AMP-activated protein kinase activation prevents denervation-induced decline in gastrocnemius GLUT-4

S. R. PAULSEN, D. S. RUBINK, AND W. W. WINDER
Department of Zoology, Brigham Young University, Provo, Utah 84602

Received 6 June 2001; accepted in final form 11 July 2001

AMP-activated protein kinase (AMPK) plays a major role in mediating the metabolic responses to insulin and contraction in skeletal muscle. AMPK is activated by AMP, which decreases in denervated muscles (1). The decrease in AMPK activity has been associated with the reduction in GLUT-4 expression in denervated muscles (4, 9, 27, 29, 36, 39). Dener-}

vation of rat hindlimbs causes rapid decline of GLUT-4 levels in skeletal muscle (4, 7, 9, 27, 39). A model for glucose transport studies, denervation also produces significant muscle atrophy (27) and insulin resistance (6, 49). Three days after denervation, GLUT-4 levels were significantly decreased by ~40% in gastrocnemius muscles and by ~30% in soleus muscles. When rats were injected with AICAR (1 mg/g body wt) for 3 days, the decline in GLUT-4 levels was prevented in denervated gastrocnemius muscles but not in denervated soleus muscles. The extent of denervation-induced muscle atrophy was similar in AICAR-treated vs. saline-treated rats. These studies provide evidence that some effects of denervation may be prevented by chemical activation of the appropriate signaling pathways.

5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; adenosine 5′-monophosphate; acetyl-coenzyme A carboxylase; glucose transport; contraction

DENERVATION OF RAT HINDLIMBS causes rapid decline of GLUT-4 levels in skeletal muscle (4, 7, 9, 27, 39). A model for glucose transport studies, denervation also produces significant muscle atrophy (27) and insulin resistance (6, 49). Three days after denervation, GLUT-4 content in rat hindlimb muscles is significantly decreased compared with the sham-operated, contralateral control muscles (4, 9, 27, 36, 39). Denervation induces a significant decline in GLUT-4 mRNA levels in the same 3-day time period (4, 14, 35, 48), suggesting transcriptional mediation of this change in GLUT-4 expression. Interestingly, insulin resistance occurs within 3 h of denervation (49), and decrease in muscle GLUT-4 protein or GLUT-4 mRNA content is not observable until 2 and 3 days after denervation (4, 7). Therefore, the insulin resistance associated with 1-day denervation is not wholly due to these changes in GLUT-4 content. Yet it is thought that the severity of insulin resistance, which is maximal at 3 days after denervation, is related to the depletion of GLUT-4 after 2–3 days (4, 9, 27, 57). One group reported an excellent correlation between the decrease in GLUT-4 and the decrease in insulin-stimulated glucose uptake (r = 0.99) in 3-day-denervated muscles (39). Denervation is a useful model for studying glucose transport and insulin resistance because sham-operated, contralateral control muscles are present in the same rat.

In muscle, GLUT-4 translocates from microparticles to the sarcolemma and t-tubular membranes in response to insulin (see Ref. 42) and contraction (18, 24). GLUT-4 translocation stimulated by contraction uses a separate pathway than does insulin (16, 23, 24, 38). Recent studies have shown that the contraction-mediated pathway may be dependent on AMP-activated protein kinase (AMPK) (23, 37). AMPK can be activated allosterically by increases in the concentration of AMP (10, 20, 47) but is also inhibited by ATP and is therefore sensitive to the AMP concentration-to-ATP concentration ratio (12, 20, 21). AMPK is inhibited by creatine phosphate (PCr) and is likely sensitive to the PCr-to-creatine ratio (44). AMP also serves to activate an upstream kinase (AMPKK), which in turn phosphorylates and thereby activates AMPK (12, 20, 22). 5-Aminoadenosine-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate (ZMP), a normal intermediate in the purine nucleotide synthetic pathway and an AMP analog, has been shown to imitate the effects of AMP and stimulate both AMPK (11, 26) and AMPKK (11). Spinal cord injury in humans leads to decreased skeletal muscle mass (1), decreased whole body insulin sensitivity (15), and, after long-standing injury, glucose intolerance and insulin resistance (2, 15). Spinal cord injury differs from denervation in that it has been shown that, despite the whole body insulin resistance and morphological changes in skeletal muscle, GLUT-4 expression (vastus lateralis) remains unchanged compared with controls (1). Although GLUT-4 content (expressed on the basis of muscle weight) does not change in response to spinal cord injury, overexpression of GLUT-4 induced by electrical stimulation has been
shown to be associated with improved glucose transport activity in human skeletal muscle from quadriplegic patients (8, 29). Because this procedure is not easily accessible to most patients, it would be beneficial if contraction-activated signaling pathways for control of expression of muscle proteins could be chemically activated.

5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) is an adenosine analog that is phosphorylated in muscle cells to become ZMP (40). Chemically induced activation of AMPK via AICAR has been shown to stimulate insulin-stimulated glucose uptake in live rats (3, 5), appearance of GLUT-4 at the cell membrane in live rats (5), glucose uptake in perfused hindlimbs (37, 40), GLUT-4 translocation in perfused hindlimbs (37), and glucose uptake in isolated epimyscealis muscles (23) via a pathway with characteristics similar to the contraction-mediated pathway. Furthermore, it has long been known that endurance training increases GLUT-4 expression in animals (17, 43, 45) and in humans (13, 32–34). More recent studies suggest that repetitive increases in AMPK activity associated with training may mediate this increase in GLUT-4 expression (30, 41, 56). These same studies have also pointed out the effectiveness of chemically activating AMPK via AICAR to mimic the effects of contraction in increasing GLUT-4 expression. This study analyzes whether increased AMPK activity induced by subcutaneous injections of AICAR can prevent the decline in GLUT-4 expression that occurs after denervation of the gastrocnemius and soleus.

**MATERIALS AND METHODS**

**Animal care.** All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Sprague-Dawley rats were housed in individual cages in a room lightly from 6 AM to 6 PM. Rats were provided with water and Harlan Tekland rodent diet (Madison, WI). All rats were ~200 ± 30 g at the beginning of the respective experiments.

**Denervation.** Rats were anesthetized with ethyl ether, and the tibial nerve of the right hindlimb was severed. The left hindlimb was sham operated to serve as a control. Both skin wounds were closed with metal clips. At the time of denervation, a jugular catheter was installed and exteriorized on the back of the neck in rats used for the acute study. This catheter was utilized for intravenous administration of anesthetic at the time of tissue sampling.

**Acute study.** The purpose of this study was to determine whether AMPK activity was acutely increased in denervated muscles after AICAR injection. Rats were denervated between 8:30 AM and 10:30 AM and were then given 25 g of food. Rats were handled twice during the day to accustom them to being handled. The rats were given a single subcutaneous injection 24 h after surgery either of AICAR (1 mg/g body wt) in 0.9% NaCl or of 0.9% NaCl and were subsequently anesthetized intravenously. Soleus and gastrocnemius muscles from both sham and denervated sides were removed and quick frozen in liquid nitrogen for analysis. AMPK activity and acetyl-CoA carboxylase (ACC) activity were determined as previously described (53). Because this analysis is done on homogenates prepared by ammonium sulfate precipitation, only those effects on AMPK activity due to phosphorylation are measured. This measurement of AMPK activity does not provide information about how AMPK might be modulated in response to AMP, ATP, or PCr allosteric effects. Phosphorylation by AMPK results in a decrease in activity of muscle ACC (53). ACC activity is measured as a reporter to give an indication of combined allosteric and covalent activation of AMPK.

**Chronic activation.** Rats were injected with either AICAR (1 mg in 0.9% NaCl/g body wt) or of 0.9% NaCl solution ~0, ~24, and ~48 h after denervation and were then killed ~72 h after denervation without injection. Rats were anesthetized with pentobarbital sodium (4.8 mg/100 g body wt), soleus and gastrocnemius muscles from both sham and denervated sides were extracted, and muscles were quick frozen in liquid nitrogen and stored for analysis.

**Dose response.** Rats were denervated as described above between 8:00 AM and 10:00 AM and were immediately injected either with 0.9% NaCl solution (n = 4) or with 0.1, 0.5 or 1 mg AICAR in 0.9% NaCl/g body wt (n = 3–4 at each dose). Rats of each treatment group were subsequently injected with the respective AICAR solutions at ~24 and ~48 h after denervation. Rats were killed ~72 h after denervation without an AICAR injection, and muscles were collected as described above.

**GLUT-4 determinations.** Muscle was ground to a powder under liquid nitrogen and homogenized (1 g muscle-9 ml buffer) 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 antitrypsin, and 1 μM apro tin, pH 7.5]. Total protein concentration was determined on these homogenates using the Bio-Rad (Bradford) protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The homogenates were then diluted with water and Laemmli’s buffer (1 vol homogenate-2 vol water-1 vol buffer) immediately before loading. Twenty microliters of this homogenate were then loaded onto a 10% SDS-PAGE minigel (Tris·HCl Ready Gels, Bio-Rad). Samples were subjected to electrophoresis at 200 V for 45 min. Proteins were transferred from gel to nitrocellulose membrane at 100 V for 24 and 100 V for 50 min. Membranes were blocked in 5% nonfat dried milk (Bio-Rad) in PBST (139 mM NaCl, 2.7 mM KH2PO4, 9.9 mM Na2HPO4, and 0.05% Tween 20) and were then left overnight with GLUT-4 polyclonal antibody (Biogenesis, Brentwood, CA). Membranes were incubated in enhanced chemiluminescence-development reagent and again being washed twice in PBST and twice in PBS (139 mM NaCl, 2.7 mM KH2PO4, and 9.9 mM Na2HPO4), the membranes were exposed to horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Sciences, Arlington Heights, IL) for 1 h at room temperature. After again being washed twice with PBST and twice with PBS, the membranes were incubated in enhanced chemoluminescence-detection reagent and then visualized on enhanced chemiluminescence hyperfilm (Amersham Life Sciences). Relative amounts of GLUT-4 were then quantified using a Hewlett Packard Scan Jet 6200C and SigmaGel software (SPSS, Chicago, IL). For GLUT-4 Western blot data, we normalized saline-treated, contralateral, innervated muscles to 100%. Data from all other muscles were compared with the saline-treated, contralateral muscles and expressed as a percentage of this control.

**Statistical analysis.** Results are expressed as means ± SE. Statistically significant differences between treatment groups were analyzed using Fisher’s least significant difference test. Statistical significance is defined as P < 0.05.
Table 1. Comparison of muscle weights in 3-day-denervated and contralateral control muscles from rats treated with AICAR or 0.9% NaCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Muscle Weight, mg</th>
<th>Soleus</th>
<th>Gastrocnemius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated denervated</td>
<td>64 ± 3.0</td>
<td>992 ± 31</td>
<td></td>
</tr>
<tr>
<td>Saline-treated control</td>
<td>82 ± 4.4</td>
<td>1,158 ± 21</td>
<td></td>
</tr>
<tr>
<td>AICAR-treated denervated</td>
<td>67 ± 3.0</td>
<td>920 ± 46</td>
<td></td>
</tr>
<tr>
<td>AICAR-treated control</td>
<td>82 ± 4.4</td>
<td>1,055 ± 45</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. AICAR, 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside. *P < 0.05 vs. saline-treated contralateral muscles.

RESULTS

Muscle atrophy. Table 1 summarizes our findings relating to muscle atrophy. We found that the denervated gastrocnemius and soleus muscles from both saline-treated (0.9% NaCl) and AICAR-treated (1 mg in 0.9% NaCl/g body wt) treatment groups were significantly atrophied (P < 0.05) compared with the contralateral, innervated controls. Furthermore, the amount of atrophy was not significantly increased or decreased in the AICAR-treated muscles vs. the saline-treated muscles.

Acute injection of AMPK. Our first objective was to ensure that AMPK was in fact being activated in response to subcutaneous AICAR injections. AMPK activity was significantly (P < 0.05) elevated in AICAR-injected rats above saline-treated controls in both innervated and denervated gastrocnemius muscles (Fig. 1). In the soleus, AICAR injection increased AMPK activity in the innervated (P < 0.05) but not in the denervated leg (Fig. 2).

Because the ammonium sulfate-precipitated homogenates do not reflect any change in AMPK activity due to allosteric effects, we also measured ACC activity as a demonstration of the in vivo action of AMPK. Although AMPK activity was not found to be significantly increased in AICAR-treated denervated soleus muscles, Fig. 3 shows that, in the physiological range of citrate concentration, ACC activity is significantly (P < 0.01) decreased in both the AICAR-treated denervated soleus and the contralateral AICAR treated soleus vs. the saline-treated contralateral innervated soleus. We were not able to collect ACC activity data from the entire citrate activation curve because of the limited amount of tissue from soleus muscles. Figure 4 graphs ACC activity in saline-treated and AICAR-treated gastrocnemius muscles at several different citrate concentrations. AICAR injection is shown to significantly decrease ACC activity in both denervated and contralateral innervated gastrocnemius muscles. The maximal velocity for the reaction as a function of increasing citrate concentration is significantly decreased (P < 0.01) from 36.7 ± 5 in the saline-treated denervated gastrocnemius to 24.7 ± 1.9 in the AICAR-treated denervated gastrocnemius. The citrate activation constant was increased (P < 0.01) from 3.6 ± 0.3 units in the saline-treated denervated gastrocnemius to 13.2 ± 1.1 units in the AICAR-treated denervated gastrocnemius.

Protein concentration. Consistent with the data of Henriksen et al. (27), we found that denervation had no significant effect on muscle protein concentration (mg/g wet weight) (P < 0.05). AICAR treatment also had no effect on muscle protein concentration (P < 0.05). Control gastrocnemius muscles contained 197 ± 10 vs. 189 ± 5 mg protein/g wet weight for denervated gastrocnemius muscles. Control soleus muscles con-
Fig. 4. Citrate dependence of ACC in denervated and contralateral innervated gastrocnemius muscles from rats acutely treated with AICAR (1 mg AICAR in 0.9% NaCl/1 g body wt) or saline (0.9% NaCl) (n = 5 for each group). Brackets denote concentration. SE values were determined but are not shown. Curves were fitted to data by using the Hill equation and Grafit software (Sigma Chemical, St. Louis, MO). Means and SEs for citrate activation constant and maximal velocity of denervated muscles are given in RESULTS.

Dose dependence of GLUT-4 in response to AICAR injection. Figure 5 shows the dose-dependent response of GLUT-4 in AICAR-treated denervated gastrocnemius muscles (curve B) and AICAR-treated contralateral innervated gastrocnemius muscles (curve A). Only the highest dose of AICAR significantly increased GLUT-4 content in denervated gastrocnemius muscles above the GLUT-4 content in the saline-treated, denervated gastrocnemius muscles (P < 0.05). The 1 mg AICAR in 0.9% NaCl/g body wt dose is also shown to be the only dose that significantly raises GLUT-4 content in innervated gastrocnemius muscles above the GLUT-4 content in saline-treated innervated gastrocnemius muscles (P < 0.05).

Total GLUT-4 increases in muscles of denervated rats in response to 3-day AICAR injection. GLUT-4 content of denervated gastrocnemius muscles was found to be 60.1 ± 4.7% of GLUT-4 content in the contralateral innervated gastrocnemius muscles (Fig. 6). GLUT-4 content in denervated gastrocnemius muscles treated with 1 mg AICAR/g body wt was significantly increased (106.6 ± 5.5%) over GLUT-4 levels in denervated gastrocnemius muscles treated with saline (P < 0.01). Furthermore, the AICAR-treated contralateral innervated muscles contained significantly increased levels of GLUT-4 (130.1 ± 7.2%) compared with saline-treated contralateral gastrocnemius muscles (P < 0.01).

A significant decrease in GLUT-4 was observed in the soleus in response to denervation, but the magnitude of the decline was less than in the gastrocnemius. Furthermore, GLUT-4 content did not significantly increase (P < 0.05) in the denervated soleus in response to 1 mg AICAR/g body wt (Fig. 7).

DISCUSSION

As explained in the introduction, the insulin resistance in 3-day-denervated muscles in part been explained by noting the high correlation between decreased insulin-stimulated glucose uptake and the decrease of total GLUT-4 in muscle cells (39, 57). Exacerbating this insulin desensitization, it has also been
shown that components of the insulin-signaling pathway are downregulated in short- and long-term-denervated muscles (28, 50, 52). The fact that chemical activation of AMPK can be accomplished via subcutaneous injection of AICAR (30) provides interesting possibilities for a unique method of improving glucose utilization in conditions such as denervation where the insulin-signaling pathway is impaired and contractile activity is impossible.

The acute studies show that injection of rats with AICAR increases AMPK in denervated gastrocnemius muscles but not in denervated soleus muscles. Furthermore, AMPK activity is significantly increased in contralateral innervated AICAR-treated gastrocnemius and soleus muscles. ACC activity is used in this investigation as a reporter enzyme to indicate the in vivo action of AMPK because the AMPK activity assay is performed on ammonium sulfate precipitated homogenates that do not reflect any modulation of AMPK activity due to allosteric effects. In muscle cells, activation of AMPK is associated with a decrease in ACC activity (25, 46, 51, 53). ACC activity is markedly decreased in the denervated and contralateral innervated AICAR-treated gastrocnemius and soleus muscles. The increase in AMPK activity in AICAR-treated denervated muscles is highlighted by the changes in kinetic properties for ACC activation in our study that mirror the changes in the kinetic properties for ACC that has been purified and phosphorylated in vitro (53).

This inhibitory influence of AMPK on ACC is significant because the product of ACC, malonyl-CoA, is an inhibitor of carnitine palmitoyltransferase 1 (CPT-1), the enzyme responsible for allowing the passage of fatty acids into the mitochondria. So, increased AMPK activity, besides being a regulatory enzyme in the pathway for contraction-mediated glucose transport, is also associated with increased fatty acid oxidation (46, 54). Previous studies have shown denervated muscle to have elevated malonyl-CoA (see Ref. 46). It follows then that chemical AMPK activation might provide a way to stimulate fatty acid oxidation in denervated muscle.

Like previous studies examining GLUT-4 content in denervated muscles (4, 7, 19, 27, 39), we found that GLUT-4 was significantly decreased in gastrocnemius muscles and soleus muscles 3 days after denervation. Subcutaneous AICAR injections resulted in ~80% increase of GLUT-4 in denervated gastrocnemius muscles vs. saline-treated denervated gastrocnemius controls. Soleus muscles of AICAR-treated rats, which exhibited a significant decrease in ACC activity but did not exhibit a significant increase in AMPK activity vs. saline-treated controls, did not manifest a significant increase in GLUT-4. This might suggest that the allosterically activated form of AMPK is active in the ACC inactivation pathway but that the phosphorylated form of AMPK is responsible for increasing GLUT-4 expression. This result is consistent with previous studies that suggest that increased AMPK activity induced by AICAR injection effects increases in total GLUT-4 content in a fiber type-specific manner, with the greatest effect being seen in white fast-twitch oxidative fibers (5, 55).

In consideration of the cause of decreased insulin-stimulated glucose uptake in denervated muscle, Megeney et al. (39) found an excellent correlation between the decrease in GLUT-4 and the decrease in insulin-stimulated glucose uptake (r = 0.99). In support of the idea that loss of GLUT-4 is responsible for reduced insulin-stimulated glucose uptake in 3-day-denervated muscles, a recent investigation examined the translocation of elements known to colocalize with GLUT-4 in intracellular compartments and translocate in response to insulin. It was shown that there was no difference in the insulin-stimulated translocation of insulin-responsive aminopeptidase, transferrin receptor, and insulin-like growth factor II/mannose 6-phosphate receptor in denervated vs. control extensor digitorum longus muscles (57). This study suggests that it is not the insulin-signaling pathway leading to GLUT-4 translocation that is impaired but rather that it is the suppressed expression of GLUT-4 that likely limits insulin-stimulated glucose transport in denervated hindlimbs.

Other investigations have shown that insulin-stimulated insulin receptor substrate-1 phosphorylation in the tibialis anterior (28), phosphatidylinositol 3-kinase activity in the tibialis anterior (28), and Akt-1 kinase activity in soleus and plantaris muscles (50) are all significantly decreased in long-term-denervated muscles. This suggests that beyond the loss of total GLUT-4 there is another level of regulation that is responsible for the decrease in insulin-stimulated glucose uptake. Because it is possible to reverse the depletion of total GLUT-4 in certain 3-day-denervated muscles as we have shown in this study, it will be possible to shed new light on the question of insulin-stimulated glucose uptake in denervated muscles. Our study did not look at glucose uptake or at GLUT-4 translocation in the denervated muscles. If decreased expression of GLUT-4 does in fact play a role in decreased insulin-stimulated glucose uptake, it will be interesting in future studies to observe whether this increase in GLUT-4 will correlate to increased insulin-stimulated glucose uptake. Further investigation will also be needed to examine what, if any, effects chemical activation of AMPK might have on the insulin-signaling pathway in denervated rats.

Two studies have shown that it is possible to increase GLUT-4 levels in paralysis patients with spinal cord injury via electrical stimulation exercise over an 8-wk period (8, 29). The congruous increases in GLUT-4 that we found by chemically activating AMPK in denervated muscles provide a foundation for the possibility of pharmacologically manipulating the glucose utilization system in such patients.

While this paper was being written, we became aware of an abstract by Holmes et al. (31) indicating that, in transgenic mice expressing the choramphenicol acetyltransferase (CAT) gene driven by various lengths of the human GLUT-4 promoter, denervation results in a 73% decrease in CAT and that AICAR
treatment increases CAT transcription 5.4-fold in denervated muscle and 3.9-fold in control muscle. These data strongly support our conclusion that chemical activation of AMPK may be an effective approach for treating glucose transport impairments. More importantly, these studies suggest that some effects of denervation may be prevented by chemical activation of the appropriate signaling pathways.

This research was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-41438.

REFERENCES
31. Holmes BF, Zheng D, MacLean PS, Olson AL, and Dohm GL. AICAR increases GLUT4 mRNA transcription in dener-


