysis. Data are presented as means ± SE. Comparison of means was made by one-way analysis of variance (ANOVA) followed by post hoc comparison with Fisher's protected least significant difference method.

RESULTS

AICAR stimulates glucose transport in the absence of insulin. We first determined if AICAR stimulates glucose transport in the isolated epitrochlearis muscles in the absence of insulin. Muscles were incubated in KRBB in the absence or presence of 2 mmol/l AICAR, and then glucose transport was measured using the protocol described above. Figure 1 shows that incubation of epitrochlearis muscles with AICAR significantly stimulates glucose transport by 4.3-fold. Insulin and contraction also increased glucose transport by 4.8- and 3.0-fold, respectively (Fig. 1).

AICAR- and contraction-stimulated glucose transport are not inhibited by wortmannin. Activation of phosphatidylinositol 3-kinase (PI 3-kinase) is necessary for insulin-stimulated glucose transport in skeletal muscle (14,15,17). In contrast, contraction does not increase PI 3-kinase activity (18), and contraction-stimulated glucose transport is insensitive to wortmannin, a PI 3-kinase inhibitor (14,15,17). Thus, if AICAR and contraction stimulate glucose transport by a similar mechanism, then AICAR-stimulated glucose transport should be wortmannin insensitive. Since high concentrations of most inhibitors can have nonspecific effects on glucose transport, we first determined the lowest concentrations in which wortmannin completely blocked insulin-stimulated glucose transport in our isolated muscle preparation (Fig. 2). This experiment revealed that 100 nmol/l of wortmannin was the lowest dose that completely inhibited insulin-stimulated glucose transport, and therefore this concentration was used for subsequent experiments. Wortmannin had no effect on basal rates of glucose transport (Fig. 2). Figure 3 shows the effects of wortmannin on AICAR-, contraction-, and insulin-stimulated glucose transport. In contrast to the effects of wortmannin to completely block insulin-stimulated glucose transport in the epitrochlearis muscles, the inhibitor did not significantly decrease AICAR- or contraction-stimulated glucose transport. Wortmannin had no effect on the development of tension during contraction (data not shown).

Insulin plus AICAR has partially additive effects on glucose transport. The combination of contraction plus a maximally effective dose of insulin has additive or partially additive effects on skeletal muscle glucose transport (12,13,19,20).

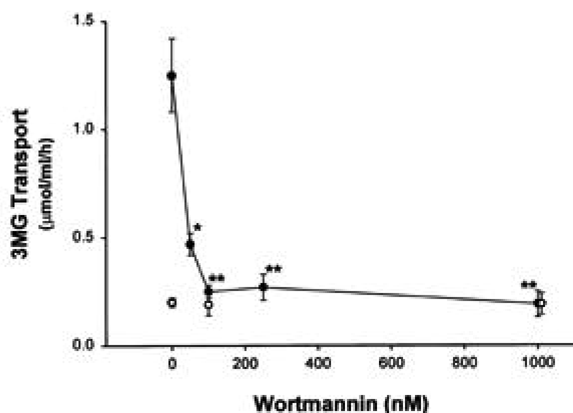

FIG. 2. Effects of wortmannin on insulin-stimulated 3MG transport. Rat epitrochlearis muscles were preincubated in KRBB in the presence of the indicated concentrations of wortmannin followed by incubation in the absence (○) or presence (●) of insulin (1 μmol/l) for 30 min. After treatment, 3MG transport was measured in the muscles as described in METHODS. $*P < 0.0002$, $**P < 0.0001$ versus insulin-stimulated 3MG transport in the absence of wortmannin. n = 3–8 per group.

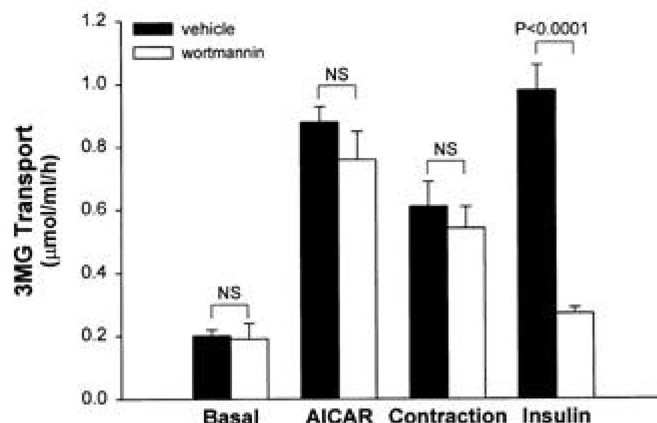

FIG. 3. Effects of wortmannin on 3MG transport. Rat epitrochlearis muscles were preincubated in KRBB in the absence (■) or presence (□) of wortmannin (100 nmol/l) followed by treatment with AICAR (2 mmol/l, 30 min), contraction (10 min), or insulin (1 μmol/l, 30 min). After treatment, 3MG transport was measured in the muscles as described in METHODS. n = 5–22 per group.

These observations are an important piece of evidence in support of the concept that contraction and insulin stimulate glucose transport by different mechanisms. Based on this reasoning, if AICAR and contraction stimulate glucose transport through the same mechanism, then the combination of AICAR plus contraction should not be additive. Furthermore, the combination of AICAR plus insulin should be partially or fully additive. To test these hypotheses, muscles were incubated and stimulated with AICAR, insulin, and/or contraction, and glucose transport was measured as described above. Figure 4 shows that the combination of AICAR plus insulin had partially additive effects on glucose transport, as did the combination of contraction plus insulin. In contrast, the combination of AICAR plus contraction on glucose transport was not additive. Taken together with the studies using wortmannin (Fig. 3), these findings strongly suggest that AICAR and contraction stimulate glucose transport by a similar, insulin-independent signaling mechanism.

AICAR and contraction increase AMPK activity in isolated skeletal muscle preparations. To insure that AICAR and contraction increase AMPK activity in our in vitro preparation, muscles were treated with AICAR, contraction, or insulin and assayed for AMPK activity using the SAMS peptide as substrate. Figure 5 shows that AICAR and contraction increased AMPK activity by 4.6- and 2.2-fold, respectively. Interestingly, similar to the somewhat greater effect of AICAR on stimulating glucose transport (Fig. 1), the effect of AICAR on AMPK activity was also greater than the contraction effect. As has previously been reported (21), insulin did not significantly increase AMPK activity, consistent with the hypothesis that AMPK is involved in mediating contraction-stimulated, but not insulin-stimulated glucose transport.

DISCUSSION

The translocation of GLUT4 from an intracellular location to the plasma membrane and transverse tubules is thought to be the major mechanism by which exercise and insulin increase skeletal muscle glucose transport. Although the signaling intermediaries that lead to exercise-stimulated GLUT4 translocation have not yet been identified, there are several lines of evidence to suggest that there are distinct mechanisms leading to insulin- and exercise-stimulated GLUT4 translocation in skeletal muscle (3). Elucidating the specific molecular mechanisms that result in exercise-induced GLUT4, translocation has been difficult because there is no known cell-surface "exercise receptor" that mediates exercise-stimulated intracellular signaling molecules. Instead, exposure of the contracting muscle fibers to various metabolic and/or mechanical stimuli (e.g., changes in intracellular pH, calcium concentrations, the AMP:ATP ratio) may be the initiating factors that lead to GLUT4 translocation and glucose transport with exercise.

On the basis of the results presented in this report, we propose that AMPK is involved in the signaling pathway leading to exercise-stimulated glucose transport in skeletal muscle. This hypothesis is based on the findings that *1*) AICAR,

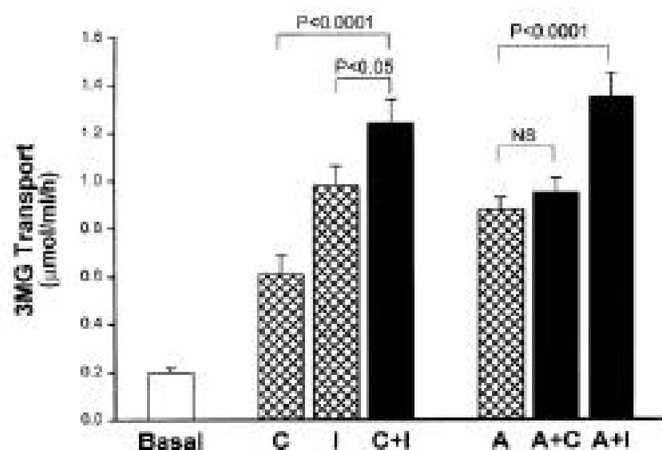

FIG. 4. Additive effects of contraction plus insulin and AICAR plus insulin on 3MG transport. Rat epitrochlearis muscles were preincubated in KRBB followed by treatment with AICAR (A, 2 mmol/l, 30 min), contraction (C, 10 min), or insulin (I, 1 μmol/l) as indicated. After treatment, 3MG transport was measured in the muscles as described in METHODS. n = 5–22 per group.

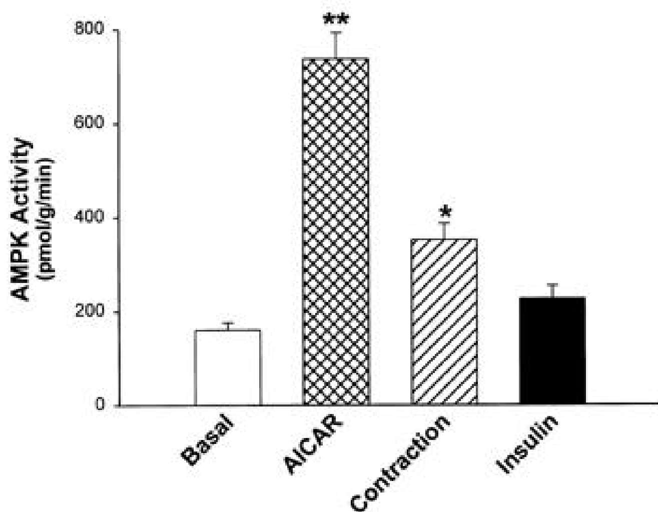

FIG. 5. Effects of AICAR, contraction, and insulin on AMPK activity. Rat epitrochlearis muscles were preincubated in KRBB and stimulated with AICAR (2 mmol/l, 30 min), contraction (10 min), or insulin (1 µmol/l, 30 min) for 30 min. Muscles were frozen in liquid nitrogen, processed, and assayed for AMPK activity as described in METHODS. *P < 0.005, **P < 0.0001 versus basal. n = 6–12 per group.

a precursor to ZMP which is a potent stimulator of AMPK, increases glucose uptake in the absence of insulin; *2*) AICAR- and contraction-stimulated glucose transport are wortmannin-insensitive; *3*) the effect of combining AICAR plus insulin on glucose transport is partially additive, but the combination of AICAR plus contraction is not additive; and *4*) contraction and treatment with AICAR increase AMPK activity in the isolated muscle preparation. A role for AMPK in contraction-stimulated glucose transport is consistent with the concept that the enzyme acts as a fuel gauge in mammalian cells, functioning to increase ATP generation under conditions of increased energy expenditure.

In the contracting muscle, an increase in intracellular AMP concentrations may act as a direct allosteric activator of AMPK and/or activate an AMPK kinase (6–8). Electrical stimulation of rat sciatic nerves to produce muscle contractions in situ for 10–20 min has been shown to increase estimated free AMP concentration by 12-fold and the AMP:ATP ratio by 2-fold in hindlimb muscles (7). These increases were accompanied by a 3-fold increase in AMPK activity (7). Furthermore, it has recently been proposed that AMPK is activated by an increase in the creatine:phosphocreatine ratio, which may occur in response to even moderate-intensity exercise (5). While changes in the AMP:ATP and creatine:phosphocreatine ratio are likely to be the major regulatory factors for the increase in AMPK activity in contracting muscle, it is unlikely that AICAR activates AMPK by altering the intracellular concentration of AMP. Previous studies have demonstrated that AICAR does not change ATP, ADP, or AMP concentrations in perfused rat hindlimb skeletal muscle (9) and in isolated rat hepatocytes (22). Instead, AICAR results in the accumulation of the monophosphorylated derivative, ZMP, which mimics multiple effects of AMP including allosteric activation of AMPK and promotion of phosphorylation by AMPK kinase (9–11,22). In addition to regulating AMPK, AICAR also has been shown to activate glycogen phosphorylase in rat skeletal muscle (23), but not phosphofructokinase (24). Thus, as with all pharmacological agents, AICAR is not completely specific for the activation of AMPK. In this regard, it is important to note that the current studies of glucose transport were done using 3-*O*-methylglucose, a nonmetabolizable hexose that should not be influenced by glucose-6-phosphate concentrations that can be increased by activated glycogenolysis.

A major source of ATP during exercise results from an increase in glycolysis, which is partially dependent on an increase in glucose uptake into the contracting muscle. A putative link between the contraction-induced increase in AMPK and the increase in glucose uptake is not known, but will be an important focus of future research in this area. For the regulation of fatty acid oxidation during exercise, recent reports suggest that activation of AMPK during exercise results in the phosphorylation and inactivation of acetyl-CoA carboxylase, controlling malonyl-CoA, increasing fatty acid oxidation, and leading to the generation of ATP (6–8). Thus, AMPK may have the dual role of controlling both fatty acid and glucose metabolism during exercise, the major fuel sources in the contracting skeletal muscles.

In summary, contraction and AICAR, two stimulators of muscle AMPK activity, increase skeletal muscle glucose transport through a similar, insulin-independent and wortmannin-insensitive mechanism. These observations provide the basis for the novel hypothesis that AMPK is an intermediary in the signaling cascade leading to contraction-stimulated glucose transport. The current experiments should provide the foundation for future studies investigating the role of AMPK in the exercise signaling pathway. These studies will include determining if AMPK is necessary for the increases in both glucose uptake and fatty acid oxidation that occur in response to exercise by using specific inhibitors to AMPK (once these compounds become available), and the identification of signaling molecules "downstream" of AMPK in the exercise signaling cascade leading to glucose transport.

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