Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle

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Holmes, B. F., E. J. Kurth-Kraczek, and W. W. Winder. Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J. Appl. Physiol. 87(5): 1990–1995, 1999.—This study was designed to determine whether chronic chemical activation of AMP-activated protein kinase (AMPK) would increase glucose transporter GLUT-4 and hexokinase in muscles similarly to periodic elevation of AMPK that accompanies endurance exercise training. The adenosine analog, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), has previously been shown to be taken up by cells and phosphorylated to form a compound (5-aminoimidazole-4-carboxamide ribonucleotide) that mimics the effect of AMP on AMPK. A single injection of AICAR resulted in a marked increase in AMPK in epitrochlearis and gastrocnemius/plantaris muscles 60 min later. When rats were injected with AICAR (1 mg/g body wt) for 5 days in succession and were killed 1 day after the last injection, GLUT-4 was increased by 100% in epitrochlearis muscle and by 60% in gastrocnemius/plantaris muscle in response to AICAR. Hexokinase was also increased 2.5-fold in the gastrocnemius/plantaris. Gastrocnemius glycogen content was twofold higher in AICAR-treated rats than in controls. Chronic chemical activation of AMPK, therefore, results in increases in GLUT-4 protein, hexokinase activity, and glycogen, similarly to those induced by endurance training.

Numerous studies have demonstrated that glucose transport is increased in response to muscle contraction (8, 15, 16, 19, 34). This effect is due to contraction-induced glucose transporter GLUT-4 translocation to the cell surface (8, 15, 16, 19, 34). Contraction-induced GLUT-4 translocation and glucose transport are mediated by different signaling mechanisms than is insulin-triggered translocation of GLUT-4 (8, 15, 16, 19). Recent studies have implicated AMP-activated protein kinase (AMPK) as being important in mediating this effect of contraction on GLUT-4 translocation and glucose transport in muscle (1, 14, 23, 25, 36). AMPK activity increases in muscles of rats running on the treadmill and in electrically stimulated muscles (35, 36), most likely in response to phosphorylation by an upstream kinase, AMPK kinase (AMPKK). Both AMPKK and AMPK are allosterically activated by 5′-AMP (12). In addition, creatine phosphate, an allosteric inhibitor of AMPK (12), decreases in response to exercise or electrically stimulated muscle contraction (12). AMPK can also be artificially activated by exposing resting muscle to 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an adenosine analog that is taken into the muscle and phosphorylated to form 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), an analog of 5′-AMP (25). ZMP activates AMPKK and AMPK similarly to AMP (12, 36). This approach has now been used in isolated perfused hindlimbs (23, 25), in incubated epitrochlearis muscle (1, 14), and in live rats (1) to demonstrate this acute effect of AMPK activation on stimulation of glucose transport. In every respect reported to date, AICAR-riboside stimulation of glucose transport resembles that induced by muscle contraction.

Endurance exercise training, which consists of more prolonged periods of muscle contraction repeated over several days, weeks, and months, has been shown to increase the total amount of the GLUT-4 and hexokinase activity in skeletal muscle of both rats and humans (3, 4, 9, 15–17, 19, 22, 26, 28, 29). This increase in GLUT-4 has been postulated to be responsible in part for the increase in insulin sensitivity that accompanies exercise training (8). In one animal model of type 2 diabetes, the fatty Zucker rat, daily treadmill running for 2 wk has been shown to increase GLUT-4 and reverse the insulin resistance of fast-twitch muscle (4). The effect on increasing GLUT-4 has been shown to be relatively short lived, and regular recruitment of the muscle is essential for maintaining the effect on insulin sensitivity (17).

Although it is clear that endurance training will result in an increase in total GLUT-4 and an increase in GLUT-4 mRNA (3, 22, 26, 28), very little is known concerning the mechanisms of coupling muscle contraction with these events. This experiment was designed to test the hypothesis that chronic AMPK activation induces an increase in GLUT-4 in skeletal muscle. If AMPK activation mediates the effect of chronic muscle contraction on GLUT-4 levels, then it should be possible to mimic the effect of training by repetitive chemical activation of this kinase. After determining acute ef-
Effects of injection of AICAR, rats were treated with AICAR for 5 days to determine whether GLUT-4 would increase in epitrochlearis and gastrocnemius/plantarlis muscles of rats.

**RESEARCH DESIGN AND METHODS**

Treatment of rats. All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Sprague-Dawley rats weighing 197 ± 5 g (Sasco, Wilmington, MA) were housed in individual cages in a temperature- (22–25°C) and light-controlled (12:12-h light-dark cycle) room and were given food (Harlan Teklad rodent diet, Madison, WI) and water ad libitum. To determine acute in vivo effects of AICAR, a jugular catheter was installed and exteriorized on the back of the neck 3 days before the day of the experiment. This catheter was implanted for the purpose of allowing rapid anesthetization of the rat and rapid blood and tissue collection. Rats were then given AICAR (1 mg/g body wt) subcutaneously in sterile 0.9% NaCl or were given 0.9% NaCl (n = 7 rats in each group). One hour after the subcutaneous injection of AICAR, rats were anesthetized by intravenous injection of pentobarbital sodium (4.8 mg/100 g body wt). The epitrochlearis and gastrocnemius/plantarlis muscles were quickly removed and rapidly frozen with stainless steel clamps at liquid nitrogen temperature. Blood was collected via the abdominal aorta, and a perchloric acid extract was prepared (0.5 ml blood to 2.0 ml 10% HClO4), neutralized, and utilized for analysis of glucose and lactate.

To determine the effect of chronic activation of AMPK, rats were injected (between 8 and 10 AM) subcutaneously with AICAR (1 mg/g body wt) or saline vehicle for 5 days in succession. This dose was shown in preliminary experiments to increase ZMP levels in the muscle to 0.57 ± 0.06 µmol/g after 15 min, to 0.79 ± 0.06 µmol/g after 60 min, to 0.69 ± 0.06 µmol/g after 90 min, and to 0.60 ± 0.06 µmol/g after 120 min (n = 3 rats at each time point). Beginning with the first injection, controls were pair fed with AICAR-injected rats. Saline-injected controls ate 17 ± 1 g, and AICAR-injected rats ate 18 ± 1 g of food during the 24-h period before blood and tissue collection. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (22–25 h after the last AICAR injection) and epitrochlearis and gastrocnemius/plantarlis muscles were collected and frozen as described above. Muscles were kept under liquid nitrogen until analyzed.

Analytic methods. Muscles from rats killed 1 h after injection of AICAR or saline were analyzed for AMPK (14, 25), citrate dependence of acetyl-CoA carboxylase (ACC) (25), malonyl-CoA (24), glycogen (13), ZMP, AICA-riboside analog of ATP (ZTP), ATP, and ADP (25). Blood glucose and lactate (10) were measured by enzymatic techniques.

Muscles from rats killed 22–25 h after the fifth AICAR injection were analyzed for glycogen and GLUT-4. For GLUT-4 measurement (see Ref. 4), muscle was ground to powder under liquid nitrogen. A homogenate (1:9 dilution) was prepared in HEPES buffer (25 mM HEPES, 1 mM EDTA, 1 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin, 1 µM aprotonin, pH 7.5). Proteins of these homogenates were separated by SDS-PAGE by using 10% resolving gels (Tris–HCl ready gels, Bio-Rad, Hercules, CA). Proteins were transferred from the gel to a nitrocellulose membrane at 100 V for 60 min. The membranes were blocked with 3% BSA in 139 mM NaCl, 2.7 mM KH2PO4, 9.9 mM Na2HPO4, and 0.05%Tween-20 (PBST) and 1% sodium azide. After two 5-min washes in 139 mM NaCl, 2.7 mM KH2PO4, 9.9 mM Na2HPO4 (PBS), membranes were incubated with GLUT-4 polyclonal antibody RaIRGT (Biogenesis, Sandown, NH) for 1 h at room temperature. After two 5-min washes in PBS and two 5-min washes in PBS, membranes were exposed to horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL) for 1 h at room temperature. After being washed twice with PBS and twice with PBS, the membranes were incubated in enhanced chemiluminescence-detection reagent and then visualized on enhanced chemiluminescence hyperfilm (Amersham Life Sciences). Relative amounts of GLUT-4 were then quantified by using a Hewlett Packard ScanJet et 6200C and SigmaGel software (SPSS, Chicago, IL). Total intensity of GLUT-4 spots on the developed hyperfilm was expressed as a fraction of intensity shown by a GLUT-4 standard run on the same gel. The GLUT-4 standard was a plasma membrane fraction prepared as described previously (23).

Hexokinase activity was determined spectrophotometrically (31) at 30°C on 700 g supernatants of the same homogenate as was used for GLUT-4 measurement. Results are expressed as means ± SE. Statistically significant differences between control and AICAR-treated rats were determined by using Student's t-test.

**RESULTS**

Acute effects of AICAR injection. Rats of this size tolerate injections of 1 mg/g body wt daily of AICAR well, with no apparent discomfort. This dose was found to increase ZMP in gastrocnemius muscle from undetectable values to 0.91 ± 0.02 µmol/g 60 min after the injection. This was in the same range as was seen in the preliminary time course experiments. ATP and ADP were not influenced by this treatment (Table 1). ZTP increased from nondetectable levels to 1.2 µmol/g (Table 1). Rats injected with AICAR were found to have significantly increased blood lactate (P < 0.001) and decreased blood glucose (P < 0.01) compared with controls (Table 1). Muscle glycogen and liver glycogen were not acutely influenced 60 min after a single injection of AICAR (Table 1).

AMPK activity was increased 2.4-fold in gastrocnemius/plantarlis muscles and fourfold in the epitrochlearis muscles 1 h after the AICAR injection (Fig. 1). Both changes were highly significant (P < 0.001).

**Table 1. Tissue and blood metabolites of rats killed 60 min after an injection of saline or AICAR**

<table>
<thead>
<tr>
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<th>Saline-Injected Rats</th>
<th>AICAR-Injected Rats</th>
</tr>
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<tbody>
<tr>
<td>Muscle glycogen, µmol/g</td>
<td>43 ± 2</td>
<td>44 ± 4</td>
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<tr>
<td>Muscle ATP, µmol/g</td>
<td>8.1 ± 0.1</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>Muscle ADP, µmol/g</td>
<td>0.97 ± 0.01</td>
<td>0.97 ± 0.02</td>
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<tr>
<td>Muscle ZMP, µmol/g</td>
<td>Not detectable</td>
<td>0.91 ± 0.02*</td>
</tr>
<tr>
<td>Muscle ZTP, µmol/g</td>
<td>Not detectable</td>
<td>1.18 ± 0.09*</td>
</tr>
<tr>
<td>Muscle malonyl-CoA, nmol/g</td>
<td>1.6 ± 0.1</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>7.4 ± 0.2</td>
<td>5.6 ± 0.6*</td>
</tr>
<tr>
<td>Blood lactate, mM</td>
<td>1.7 ± 0.1</td>
<td>6.8 ± 0.5*</td>
</tr>
<tr>
<td>Liver glycogen, µmol/g</td>
<td>333 ± 16</td>
<td>344 ± 44</td>
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</tbody>
</table>

Values are means ± SE, n = 7 rats/group. Muscles are gastrocnemius/plantarlis. AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside. ZMP, phosphorylated form of AICA-riboside, analog of AMP; ZTP, AICA-riboside, analog of ATP. *Significantly different from saline-injected rats, P < 0.01.
Gastrocnemius/plantarlis ACC activity was markedly influenced by AICAR injection (Fig. 2). The maximal velocity of the reaction ($V_{\text{max}}$) as a function of citrate concentration was reduced from 60.1 ± 1.3 to 32.4 ± 1.3 nmol·g$^{-1}$·min$^{-1}$ ($P < 0.001$). The citrate activation constant ($K_a$) was increased from 3.1 ± 0.1 to 13.0 ± 0.3 mM ($P < 0.001$). Because of the limited amount of tissue, the entire citrate activation curve could not be determined for epitrochlearis muscle, but the activity of ACC at a physiological concentration of citrate (0.2 mM) was reduced from 0.50 ± 0.10 to 0.10 ± 0.03 nmol·g$^{-1}$·min$^{-1}$. Gastrocnemius/plantarlis malonyl-CoA was, similarly, significantly ($P < 0.001$) lower in the AICAR-injected rats compared with controls 1 h after the injection (Table 1).

Total GLUT-4, hexokinase activity, and glycogen increase in muscles of 5-day AICAR-injected rats. Figures 3 and 4 show marked increases in GLUT-4 in both epitrochlearis and in gastrocnemius/plantarlis muscles of rats in response to injection with AICAR for 5 days. We noted also in preliminary experiments that larger rats (weighing 350–450 g) responded to AICAR injections (0.5 mg/g body wt) with a significant increase (0.76 ± 0.08 vs. 0.32 ± 0.05 arbitrary units, $P < 0.02$, $n = 5$ rats/group) in total GLUT-4.

Hexokinase activity increased markedly in response to 5 days of AICAR injections (Fig. 5). The increase was ~2.8-fold over control values in both the epitrochlearis and the gastrocnemius/plantarlis muscles. Both increases were highly significant, $P < 0.001$.

In rats killed 24 h after the last of five daily injections of AICAR, gastrocnemius/plantarlis glycogen was 87 ± 4 compared with 43 ± 2 μmol glucose units/g. This difference was highly significant ($P < 0.001$).

DISCUSSION

The acute studies clearly demonstrate that AMPK is activated in epitrochlearis and gastrocnemius/plantarlis muscles of rats injected with AICAR. Previous studies in perfused hindlimbs, in isolated muscles incubated in vitro, and in live animals have demonstrated an acute effect of AICAR on increasing glucose uptake and in increasing fatty acid oxidation in the muscle (1, 14, 25, 36). Additional evidence of activation of AMPK is provided by the fact that the kinetic properties of ACC change similarly to what is seen when purified ACC is phosphorylated in vitro (35). ACC is a downstream target protein for AMPK. Phosphorylation of ACC by AMPK during exercise has been postulated to be responsible for decreasing the muscle con-
tent of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase 1 and allowing, therefore, an increase in oxidation of long-chain fatty acids as they become available (25, 29, 35, 36). Results from the present study show that these effects, normally occurring during exercise, can also be triggered in vivo by chemical activation of the AMPK. One of the postulated causes of insulin resistance in the type 2 diabetes is elevated muscle malonyl-CoA, which would inhibit fatty acid oxidation and increase long-chain acyl-CoA concentrations in the cells (29). Although no data are currently available on malonyl-CoA in muscle of human diabetic patients, these studies clearly demonstrate the feasibility of manipulation of malonyl-CoA by drugs designed to activate the AMPK-signaling system in vivo.

The decrease in blood glucose in response to a single injection of AICAR is consistent with either an increase in glucose uptake into peripheral tissues and/or a decrease in glucose production by the liver. Previous reports from this laboratory using the perfused hindlimb system indicate that the increase in glucose uptake stimulated by AICAR results in increased rates of lactate production in the resting muscle (23, 25). The increase in concentration of blood lactate in the AICAR-injected rats is consistent with the idea that glucose uptake is enhanced, resulting in increased glycolytic flux. Liver glycogenolysis or glycogen synthesis did not appear to be influenced 60 min after an AICAR injection. Previous reports indicate that hepatic gluconeogenesis may be inhibited at the fructose-1,6-bisphosphatase reaction (32, 33). A reduction in utilization of lactate for glucose production by the liver may also have contributed to the decrease in blood glucose and increase in blood lactate.

Numerous studies have demonstrated beneficial effects of regular endurance exercise in increasing insulin sensitivity of muscle (8, 15, 16, 19, 34). In both animals and humans, a few bouts of exercise will increase total GLUT-4 and increase insulin sensitivity (3, 4, 9, 15–17, 19, 22, 26, 28, 29). Patients with type 2 diabetes do not have a deficiency in total GLUT-4 in muscle (6, 11, 27), but insulin-induced translocation of GLUT-4 to the cell surface is defective (7, 20). Etgen et al. (4) demonstrated that 2 wk of exercise (1 h/day) would increase total GLUT-4 and compensate for this defect in the fatty Zucker rat, an animal model of type 2 diabetes. They postulated that this adaptation to training increased insulin-recruitable GLUT-4 in the muscle fibers, thereby allowing increased glucose transport in response to insulin (4). The mechanisms coupling the periodic muscle contraction of endurance training with the increase in GLUT-4 have not been determined. Data from the present study provide the first suggestion that it is the chronic contraction-induced activation of AMPK that triggers the increase in GLUT-4.

Previous studies have demonstrated that GLUT-4 mRNA is increased in muscle in response to endurance...
exercise training (3, 26, 28). The GLUT-4 gene has what has been termed “an exercise response element” (5, 26, 30) residing between 442 and 1,000 base pairs upstream from the transcription start site. Nuclear run-on analyses have clearly demonstrated an increase in muscle GLUT-4 mRNA synthesis with training (26). In animals that had been exercising for several days, an increase in GLUT-4 message was observed 3 h after a bout of exercise. An increase in GLUT-4 gene transcription was not observed in nontrained rats after a single bout of exercise, implying that other mechanisms besides regulation of transcription may be operative in causing the initial increase in GLUT-4 (26). Others (28) have reported an increase in GLUT-4 mRNA in response to a single bout of exercise. Our present data suggest the possibility that what has been called the exercise response element of the GLUT-4 gene may actually be an AMPK response element. It will be important now to determine whether GLUT-4 and hexokinase gene expression increase in response to AMPK activation. If that is found to be the case, transcription factors binding to that region of the gene can be screened to see whether they have target sites for phosphorylation by AMPK. Previous studies provide evidence of regulation of transcription of hepatocyte genes by AMPK. Treatment of isolated hepatocytes with AICAR has been shown to decrease pyruvate kinase and fatty acid synthetase gene expression (see Ref. 36).

It is well documented that, concurrent with an increase in GLUT-4, an increase in muscle hexokinase activity is also seen in response to endurance exercise training (see Refs. 16, 19). The finding of increased hexokinase activity with chronic activation of AMPK with AICAR in sedentary rats lends credence to the idea that repetitive AMPK activation is mediating the effect of chronic muscle contraction on these training adaptations.

Glycogen supercompensation is another well-established effect of endurance exercise training that appears to occur concurrently with an increase in muscle GLUT-4 (18). Although there are other factors that may be responsible for the elevated glycogen in muscles of the rats chronically treated with AICAR (but killed 1 day after the last injection), it is tempting to postulate that the increased GLUT-4 in the muscle allows increased glucose uptake (after the acute effects of AICAR are gone) and the accumulation of more than double the amount of glycogen seen in the saline-injected controls.

As with all studies using the in vivo model, there exists the possibility of nonspecific effects of AICAR on changes in blood hormone concentrations or local changes in the muscle that would secondarily affect GLUT-4 and hexokinase levels in the muscle. It will be important to demonstrate the AICAR-induced increase in GLUT-4 in culture systems where the environment can be more precisely controlled. Similarly, development of specific inhibitors of AMPK will be helpful in confirming these mechanisms. The present studies are important from a practical standpoint, however, demonstrating a new way to alter GLUT-4 and malonyl-CoA levels in the sedentary state in vivo.

Relatively few agents have been described that are effective in manipulating GLUT-4 levels in muscle (5, 30). The data from this study (showing inactivation of ACC, decrease in malonyl-CoA, and increase in total GLUT-4) suggest the possibility of targeting the AMPK signaling system for treatment of insulin resistance. This can be done naturally with exercise, but for those who are unable to exercise, pharmacological manipulation of this signaling system appears feasible. The chronically elevated muscle glycogen in response to chemical AMPK activation may, however, prevent the elevated GLUT-4 from having a beneficial effect on insulin resistance. The increase in glycogen utilization may be an essential component of treatment of insulin resistance with exercise. It is clear from a recent fascinating report (21) that at least some patients with type 2 diabetes respond to an acute bout of exercise with translocation of GLUT-4 to plasma membranes. It now appears that chronic periodic activation of AMPK with chemical activators may prove useful in manipulating GLUT-4. In addition, these studies may provide the rationale for searching for possible defects in the AMPK signaling system as a cause of insulin resistance and dyslipidemia in some forms of type 2 diabetes.

In summary, chronic activation of skeletal muscle AMPK by injection of AICAR into sedentary rats results in significant increases in total GLUT-4 and hexokinase activity, similar to the changes induced by endurance exercise training. Previous studies have demonstrated that muscle contraction occurring during exercise or in response to electrical stimulation increases AMPK activity. These observations together are consistent with the hypothesis that the increases in skeletal muscle GLUT-4 and hexokinase induced by training are mediated by AMPK activation.

Technical assistance was provided by B. Heaps, A. Perry, and D. Rubink. This work was supported by the National Institute of Arthritis Musculoskeletal and Skin Diseases Grant AR-41438 to W. W. Winder. Address for reprint requests and other correspondence: W. W. Winder, 545 WIDB, Dept. of Zoology, Brigham Young University, Provo, UT 84602 (E-mail: william_winder@byu.edu).

Received 23 July 1999; accepted in final form 16 August 1999.

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