

# 5' AMP-Activated Protein Kinase Activation Causes GLUT4 Translocation in Skeletal Muscle

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It has previously been reported that exercise causes an increase in glucose uptake in skeletal muscle and also an increase in 5' AMP-activated protein kinase (AMPK) activity. 5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICA-riboside), an analog of adenosine, is taken up into cells and phosphorylated to form AICA-riboside monophosphate (ZMP), which can also activate AMPK. This study was designed to determine whether the increase in glucose uptake observed with AMPK activation by AICA-riboside is due to GLUT4 translocation from an intracellular location to the plasma membranes, similar to that seen in response to contraction. Rat hindlimbs were perfused with Krebs-Henseleit bicarbonate containing 4% bovine serum albumin, washed bovine erythrocytes, 8 mmol/l glucose, and  $\pm 2$  mmol/l AICA-riboside or  $\pm 60$  nmol/l insulin. Perfusion medium containing AICA-riboside was found to significantly increase AMPK activity, glucose uptake, and GLUT4 translocation in skeletal muscle above basal levels. Insulin-perfused muscles showed significant increases in glucose uptake and GLUT4 translocation, but AMPK activation was not significantly changed from basal levels. These results provide evidence that the increased glucose uptake observed with AMPK activation by AICA-riboside in perfused rat hindlimb muscles is due to an increase in the translocation of GLUT4 to surface membranes. *Diabetes* 48:XXX-XXX, 1999

**M**uscle contraction has been shown to have an insulin-like effect on glucose uptake in rat skeletal muscle, causing an increase in glucose uptake and GLUT4 translocation, although the mechanism is not well understood (1-5). There are now considerable data demonstrating that insulin and contractions

use distinct intracellular signaling mechanisms to stimulate GLUT4 translocation and increase glucose uptake (3-7).

We have recently provided evidence that 5' AMP-activated protein kinase (AMPK) may be part of the signaling mechanism by which muscle contraction increases glucose transport (7,8). AMPK is activated by a decrease in phosphocreatine (9) and an increase in the AMP-to-ATP ratio through a complex mechanism involving allosteric regulation of AMPK and phosphorylation of AMPK by the upstream kinase AMPK kinase (AMPKK) (10,11). AMPK is activated by physical exercise (12), electrical stimulation producing *in situ* muscle contractions (13,14), and contraction of isolated muscles *in vitro* (7).

It was previously proposed that AMPK activation may have a role in regulating skeletal muscle glucose uptake when it was noted that 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranoside (AICA-riboside) in the presence of insulin (200  $\mu$ U/ml) activated AMPK and stimulated both fatty acid oxidation and glucose uptake in the perfused rat hindlimb preparation (8). AICA-riboside is an analog of adenosine that is taken up into the cell and phosphorylated to form AICA-riboside monophosphate (ZMP). ZMP mimics the effects of AMP on AMPK, causing allosteric activation and promoting phosphorylation and activation by the upstream kinase, AMPKK (15). Further study provided evidence for AMPK's role in regulating skeletal muscle glucose transport. We observed that in incubated epitrochlearis muscles, AICA-riboside treatment activates AMPK and increases glucose uptake through a wortmannin-insensitive pathway. In this respect, the effect of AICA-riboside is similar to that of contractions but not to insulin in stimulation of glucose uptake by muscle (7). However, the mechanism for the increase in glucose uptake remained unelucidated.

This study was designed to determine whether the increase in glucose uptake following activation of AMPK (with AICA-riboside) is due to GLUT4 translocation in rat skeletal muscle. Such data will provide additional evidence for AMPK's possible role in the intracellular signaling pathway for contraction-induced glucose uptake.

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Received for publication 23 April 1999 and accepted in revised form 19 May 1999. Posted on the World Wide Web at [www.diabetes.org/diabetes](http://www.diabetes.org/diabetes) on <<date>>.

AICA-riboside, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; AMPK, 5' AMP-activated protein kinase; AMPKK, 5' AMP-activated protein kinase kinase; BSA, bovine serum albumin; IMP, inosine monophosphate; KHB, Krebs-Henseleit buffer; TBS, Tris-buffered saline; ZMP, AICA-riboside monophosphate; ZTP, AICA-riboside triphosphate.

## RESEARCH DESIGN AND METHODS

**Animals.** Male Sprague-Dawley rats (Sasco, Omaha, NE) weighing  $252 \pm 4$  g were housed in individual cages in a temperature-controlled (20-22°C) and light-controlled (12:12-h light-dark cycle) room. Rats were fed *ad libitum* with Harlan Teklad (Madison, WI) rodent diet and had unlimited access to water. The evening before the perfusions, the rats were given only 3-5 g of food.

**Hindlimb perfusion.** All procedures were approved by the Institutional Animal Care and Use Committee. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The surgical and perfusion techniques used were similar to those

originally described by Ruderman et al. (16). Briefly, catheters were inserted into the abdominal aorta and inferior vena cava, and the tips were advanced to the level of the iliac arteries and veins. Blood vessels to the tail and abdominal region were ligated. After flushing the vessels with Krebs-Henseleit buffer (KHB) plus heparin (80 U.S. Pharmacopeia [USP] units i.a.), the hindlimbs were perfused with media (37°C) composed of washed bovine erythrocytes and KHB containing 4 g/100 ml bovine serum albumin (BSA) (Sigma, St. Louis, MO) and 8 mmol/l glucose. The hindlimbs were perfused for a 25-min washout period before starting the timed perfusion. AICA-riboside (Sigma) was added to the perfusate 5 min before the end of the washout period (time required for the media to transverse the tubing from the reservoir to the rat). The final concentration of AICA-riboside in the perfusate was 2 mmol/l. In the insulin-treated muscles, 60 nmol/l insulin (Humulin; Eli Lilly, Indianapolis, IN) was present in the media during the washout period. The KHB/BSA was filtered sequentially through 5- and 1- $\mu$ m filters before the day of the perfusion and filtered through a 0.45- $\mu$ m filter on the day of the perfusion just before combination with the erythrocytes. The final hematocrit was  $37 \pm 2\%$ . The pH of the perfusate was  $7.41 \pm 0.05$ .

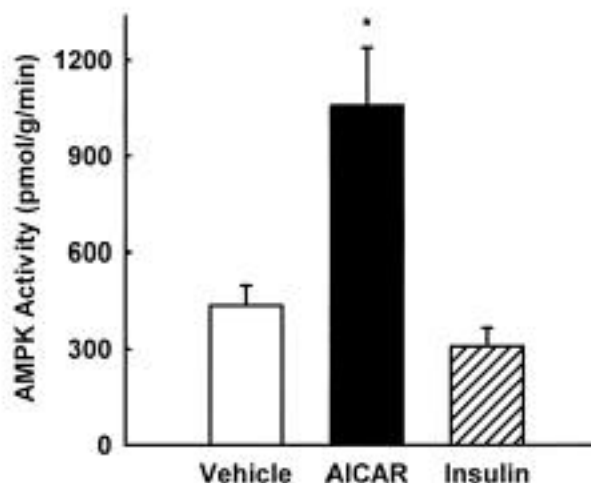
The perfusions were carried out in a plexiglass perfusion chamber (37°C), similar to that described by Gorski et al. (17). The perfusate was oxygenated as it traversed a silastic tubing coil (6 m, 0.16 cm inside diameter, 0.24 cm outside diameter) in a 1-L chamber equilibrated with 95% oxygen and 5% carbon dioxide. Rats were killed with an overdose of pentobarbital directly to the heart once the perfusion was started. Hindlimbs were perfused at a rate of 9 or 10 ml/min, depending on the size of the rat. Arterial and venous perfusate samples were taken at 0, 15, 30, and 45 min. Oxygen hemoglobin saturation was determined on arterial and venous samples with an OSM 2 Hemoximeter (Radiometer, Copenhagen). Oxygen consumption of hindlimbs perfused with vehicle (KHB/BSA/bovine erythrocytes/glucose), 2 mmol/l AICA-riboside, or 60 nmol/l insulin were  $29.3 \pm 0.9$ ,  $31.6 \pm 1.1$ , and  $38.2 \pm 1.4$   $\mu$ mol O<sub>2</sub>/min, respectively. At the end of the 45-min perfusion, the gastrocnemius/plantaris and quadriceps muscle groups were quickly removed and clamp-frozen with stainless steel block tongs cooled to liquid nitrogen temperature.

**Analytical methods.** Glucose uptake (18) and lactate production (19) were determined by arterial/venous difference using neutralized perchloric acid extracts of the medium. Extracts were stored at  $-20^\circ\text{C}$  until analyzed.

The muscles were stored under liquid nitrogen until analysis, when they were first ground to powder under liquid nitrogen. For glucose-6-phosphate, fructose-6-phosphate, and adenine nucleotide determination, perchloric acid was added to powdered frozen tissue (400 mg tissue powder per 2.0 ml 6% perchloric acid), and this was rapidly homogenized. A portion of the perchloric acid extract was neutralized for glucose-6-phosphate and fructose-6-phosphate determination (20). Adenosine, ATP, ADP, AICA-riboside triphosphate (ZTP), ZMP, inosine monophosphate (IMP), and AICA-riboside concentrations were determined using a Beckman (Redmond, WA) high-performance liquid chromatography system as previously described (21). AMPK activity was determined using homogenates prepared from powdered (under liquid nitrogen) muscle and precipitated with ammonium sulfate, as described by Davies et al. (22). Muscle glycogen was determined by the anthrone method (23).

Muscle sarcolemma and intracellular membrane fractions were isolated using a modification of the method described by Hirshman et al. (24). Briefly, the gastrocnemius/plantaris and quadriceps muscle groups (~5 g of tissue) were ground to powder under liquid nitrogen. The powdered muscle was first homogenized on ice to a 25% homogenate in sucrose/EDTA buffer (255 mmol/l sucrose, 0.2 mmol/l EDTA, 100 mmol/l Tris, pH 7.6), using a Kinematica AG polytron (Brinkmann, Westbury, NY) set at 12,000 rpm, and then in a glass-on-glass homogenizer. The homogenates were then processed as described by Hirshman et al. (24). Isolated membrane fractions were stored at  $-75^\circ\text{C}$  until analysis. Protein concentrations (determined using a Bio-Rad [Hercules, CA] protein assay kit) and the specific activity of the plasma membrane marker enzyme, 5'-nucleotidase (25), were determined on aliquots of the homogenate, plasma membrane, and intracellular membrane fractions.

Western blot analysis for GLUT4 was done by loading 30  $\mu$ g of protein from the plasma membrane and intracellular membrane fractions onto a 10% SDS-PAGE mini gel. Samples were subjected to electrophoresis at 200 V for 45 min. Samples were transferred from the gel to a nitrocellulose membrane at 100 V for 60 min. The nitrocellulose membranes were placed in blocking solution (Tris-buffered saline [TBS: 10 mmol/l Tris, 0.25 mol/l NaCl, pH 7.5], 3% BSA, 0.05% Tween20, and 0.01% sodium azide) and then incubated overnight with GLUT4 polyclonal antibody (Biogenesis, Sandown, NH) in blocking solution at a 1:500 dilution. Membranes were washed three times with TBS/NP-40 solution (TBS, 0.1% tergitol type NP-40; Sigma, St. Louis, MO) and once with TBS. The nitrocellulose was incubated with <sup>125</sup>I-labeled (0.3  $\mu$ Ci/ml) protein-A (ICN, Irvine, CA) in blocking solution and then washed as described above. GLUT4 was quantified by exposing the nitrocellulose to Kodak XAR-5 film and quantifying the bands produced using a Multi-Image Light Cabinet (Alpha Innotech, San Leandro, CA) supported by Alpha-



**FIG. 1.** AMPK activity (picomoles per gram per minute) in gastrocnemius/plantaris muscles perfused with medium containing vehicle ( $\square$ ), 2 mmol/l AICA-riboside (AICAR) ( $\blacksquare$ ), or 60 nmol/l insulin ( $\square$ ). Values are expressed as mean  $\pm$  SE ( $n = 11$  per group for vehicle and AICA-riboside groups and  $n = 5$  for insulin). \*Significantly different from muscles perfused with vehicle ( $P < 0.005$ ).

Imager 4.0 software. Western blot data are expressed as arbitrary units where individual values were divided by the mean basal (perfused with vehicle) GLUT4 value.

Results are expressed as means  $\pm$  SE. Statistically significant differences were determined using one-way analysis of variance and Fisher's least significant difference test.

## RESULTS

Figure 1 shows AMPK activity in gastrocnemius/plantaris muscle groups perfused for 45 min with vehicle ( $n = 11$ ), 2 mmol/l AICA-riboside ( $n = 11$ ), or 60 nmol/l insulin ( $n = 5$ ). AMPK activity in the AICA-riboside-perfused muscles was significantly higher than that in muscles perfused without AICA-riboside ( $P < 0.005$ ). There was no significant difference in AMPK activity between vehicle- and insulin-perfused muscles.

The concentrations of adenosine, IMP, ADP, ATP, AICA-riboside, ZMP, and ZTP in the gastrocnemius/plantaris muscle groups after 45 min of perfusion with vehicle ( $n = 11$ ), 2 mmol/l AICA-riboside ( $n = 11$ ), or 60 nmol/l insulin ( $n = 5$ ) are shown in Table 1. There was no significant difference between adenosine, ATP, or ADP concentrations among treatment groups. IMP was significantly higher in the AICA-ribo-

**TABLE 1**  
Concentrations of adenine nucleotides (micromoles per gram of tissue) in gastrocnemius/plantaris muscle groups perfused for 45 min with vehicle, 2 mmol/l AICA-riboside, or 60 nmol/l insulin

	Vehicle	AICA-riboside	Insulin
Adenosine	$0.056 \pm 0.004$	$0.054 \pm 0.004$	$0.049 \pm 0.003$
IMP	$0.005 \pm 0.001$	$0.176 \pm 0.025^*$	$0.003 \pm 0.001$
ADP	$1.08 \pm 0.04$	$1.09 \pm 0.05$	$1.07 \pm 0.05$
ATP	$8.10 \pm 0.18$	$7.53 \pm 0.25$	$8.08 \pm 0.27$
AICA-riboside	ND	$1.30 \pm 0.08$	ND
ZMP	ND	$1.78 \pm 0.07$	ND
ZTP	ND	$1.87 \pm 0.20$	ND

Data are means  $\pm$  SE.  $n = 5$ –11 per group. \*Significantly different from muscles perfused with vehicle ( $P < 0.001$ ). ND, not detectable.

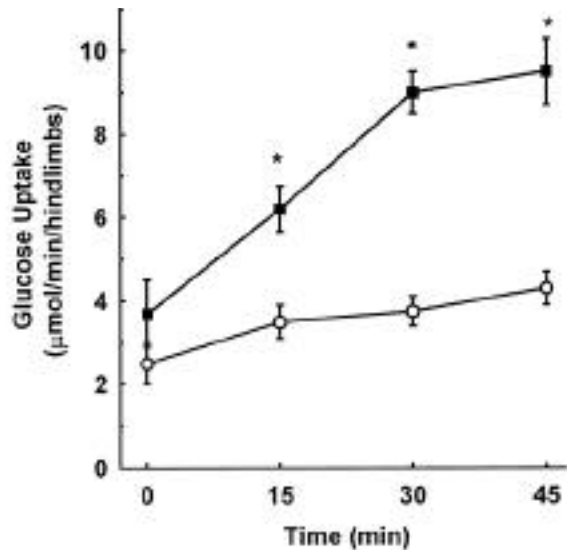


FIG. 2. Glucose uptake (micromoles per hindlimb per minute) during rat hindlimb perfusion with vehicle (○) or 2 mmol/l AICA-riboside (■). Values are expressed as means  $\pm$  SE ( $n = 11$  per group). \*Significantly different from muscles perfused with vehicle ( $P < 0.005$ ).

side-treated muscles ( $P < 0.001$ ). The most likely reason for the increase is that IMP is a breakdown product of ZMP, which was increased in the AICA-riboside-treated muscles. AICA-riboside, ZMP, and ZTP were observed in AICA-riboside-perfused muscles, but they were not detected in vehicle- or insulin-perfused muscles.

Figure 2 shows glucose uptake by the rat hindlimbs perfused with vehicle ( $n = 11$ ) or 2 mmol/l AICA-riboside ( $n = 11$ ). Muscles perfused with 2 mmol/l AICA-riboside in the absence of insulin showed a significant increase in glucose uptake from basal levels with 15 min of exposure to AICA-riboside ( $P < 0.005$ ) and continued to increase at 30 and 45 min with maximal uptake rates of  $9.7 \pm 0.6 \mu\text{mol} \cdot \text{hindlimb}^{-1} \cdot \text{min}^{-1}$  in AICA-riboside-treated rats ( $P < 0.001$ ). In comparison, insulin perfusion for 45 min increased glucose uptake to  $25.8 \pm 1.7 \mu\text{mol} \cdot \text{hindlimb}^{-1} \cdot \text{min}^{-1}$  ( $n = 5$ ).

Glucose-6-phosphate and fructose-6-phosphate concentrations in gastrocnemius/plantaris muscle groups perfused for 45 min with vehicle ( $n = 11$ ), 2 mmol/l AICA-riboside

TABLE 2  
Concentrations of glucose-6-phosphate, fructose-6-phosphate, glycogen, and lactate production in gastrocnemius/plantaris muscle groups perfused for 45 min with vehicle, 2 mmol/l AICA-riboside, or 60 nmol/l insulin

	Vehicle	AICA-riboside	Insulin
Glucose-6-phosphate (nmol/g)	$309 \pm 23$	$1,140 \pm 73^*$	$418 \pm 54$
Fructose-6-phosphate (nmol/g)	$35 \pm 5$	$161 \pm 13^*$	$54 \pm 13$
Glycogen concentration ( $\mu\text{mol}$ glucose units/g)	$44.4 \pm 5.0$	$47.2 \pm 6.1$	$83.8 \pm 7.8^\dagger$
Lactate production ( $\mu\text{mol} \cdot \text{hindlimb}^{-1} \cdot \text{min}^{-1}$ )	$4.2 \pm 0.5$	$7.6 \pm 0.8^\ddagger$	$8.8 \pm 1.3^\ddagger$

Data are means  $\pm$  SE.  $n = 5$ –11 per group. Significantly different from muscles perfused with vehicle: \* $P < 0.001$ ,  $^\dagger P < 0.01$ , and  $^\ddagger P < 0.02$ .

( $n = 11$ ), or 60 nmol/l insulin ( $n = 5$ ) were approximately three times higher in muscles perfused with AICA-riboside ( $P < 0.001$ ) (Table 2). There was no significant difference in glucose-6-phosphate or fructose-6-phosphate concentrations between vehicle- and insulin-perfused muscles. Insulin perfusion increased glycogen concentrations in the muscle ( $P < 0.01$ ), whereas there was not a significant difference in glycogen concentrations between the AICA-riboside- and vehicle-perfused muscles (Table 2). Lactate production in hindlimbs perfused with vehicle was significantly lower than those perfused with AICA-riboside or with insulin ( $P < 0.02$ ) (Table 2).

Figure 3 shows the GLUT4 protein content in the plasma membrane (Fig. 3A) and intracellular membrane fractions (Fig. 3B) of muscles perfused for 45 min with vehicle, 2 mmol/l AICA-riboside, or 60 nmol/l insulin. The high insulin concentration (60 nmol/l,  $\sim 100$  times higher than a high physiologic insulin concentration) was chosen to compare the maximal insulin-stimulated response of glucose uptake and GLUT4 translocation with the response of AMPK

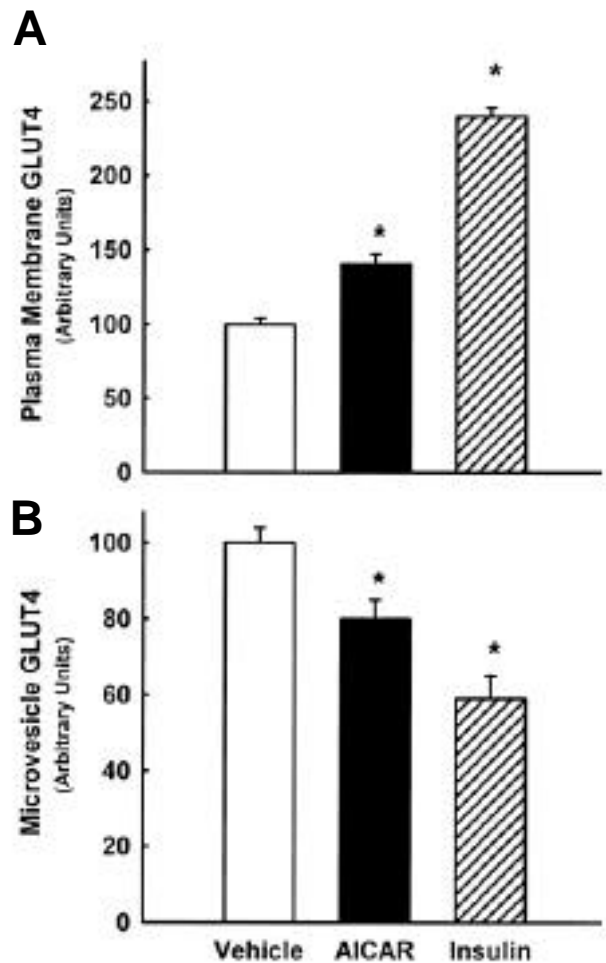


FIG. 3. GLUT4 protein content in the plasma membrane fraction (A) and the intracellular membrane fraction (B) in gastrocnemius/plantaris muscles perfused for 45 min with vehicle (□), 2 mmol/l AICA-riboside (AICAR) (■), or 60 nmol/l insulin (▨). Data are expressed as arbitrary units, where individual values are divided by the mean basal (perfused with vehicle) GLUT4 value. Values are expressed as means  $\pm$  SE ( $n = 5$ –11 per group). \*Significantly different from muscles perfused with vehicle ( $P < 0.001$ ).

activation by 2 mmol/l AICA-riboside. Our results show a significant 40% increase of GLUT4 in plasma membranes with AICA-riboside and a 140% increase with maximal insulin stimulation compared with the basal level ( $P < 0.001$ ). We observed a 40% decrease in intracellular GLUT4 with insulin stimulation and a 20% decrease with AICA-riboside stimulation ( $P < 0.001$ ). Therefore, the disappearance of GLUT4 from the intracellular fraction is concomitant to the relative appearance of GLUT4 in the plasma membranes. These values are comparable to previously reported values for insulin and contraction-stimulated GLUT4 translocation using subcellular fractionation (1). Activity of 5'-nucleotidase, a plasma membrane marker enzyme, indicated that ~7% of plasma membranes were recovered and that this fraction was enriched by ~25-fold compared with the homogenates. Plasma membrane recovery and enrichments were not different among the groups.

## DISCUSSION

AMPK has been designated the "fuel gauge" of the mammalian cell (10). Phosphorylation of several target proteins results in inactivation of anabolic pathways such as lipogenesis (26), cholesterologenesis (10,11), and activation of catabolic pathways that increase ATP production, such as fatty acid oxidation (8,21,27). In skeletal muscle, AMPK is activated by a decrease in creatine phosphate concentration and a concurrent increase in free AMP concentration, such as occurs during muscle contraction (9,13). An increase in AMP activates AMPK by both allosteric and covalent mechanisms (10,11,22). AMPK activity is increased in muscle of rats running on the treadmill, as well as in muscle electrically stimulated to contract (7,12–14). AICA-riboside also increases AMPK activity in skeletal muscle as it is taken up into the cell and phosphorylated to form ZMP, which mimics the effects of AMP on activation of AMPK and AMPKK (7–8,28). AICA-riboside can thus be used to mimic the effect of contraction to study the effects of AMPK activation in isolated or perfused tissues.

The translocation of GLUT4 into the plasma membranes and T-tubules from an intracellular location is the major mechanism responsible for the increase in skeletal muscle glucose uptake seen with insulin and contraction stimulation, but the exact mechanism is not fully understood (3). There is strong evidence that insulin and contractions do not use the same intracellular signaling pathway to achieve GLUT4 translocation (3–7). We have hypothesized that AMPK activation is an early event in the intracellular signal transduction pathway for contraction-stimulated GLUT4 translocation. AMPK activation having a critical role in the cell signaling cascade for contraction-stimulated glucose uptake is consistent with AMPK's role as an ATP-conserving energy-producing mechanism in the cell. Previous studies provide evidence that contraction and AMPK activation by incubation with AICA-riboside increase glucose uptake through a similar cell signaling pathway, a pathway separate from that of insulin (7). The current study clearly demonstrates that activation of AMPK with AICA-riboside causes GLUT4 translocation in skeletal muscle and indicates that this translocation could be responsible for the increase in glucose uptake. These observations do not preclude the existence of other mechanisms, such as increases in activity of existing sarcolemmal/T-tubule glucose transporters.

In this study, AMPK was activated by AICA-riboside treatment, but there was no increased energy expenditure in the cells because the muscles were not contracting. The concentration of glucose-6-phosphate, an inhibitor of hexokinase (4), was about three times higher in the AICA-riboside-perfused muscles. The rate of glucose uptake in the AICA-riboside-treated muscles may have been inhibited at the level of hexokinase because of the high concentration of glucose-6-phosphate. Glucose-6-phosphate would be rapidly metabolized during muscle contraction because of the increased rate of utilization of glycolysis products. Since total energy demands are not increased with AICA-riboside treatment, glucose entering the muscle fiber is phosphorylated, with consequent glucose-6-phosphate accumulation. It is likely that the accumulation of glucose-6-phosphate is the driving force that increases glycolytic flux to lactate in the AICA-riboside-treated resting muscle.

The fate of glucose taken into muscles treated with insulin included glycogen synthesis, oxidation, and lactate production. The glucose taken into the cells by the AMPK-stimulated pathway was not converted to glycogen, since the glycogen concentration in the AICA-riboside-perfused muscles was not significantly different from basal levels. Apparently, lactate production and oxidation were the principal routes of metabolism in the AICA-riboside-treated muscles.

It has been previously shown that AMP concentrations are unchanged by treatment with AICA-riboside (8,15); therefore, the activation of AMPK observed is due to ZMP accumulation. ZMP may mimic the effects of AMP on other AMP-regulated enzymes, such as activating glycogen phosphorylase (28) and acting on fructose-1,6-bisphosphatase to inhibit gluconeogenesis (29). We saw no evidence that AICA-riboside activated phosphorylase in this study, since muscle glycogen concentrations in AICA-riboside-perfused muscles were not significantly different from basal levels. Evidence that AICA-riboside treatment causes activation of other protein kinases besides the AMPK cascade has not yet been observed (30).

It has been suggested that stimulation of sarcolemma adenosine receptors is associated with enhanced GLUT4 translocation and glucose uptake in skeletal muscle (31,32). In the current study, we observed no significant change in muscle adenosine concentrations between treatment groups. Bergeron et al. (33) found that the presence of 8-(p-sulphophenyl)-theophylline, an adenosine receptor antagonist, did not influence glucose uptake in epitrochlearis muscles incubated with AICA-riboside. We, therefore, consider it unlikely that the effects of AICA-riboside on glucose uptake and GLUT4 translocation are mediated by the adenosine receptor.

In summary, AICA-riboside stimulates an increase in skeletal muscle AMPK activity, an increase in glucose uptake, and an increase in the translocation of GLUT4 from an intracellular location to the plasma membranes. These findings are consistent with the hypothesis that AMPK activation may be an early step in the intracellular signaling pathway for contraction-stimulated GLUT4 translocation and glucose uptake. Increased activation of the AMPK signaling pathway, by either exercise or pharmaceutical agents, may be an effective treatment for some forms of type 2 diabetes (30). Further study is needed to determine whether recruitment of the AMPK signaling pathway could contribute to correction of insulin resistance through bypassing the insulin-regulated system for GLUT4 translocation.

## ACKNOWLEDGMENTS

This work was supported by the National Institute of Musculoskeletal and Skin Diseases Grants AR-41438 (to W.W.W.) and AR-45670 (to L.J.G.).

Technical assistance was provided by B. Holmes, C. Hancock, and A. Perry.

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