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

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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 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 phosphatidyl-inositol 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 in vivo cyclohexamide in the incubation medium for 3.5 h after exercise did not prevent the increase in insulin sensitivity. Contraction, 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.

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). [1-14C]mannitol and [γ-32P]ATP were obtained from Perkin-Elmer Life Sciences (Boston, MA). A polyclonal antibody specific for phosphoserine-473 of protein kinase B (PKB) was purchased from Cell Signaling Technology (Boston, MA). A polyclonal antibody specific for phosphoserine-473 of protein kinase B (PKB) was purchased from Cell Signaling Technology (Boston, MA). SAMS peptide (SAMSGLHLVKRR, the substrate for AMPK assays) was purchased from Invitrogen (Carlsbad, 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 epitochalears 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.

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 immediately after exercise. All rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitochalears muscles were excised.

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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 followed by 30 min of incubation in the same medium with or without 30 μU/ml insulin.

A serum factor has been found to be necessary to elicit the contractile activity-induced enhancement of sensitivity of glucose transport to stimulation by insulin (7). To determine whether serum is also required for the AICAR-induced increase in insulin sensitivity, some muscles from sedentary animals were incubated in KHB containing 2 mM AICAR, 8 mM glucose, 32 mM mannitol, and 0.1% BSA in the absence of serum. In parallel experiments, muscles were made hypoxic while incubated without serum.

After muscles were removed from the sedentary rats used in this and other studies, blood was collected from the descending aorta to provide serum for subsequent experiments. Serum was stored at −20°C.

Effects of in vitro muscle contraction. Some muscles were electrically stimulated to contract in vitro, as described previously (7, 28). Ten tetanic contractions were elicited by stimulation at 100 Hz for 10 s at a rate of 1 contraction/min for 10 min. Some muscles were stimulated to contract while incubated in KHB (in the absence of serum), whereas other muscles were incubated in serum during contractions. 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 before 3-MG transport assays.

Glucose transport. Muscle glucose transport activity was assessed using 3-MG, as previously described (33). Muscles were transferred to 1.5 ml of KHB containing 8 mM 3-[[3H]MG (2 μCi/ml), 32 mM [4C]mannitol (0.2 μCi/ml), 0.1% BSA, and insulin, if it was present during the previous incubation, and incubated for 10 min at 30°C. Intracellular 3-MG accumulation was determined as described previously (33) and is expressed as micromoles per milliliter intracellular water in 10 min.

AMPK activity. AMPK activity was measured as described by Winder and Hardie (29) in resting muscles or muscles frozen immediately after 1 h of incubation in the presence of 2 mM AICAR, after 80 min of hypoxia, or after 10 min of in vitro contractile activity. Frozen muscle samples were pulverized under liquid nitrogen and homogenized in buffer containing 100 mM mannitol, 50 mM NaF, 10 mM Tris, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.5, and protease inhibitors (5 μg/ml each of aprotinin, leupeptin, and antitrypsin). The homogenates were centrifuged for 30 min at 48,000 g. AMPK was precipitated from the supernatant by addition of 144 mg ammonium sulfate/ml. After the ammonium sulfate suspension was stirred for 30 min on ice, the precipitate containing AMPK was pelleted by centrifugation at 48,000 g for 30 min. The pellet was dissolved in homogenizing buffer and centrifuged to remove insoluble (protein). AMPK assays were performed for 10 min at 37°C in buffer containing 40 mM HEPES, pH 7.0, 0.2 mM SAMS peptide, 0.2 mM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM MgCl2, and 0.2 mM ATP (0.08 μCi/ml [32P]ATP). Aliquots of assay mixtures were spotted on Whatman P81 filter paper, washed six times in 1% phosphoric acid, rinsed in acetone, and air-dried before measurement of radioactivity by scintillation counting.

PI 3-kinase activity. After the 30-min incubation in the presence or absence of insulin 3 h after exercise or exposure to AICAR, muscles were blotted and then clamp-frozen for assay of phosphotyrosine-associated PI 3-kinase activity (9, 17). Muscle samples were homogenized in 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1.0 mM aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (0.5 μg/ml), and 2 mM phenylmethylsulfonyl fluoride. Homogenates were incubated with end-over-end rotation at 4°C for 60 min and then centrifuged at 200,000 g for 50 min at 4°C. Supernatants were preclarified by incubation for 1 h with protein A-Sepharose. For analysis of PI 3-kinase activity associated with phosphorylated tyrosine, aliquots of supernatant containing 750 μg of protein were immunoprecipitated overnight with end-over-end rotation at 4°C in the presence of 40 μl of monoclonal anti-phosphotyrosine antibody coupled to agarose. Immunocomplexes were collected by centrifugation, washed, suspended in assay medium, and analyzed for PI 3-kinase activity as described by Goodyear et al. (9).

Phosphorylated PKB. Phosphorylation of PKB during insulin stimulation was determined in muscles from sedentary or exercised rats that had recovered for 3 h before 30 min of incubation with 30 μU/ml insulin. Phosphorylation of serine-473 on PKB is a marker for activation of PKB (1). For Western blot analysis of serine-phosphorylated PKB, samples of the 200,000-g supernatants described above for the PI 3-kinase assay were mixed with Laemmli sample buffer. Protein in Laemmli sample buffer (50 μg) with dithiothreitol was subjected to SDS-PAGE on 10% gels, electrophoretically transferred to nitrocellulose, and incubated with primary antibodies against phosphoserine-473 PKB and secondary antibodies linked to horseradish peroxidase. Serine-phosphorylated PKB was quantitated by densitometry after enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Glycogen. Glycogen concentration was measured fluorometrically in muscle samples homogenized in 0.3 M perclo-

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ric acid (22). Glycogen levels were measured for muscles 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 control muscles (Fig. 1). In our epitrochlearis muscle preparation, 3-MG transport was then measured. Each bar represents the mean ± SE for 12–18 muscles. *P < 0.05 vs. basal. †P < 0.001 and ‡P < 0.005 vs. insulin-stimulated control.

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 ± 0.3 μmol·ml⁻¹·10 min⁻¹ in the controls and 0.47 ± 0.07 μmol·ml⁻¹·10 min⁻¹ in the AICAR without serum group).

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, hypoxia, and contractile activity stimulate AMPK activity. As shown in Fig. 4, the stimuli demonstrated to cause a twofold enhancement in the sensitivity of glucose transport to stimulation by insulin also produce a two- to threefold increase in AMPK activity above the resting activity level.

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 increase in PI 3-kinase activity by 30 μU/ml insulin.

Fig. 4. AICAR, hypoxia, and contractions increase AMP kinase (AMPK) activity. Muscles were frozen at rest or immediately after 1 h of incubation with 2 mM AICAR, 80 min of hypoxia, or 10 min of in vitro contractions for subsequent measurement of AMPK activity. Values are means ± SE for 6 muscles/group (n = 3 for hypoxia). *P < 0.005 vs. resting value.

Fig. 5. Prior exercise or AICAR treatment does not modify phosphatidylinositol 3-kinase (PI 3-kinase) activity after 3 h of recovery. Epitrochlearis muscles were taken from rats immediately after a 2-h swim. Muscles from sedentary rats were incubated in the absence or presence of 2 mM AICAR in rat serum for 1 h. All 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 or 2 mM insulin. A: PI 3-kinase activity in arbitrary units. B and C: autoradiographs of [32P]-labeled phosphatidylinositol 3-phosphate (PI3-P) from exercised (B) or AICAR-treated (C) muscles.
PKB, phosphoserine-473.

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.

increase 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.
REFERENCES


