Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin

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Fisher, Jonathan S., Jiaping Gao, Dong-Ho Han, John O. Holloszy, and Lorraine A. Nolte. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab 282: E18–E23, 2002.—Evidence has accumulated that activation of AMP kinase (AMPK) mediates the acute increase in glucose transport induced by exercise. As the exercise-induced, insulin-independent increase in glucose transport wears off, it is followed by an increase in muscle insulin sensitivity. The major purpose of this study was to determine whether hypoxia and 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), which also activate AMPK and stimulate glucose transport, also induce an increase in insulin sensitivity. We found that the increase in glucose transport in response to 30 μU/ml insulin was about twofold greater in rat epitrochlearis muscles that had been made hypoxic or treated with AICAR 3.5 h previously than in untreated control muscles. This increase in insulin sensitivity was similar to that induced by a 2-h bout of swimming or 10 min of in vitro electrically stimulated contractions. Neither phosphatidylinositol 3-kinase activity nor protein kinase B (PKB) phosphorylation in response to 30 μU/ml insulin was enhanced by prior exercise or AICAR treatment that increased insulin sensitivity of glucose transport. Inhibition of protein synthesis by inclusion of cycloheximide in the incubation medium for 3.5 h after exercise did not prevent the increase in insulin sensitivity. Contractions, hypoxia, and treatment with AICAR all caused a two- to three-fold increase in AMPK activity over the resting level. These results provide evidence that the increase in insulin sensitivity of muscle glucose transport that follows exercise is mediated by activation of AMPK and involves a step beyond PKB in the pathway by which insulin stimulates glucose transport.

cycloheximide; exercise; hypoxia; insulin signaling; 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside

CONTRACTILE ACTIVITY STIMULATES glucose transport in skeletal muscle (15). This effect is mediated by a signaling pathway that is separate from and independent of the insulin signaling pathway (15). As the acute increase in glucose transport reverses after cessation of contractile activity, there is a marked increase in the sensitivity of muscle to insulin (4, 5, 8, 25, 34). Although the mechanism for increased insulin sensitivity after exercise, which was first described by Richter et al. (25) in 1982, is unknown, it has been suggested to be related to increased activation of phosphatidylinositol 3-kinase (PI 3-kinase) by insulin (35). The increase in insulin sensitivity can persist for a number of days as long as glycogen repletion is prevented by means of a carbohydrate-deficient diet (5).

Hypoxia appears to stimulate muscle glucose transport via the same pathway as contractions (3). There is evidence that activation of the AMP-activated protein kinase (AMPK) by the decreases in phosphocreatine and ATP and the increase in AMP induced by exercise or hypoxia mediates the stimulation of glucose transport (14, 19, 30). In this context, the major purpose of this study was to determine whether activation of AMPK by hypoxia or the adenosine analog 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR; see Ref. 30) also causes an enhancement of muscle insulin sensitivity. Additional aims of this research were to determine whether protein synthesis is involved in the development of the increase in insulin sensitivity and to further evaluate the possibility that enhanced insulin signaling is involved.

METHODS

Materials. Purified porcine insulin was purchased from Eli Lilly (Indianapolis, IN). AICAR was obtained from Toronto Research Chemicals (North York, Ontario, Canada). 3-O-methyl-d-[3H]glucose (3-MG) was obtained from American Radiolabeled Chemicals (St. Louis, MO). d-[1-14C]mannitol and [γ-32P]ATP were obtained from Perkin-Elmer Life Sciences (Boston, MA). SAMS peptide (HMR-SAMSGHLVKRR, the substrate for AMPK assays) was purchased from Zinsser Analytic (Maidenhead, Berkshire, UK). A polyclonal antibody specific for phosphoryserine-473 of protein kinase B (PKB) was purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Reagents for SDS-PAGE were obtained from Bio-Rad ( Hercules, CA). Other chemicals, including protein A-Sepharose and agarose beads coated with monoclonal anti-
phosphotyrosine antibody, were purchased from Sigma Chemical (St. Louis, MO).

**Animals.** This research was approved by the Animal Studies Committee of Washington University School of Medicine. Male Wistar rats (120–140 g) were given free access to Purina rat chow and water until the night before an experiment, when food was removed at 5:00 P.M. The next morning, one group of rats was exercised by means of swimming for 2 h as described previously (5) and was anesthetized immediately after exercise. All rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitrochlearis muscles were excised.

**Muscle incubation after exercise.** Muscles of exercised rats and sedentary controls were incubated with shaking for 3 h at 35°C in Erlenmeyer flasks containing 2 ml of oxygenated Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA. Muscles were then transferred to KHB containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA, with or without 30 μU/ml insulin, for 30 min at 35°C. To determine whether postexercise insulin sensitivity requires protein synthesis, muscles from some exercised animals were incubated for 3 h in the recovery medium with or without 75 μM cycloheximide. This concentration of cycloheximide prevents 94% of protein synthesis in our muscle preparation (33). Cycloheximide was also included during the 30-min incubation with and without 30 μU/ml insulin but not in the 3-MG transport assay medium.

**Effect of AICAR.** Muscles from sedentary animals were incubated for 1 h with or without 2 mM AICAR in 100% rat serum or KHB. Muscles were then allowed to recover in KHB containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA for 3 h, followed by 30 min of incubation in the same medium with or without 30 μU/ml insulin.

**Effect of hypoxia.** Muscles were made hypoxic by incubation in serum with a gas phase of 95% N2-5% CO2 for 80 min (3), followed by a 3-h recovery period in oxygenated KHB containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA for 3 h, followed by 30 min of incubation in the same medium with or without 30 μU/ml insulin.

**Glycogen.** Glycogen concentration was measured fluorometrically in muscle samples homogenized in 0.3 M perchlo-
ric incubated in the absence or presence of 2 mM AICAR in rat serum as described in Effect of AICAR. Muscle samples were frozen after the full 4-h 50-min incubation period (1 h incubation + AICAR, 3 h recovery, 30 min insulin stimulation, 10 min rinse, 10 min glucose transport assay).

Statistical analysis. Data are presented as means ± SE. Analysis of differences between groups was performed with one-way ANOVA (P < 0.05 was considered to be significant) followed by Fisher’s least significant difference post hoc tests when appropriate.

RESULTS

Cycloheximide does not prevent the increase in insulin sensitivity after exercise. As in previous studies (4, 5, 7, 11), the increase in 3-MG transport induced by 30 μU/ml insulin was approximately twofold greater in muscles that had recovered from exercise for 3.5 h than in control muscles (Fig. 1). In our epitrochlearis muscle preparation, 30 μU/ml insulin normally induces ~33% of the maximal effect of insulin on 3-MG transport (12). Cycloheximide, at a concentration that blocks muscle protein synthesis in our epitrochlearis muscle preparation (33), did not prevent the increase in muscle insulin sensitivity after exercise.

Hypoxia induces an increase in muscle insulin sensitivity. Muscles that had recovered from hypoxia for 3.5 h had an approximately twofold increase in the stimulation of 3-MG transport by 30 μU/ml insulin compared either with control muscles that were not made hypoxic (Fig. 2) or with muscles made hypoxic in the absence of serum. The 3-MG transport rate in muscles stimulated with 30 μU/ml insulin in the absence of serum was approximately twofold (difference not statistically significant) in muscle in response to 30 μU/ml insulin, 30 min of incubation in the absence or presence of 75 μM AICAR, 3 h recovery, 30 min insulin stimulation, 10 min rinse, 10 min glucose transport assay). Contractile activity (10 min) induces the full effect of exercise on insulin sensitivity. As in our previous study (7), the induction of insulin sensitivity of glucose transport by in vitro muscle contractions (Fig. 3) was similar to the effect of 2 h of swimming. As with swimming, muscle contractions caused a twofold potentiation of the insulin-stimulated increase in 3-MG transport above basal transport. There was no increase in insulin sensitivity in muscles stimulated to contract in KHB instead of serum.

AICAR induces an increase in muscle insulin sensitivity. As shown in Fig. 2, the effect of 30 μU/ml insulin on 3-MG transport was approximately twofold greater in muscles that had been incubated with AICAR and serum 3.5 h earlier. This increase in insulin sensitivity depended on the presence of serum during the 60-min incubation with AICAR, as muscles incubated with AICAR in the absence of serum showed no enhancement of insulin action (3-MG transport averaged 0.51 ± 0.03 μmol·ml⁻¹·10 min⁻¹ in the controls and 0.42 ± 0.05 μmol·ml⁻¹·10 min⁻¹ in muscles that had been made hypoxic in the absence of serum).

Insulin signaling. As shown in Fig. 5, phosphotyrosine-associated PI 3-kinase activity increased approximately twofold (difference not statistically significant) in muscle in response to 30 μU/ml insulin. Neither exercise nor AICAR, under conditions that induced an increase in insulin sensitivity of glucose transport, had any effect on the magnitude of the
increase in PI 3-kinase activity induced by 30 μU/ml insulin.

In contrast to the large increase in PKB phosphorylation on serine-473 induced by 2 mU/ml insulin, the increase in serine-473-phosphorylated PKB induced by 30 μU/ml insulin was small (not statistically different from baseline) and not enhanced by prior exercise (Fig. 6).

Glycogen. Incubation of muscles with AICAR (in serum) did not reduce glycogen levels compared with muscles incubated in KHB (control 12.7 ± 1.0 μmol glucosyl units/g, AICAR 14.0 ± 1.4 μmol glucosyl units/g, n = 7–8 muscles/group).

**DISCUSSION**

The novel information provided by this study is that, like contractile activity, hypoxia and AICAR induce an increase in PI 3-kinase activity.

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were performed with an antibody specific for phosphoserine-473 of insulin receptor substrate-1. Western blots were performed with an antibody specific for phosphoserine-473 of PKB.

Fig. 6. Prior exercise does not increase insulin-stimulated phosphorylation of protein kinase B (PKB). Epitrochlearis muscles were taken from sedentary rats or from animals immediately after a 2-h swim. Muscles recovered for 3 h in vitro in the absence of insulin before a 30-min incubation in the absence or presence of 30 μU/ml insulin. Additional muscles from sedentary rats were incubated in the presence of 2 mU/ml insulin after the 3-h recovery period. Western blots were performed with an antibody specific for phosphoserine-473 of PKB.

increases in muscle insulin sensitivity. We previously found that the increase in insulin sensitivity depends on the presence of serum during the period in which muscles are stimulated to contract (7). Similarly, the hypoxia- and AICAR-induced increases in insulin sensitivity occurred only when the muscles were exposed to serum during the treatment periods. Contractile activity, hypoxia, and AICAR all stimulate muscle glucose transport acutely (14, 15, 19). There is considerable evidence that activation of AMPK plays a key role in the activation of glucose transport by these stimuli (13). AICAR is taken up by muscles and converted to the AMP analog AICAR 5'-monophosphate and thus activates AMPK (30). Activation of AMPK appears to be the only action of exercise and hypoxia that is mimicked by AICAR (13, 14). It therefore seems probable that, like the acute stimulation of glucose transport, the increase in muscle insulin sensitivity that develops as the acute effect on glucose transport wears off is also mediated by AMPK.

In addition to acutely stimulating glucose transport and enhancing insulin sensitivity, exercise induces an increase in GLUT-4 protein expression in muscle, resulting in an increase in insulin responsiveness (10, 23, 26, 27). This effect of exercise is also mimicked by AICAR, as injection of rats with AICAR (16) or exposure of muscles in vitro to AICAR (21) induces an increase in muscle GLUT-4 protein. Although the increase in insulin sensitivity is unrelated to and precedes the increase in GLUT-4 (12), it seemed possible that the increase in insulin sensitivity might be mediated by increased expression of another protein with a short half-life involved in the regulation of glucose transport. This possibility appears to be ruled out by the present finding that cycloheximide did not prevent the exercise-induced increase in insulin sensitivity. A change in protein expression during contractile activity is probably not a mechanism for increased insulin sensitivity because of the short time period, i.e., 10 min of in vitro contractile activity produces the same effect on insulin sensitivity as does 2 h of swimming. The signals that mediate enhanced insulin sensitivity after exercise are probably only present during contractile activity, and not during the hours of recovery afterwards, because AMPK activity falls back to baseline within 15 min after exercise (24), and the serum factor necessary to evoke the effect of contractile activity, hypoxia, and AICAR on insulin sensitivity only has to be present during the stimulation/treatment period.

The increased sensitivity of glucose transport to stimulation by insulin after exercise is mediated by translocation of more of the GLUT-4 from the intracellular pools to the cell surface (12), suggesting amplification of the insulin signal. One study has shown increased activation of PI 3-kinase by a maximally effective insulin concentration immediately after exercise (35), i.e., an increase in insulin responsiveness, not sensitivity. However, other previous studies have found no increases in insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, PI 3-kinase activity, or PKB phosphorylation in response to a submaximal insulin stimulus after a single bout of exercise (9, 12, 20, 31, 32). These findings provide evidence that the persistent effect of exercise on insulin sensitivity is mediated at a point beyond PKB in the pathway by which insulin stimulates glucose transport. The findings in the present study that the effects of 30 μU/ml of insulin on PI 3-kinase activity and PKB phosphorylation were not enhanced after exercise or AICAR treatment are in keeping with this conclusion.

It has been hypothesized that some of the GLUT-4 vesicles in muscle are associated with glycogen and that the increase in insulin sensitivity after exercise is the result of a larger available pool of free GLUT-4 vesicles because of glycogen depletion (6). The finding that treatment with AICAR induces an increase in insulin sensitivity provides evidence that glycogen depletion is not involved, because AICAR did not cause a decrease in muscle glycogen in the present study, as has also been shown previously (2, 13, 16, 18).

In conclusion, hypoxia and AICAR treatment, like exercise, are followed by increases in muscle insulin sensitivity. Although it is possible that enhancement of insulin sensitivity by exercise, hypoxia, and AICAR occurs through some pathway other than AMPK signaling, the only acute effect of exercise and hypoxia that is known to be mimicked by AICAR is activation of AMPK. This leads to the conclusion that activation of AMPK initiates the process that leads to increased insulin sensitivity. In this context, it appears that increased serine phosphorylation of a protein by AMPK (AMPK phosphorylates its targets on serine residues; see Ref. 30) is involved in the events that lead to translocation of more GLUT-4 to the cell surface in response to a given submaximal insulin stimulus.
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