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α2 Isoform–specific activation of 5′ adenosine monophosphate–activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle

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Abstract

5′ Adenosine monophosphate–activated protein kinase (AMPK) has been implicated in exercise-induced stimulation of glucose metabolism in skeletal muscle. Although skeletal muscle expresses both the α1 and α2 isoforms of AMPK, the α2 isoform is activated predominantly in response to moderate-intensity endurance exercise in human and animal muscles. The purpose of this study was to determine whether activation of α2 AMPK plays a role in increasing the rate of glucose transport, promoting glucose transporter 4 (GLUT4) expression, and enhancing insulin sensitivity in skeletal muscle. To selectively activate the α2 isoform, we used 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), which is metabolized in muscle cells and preferentially stimulates the α2 isoform. Subcutaneous administration of 250 mg/kg AICAR activated the α2 isoform for 90 minutes, but not the α1 isoform in hind limb muscles of the C57/B6J mouse. The maximal activation of the α2 isoform was observed 30 to 60 minutes after administration of AICAR and was similar to the activation induced by a 30-minute swim in a current pool. The increase in α2 activity paralleled the phosphorylation of Thr^{172}, the essential residue for full kinase activation, and the activity of acetyl-coenzyme A carboxylase β, a known substrate of AMPK in skeletal muscle. Subcutaneous injection of AICAR rapidly increased, by 30%, the rate of 2-deoxyglucose (2DG) transport into soleus muscle; 2DG transport increased within 30 minutes and remained elevated for 4 hours after administration of AICAR. Repeated intraperitoneal injection of AICAR, 3 times a day for 4 to 7 days, increased soleus GLUT4 protein by 30% concomitant with a significant 20% increase in insulin-stimulated 2DG transport. These data suggest that moderate endurance exercise promotes glucose transport, GLUT4 expression, and insulin sensitivity in skeletal muscle at least partially via activation of the α2 isoform of AMPK.
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1. Introduction

Physical exercise is a potent stimulator of glucose transport and glucose transporter 4 (GLUT4) expression in skeletal muscle. An acute bout of exercise increases the rate of glucose transport into contracting muscles by inducing translocation of GLUT4 to the cell surface via an insulin-independent mechanism (contraction-stimulated glucose transport) [1]. Acute exercise also activates expression of GLUT4 protein, and the GLUT4 protein expression is elevated with repeated bouts of acute exercise [2]. The exercise-induced increase in GLUT4 is associated with improved insulin sensitivity (ie, increased rates of insulin-stimulated GLUT4 translocation and glucose transport into

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skeletal muscle) [3]. These mechanisms of enhanced glucose transport help improve glycemic control in patients with diabetes and may help prevent nondiabetic subjects from developing glucose intolerance.

Recent studies have suggested that 5′ adenosine monophosphate–activated protein kinase (AMPK) is an important signaling intermediary leading to contraction-stimulated GLUT4 translocation and glucose transport [4-9] and GLUT4 expression [10-15] in skeletal muscle. AMPK is a heterotrimeric protein composed of a catalytic α subunit and regulatory subunits, β and γ. Although the α subunit exists in different isoforms in skeletal muscle [16], α1 and α2, the α2 isoform–containing AMPK is preferentially activated in response to exercise. For example, cycle ergometer exercise at 50% of maximum energy consumption ($\dot{V}O_2$max) does not change α2 or α1 activity, and exercise at 60% to 75% of $\dot{V}O_2$max increases α2, but not α1, activity in biopsy samples of vastus lateralis muscle from healthy subjects [17-19]. Similar activation of α2 occurs in response to cycle ergometer exercise at 70% of $\dot{V}O_2$max in patients with type 2 diabetes mellitus who have similar protein expression of α isoforms as healthy subjects [20]. In contrast, both isoforms are significantly activated in response to high-intensity exercise such as sprint exercise requiring power output 2- to 3-fold greater than that attained during maximal aerobic exercise [21]. In rat skeletal muscle, voluntary treadmill running exercise increases only α2 activity, whereas high-intensity contractions, such as electrically induced tetanic contractions, increase the activities of both isoforms in isolated rat skeletal muscle [8]. These observations in human and animal muscles suggest that regulation of the α isoforms is intensity-dependent in contracting skeletal muscle, and that the α2 isoform, rather than α1, is involved in the metabolic responses to moderate-intensity endurance exercise.

We explored the physiological relevance of the predominant α2 activation in skeletal muscle, focusing particularly on glucose transport, GLUT4 expression, and insulin sensitivity by selectively activating α2 AMPK using the AMPK-stimulating agent, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR).

2. Materials and methods

2.1. Materials

AICAR was obtained from Sigma (St Louis, MO). Phosphospecific antibody directed against AMPKα Thr^{172} was obtained from Cell Signaling Technology (Beverly, MA) and that directed against acetyl-coenzyme A carboxylase β (ACCβ) Ser^{79} from Upstate Biotechnology (Lake Placid, NY). Anti-GLUT4 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All radioactive materials were purchased from NEN Life Science Products (Boston, MA). Reagents for the protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Sigma or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

2.2. Animals

Male C57/B6 mice, aged 7 to 10 weeks, were obtained from Shimizu Breeding Laboratories (Kyoto, Japan) and fed standard laboratory chow and water ad libitum. They were housed in plastic cages in an environmentally controlled room maintained at 23°C with a 12-hour light-dark cycle. Mice were fasted for 8 to 10 hours before the experiments, except as otherwise described. Blood samples were collected from the tail vein. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan.

2.3. Administration of AICAR

For studies of a single administration of AICAR, AICAR was dissolved in saline (20 g/L) and injected subcutaneously or intraperitoneally without anesthesia at a dose of 250 mg/kg body weight. Mice were then killed by cervical dislocation at the indicated time points, and either hind limb muscles (gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus [EDL] muscles) or soleus and EDL muscles were dissected. For studies of repeated injections of AICAR, 250 mg/kg of AICAR was dissolved in saline (20 g/L) and injected into fed mice intraperitoneally 3 times a day for up to 8 days. Mice were killed by cervical dislocation 12 to 16 hours after the last injection, and the hind limb or soleus and EDL muscles were collected. The muscles were either processed fresh to measure 2-deoxyglucose (2DG) transport or frozen and stored in liquid nitrogen for later assays. Saline was injected as a control condition in the studies using the single and repeated administration of AICAR.

2.4. Swimming exercise

Mice swam in groups of 6 or less at a time at ~60% of $\dot{V}O_2$max (5 L/min flow rate) for 30 minutes during the dark cycle as described previously [22]. A large adjustable-current pool (90 × 45 × 45 cm) filled to a depth of 38 cm [22] allowed each mouse to swim without interference with other mice. A constant current was generated by circulating water with a pump, and the flow was monitored by a water flow meter, which was used to adjust the strength of the current. The temperature of the water was maintained at 34°C with a water heater and thermostat. For studies involving a single bout of exercise, mice were killed by cervical dislocation immediately after swimming, the hind limb muscles were dissected, and the muscles were frozen and stored in liquid nitrogen. For studies involving repeated bouts of exercise, fed mice swam for 30 minutes during the dark cycle twice a day for up to 7 days. Twelve to 16 hours after the last exercise session, the mice were killed and muscle samples were dissected, frozen, and stored in liquid nitrogen.

2.5. Intraperitoneal glucose test and insulin tolerance test

The intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as described [23], with modifications. For the GTT, glucose (2.0 g/kg body weight) was administered intraperitoneally to conscious animals 12 to 16 hours after the last injection of AICAR or saline, or swimming exercise. For the ITT, human recombinant insulin (Eli-Lilly, Indianapolis, IN) (1.2 U/kg body weight diluted with saline) was injected intraperitoneally to fed conscious mice.

2.6. Isoform-specific AMPK activity

Isoform-specific AMPK activity was determined as described [24], with modifications. Frozen muscles were weighed and then homogenized in ice-cold lysis buffer (1:100 wt/vol) containing 20 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L soybean trypsin inhibitor, 0.1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride, and centrifuged at 14 000*g* for 30 minutes at 4°C. Supernatants (200 μg protein) were immunoprecipitated with specific antibodies against the α1 or α2 catalytic subunit [24] and protein A/G agarose beads (Pierce, Rockford, IL). Immunoprecipitates were washed twice in lysis buffer and twice in wash buffer containing 240 mmol/L HEPES (pH 7.0) and 480 mmol/L NaCl. The kinase reaction, which was started by adding 0.1 mmol/L SAMS peptide with the sequence HMRSAMSGLHLVKRR, contained 40 mmol/L HEPES (pH 7.0), 0.2 mmol/L AMP, 80 mmol/L NaCl, 0.8 mmol/L dithiothreitol, 5 mmol/L $MgCl_2$, and 0.2 mmol/L ATP [2 μCi (γ-^{32}P)ATP] at 30°C for 20 minutes in a final volume of 40 μL. At the end of the reaction, a 15-μL aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, UK). The papers were washed 6 times in 1% phosphoric acid and once in acetone. ^{32}P incorporation was quantified with a scintillation counter, and kinase activity was expressed as fold increases relative to basal levels.

2.7. 2-Deoxyglucose transport activity

The amount of 2DG transport was determined as described [25], with modifications. Tendons from both ends of dissected soleus and EDL muscles were tied with sutures (silk 3-0, Natsume Seisakusho, Tokyo, Japan), and the muscles were mounted on an incubation apparatus to maintain resting length. To measure 2DG transport after a single injection of AICAR, muscles were incubated for 10 minutes in 7 mL of incubation buffer containing Krebs-Ringer bicarbonate (KRB) buffer (117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 2.4 mmol/L KH_2PO_4, 2.4 mmol/L $MgSO_4$, and 24.6 mmol/L $NaHCO_3$) with 2 mmol/L pyruvate and gassed continuously with 95% O_2 and 5% CO_2. Muscles were then transferred to 2 mL of transport buffer containing KRB buffer with 1 mmol/L 2-deoxy-D-[^{3}H]glucose (1.5 mCi/L) and 7 mmol/L D-[^{14}C]mannitol (0.45 mCi/L) at 30°C and incubated for 10 minutes. To measure basal- and insulin-stimulated 2DG transport after repeated AICAR treatment, dissected muscles were preincubated in the incubation buffer for 40 minutes and then incubated in the incubation buffer with or without 5000 mU/L insulin for 40 minutes. Muscles were then transferred to 2 mL of the transport buffer with or without 5000 mU/L insulin and incubated for 10 minutes. Transport was terminated by dipping muscles in KRB at 4°C, and the muscles were frozen in liquid nitrogen. Frozen muscles were weighed and then processed by incubating in 300 μL of 1 mol/L NaOH at 80°C for 10 minutes. Digestates were neutralized with 300 μL of 1 mol/L HCl. Radioactivity in aliquots of the digestates was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated.

2.8. Muscle glycogen content

Glycogen content was assayed as described [26], with modifications. Frozen muscles were weighed and digested in 1 mol/L NaOH (1:9 wt/vol) at 85°C for 10 minutes. At the end of the incubation, tubes were shaken by hand to facilitate digestion. After cooling to room temperature, digestates were neutralized with 1 mol/L HCl (1:9 wt/vol), and then 5 mol/L HCl was added to obtain a final concentration of 2 mol/L HCl. The digestates were incubated again at 85°C for 2 hours and then neutralized with 5 mol/L NaOH. The concentration of hydrolyzed glucose residues was measured enzymatically using the hexokinase glucose assay reagent (Sigma). Glycogen content was expressed as micromoles of glucose units per gram (wet weight) of muscle.

2.9. Glycogen synthase activity

Glycogen synthase activity was assayed as described [26], with modifications. Frozen muscles were homogenized in buffer containing 20 mmol/L HEPES (pH 7.4), 1% Triton X-100, 50 mmol/L NaCl, 2 mmol/L EGTA, 50 mol/L NaF, 50 mol/L β-glycerophosphate, 10 mg/L aprotinin, 3 mol/L benzamidine, 4 mg/L leupeptin, and 0.5 mol/L phenylmethylsulfonyl fluoride, and centrifuged at 14 000*g* for 30 minutes at 4°C. The supernatants (40 μg of protein) were added to 80 μL of reaction solution containing 50 mmol/L Tris-HCl (pH 7.8), 5 mol/L EDTA, 6.7 mmol/L UDP-[^{14}C]glucose (100 μCi/mmol/L), 10 g/L glycogen, 50 mol/L β-glycerophosphate, and 50 mmol/L NaF in the presence or absence of 6.7 mmol/L glucose-6-phosphate at 30°C to measure the glucose-6-phosphate–independent (I-form) and the total glycogen synthase activities, respectively. The reaction was terminated after 15 minutes by spotting the reaction mixture on filter papers; after extensive washing with 66% (vol/vol) ethanol, the samples were counted in a scintillation counter to measure ^{14}C incorporated into glycogen. The enzyme activity was calculated as the ratio of the I-form activity to total activity.

2.10. Immunoblotting

Immunoblotting was performed as described [26], with modifications. Frozen muscles were homogenized in 10 volumes (1:10, wt/vol) of a solution containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L β-glycerophosphate, 2mmol/L EGTA, 1% Triton X-100, 10% glycerol, 1 mmol/L dithiothreitol, 3 mmol/L benzamidine, 1 mmol/L $NaVO_4$, 0.5 mmol/L phenylmethylsulfonyl fluoride, 200 mg/L of soybean trypsin inhibitor, 10 mg/L aprotinin, and 10 mg/L leupeptin. The homogenates were centrifuged at 14000g at 4°C for 30 minutes. The supernatants were then diluted with water and Laemmli buffer and boiled at 80°C for 2 minutes. Denatured lysates (20-30 μg protein) were separated on a 10% polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane (PolyScreen; Perkin-Elmer, Boston, MA) at 100 V for 1 hour. The membranes were blocked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan) and left to incubate overnight with antibodies. The membranes were then washed, reacted with antirabbit immunoglobulin G coupled to peroxidase (Santa Cruz Biotechnology), and developed with an enhanced chemiluminescence reagent (Hyperfilm) according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The signal on the blot was detected and quantified with a Lumino-Image Analyzer LAS-1000 System (Fuji Photo Film, Tokyo, Japan). Data were expressed relative to control values.

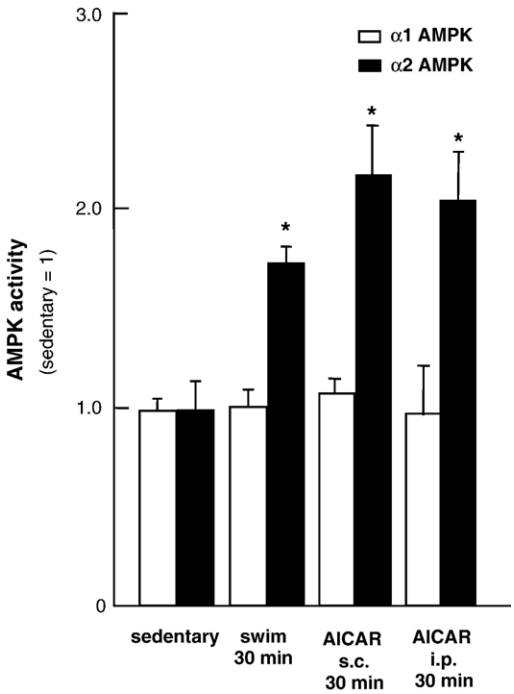

Fig. 1. Effects of exercise and AICAR on α1 and α2 AMPK activities in hind limb muscles. After a 30-minute bout of swimming or 30 minutes after subcutaneous (s.c.) or intraperitoneal (i.p.) injection of 250 mg/kg AICAR, hind limb muscles (gastrocnemius, soleus, tibialis anterior, and EDL) were removed, and isoform-specific AMPK activities were determined. Results are means ± SE (n = 7-10 per group). *P < .05 compared with muscles from sedentary animals.

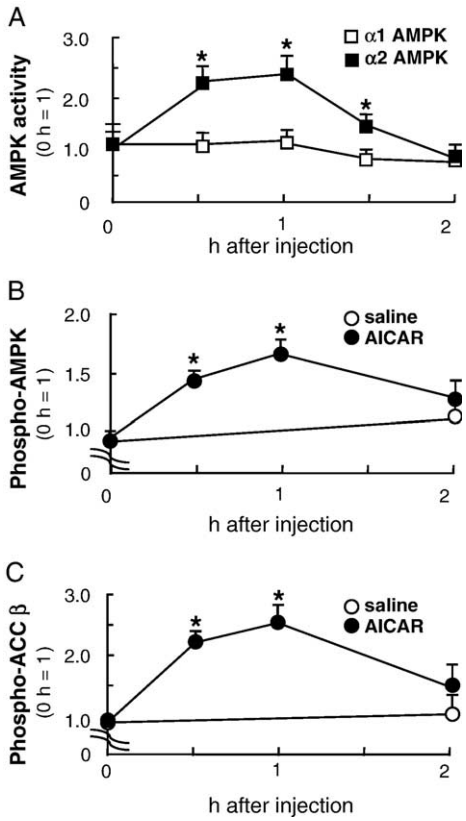

Fig. 2. Time course of changes in isoform-specific AMPK activity (A), AMPK phosphorylation (B), and ACCβ phosphorylation (C) in hind limb muscles after subcutaneous AICAR injection (250 mg/kg). Results are means ± SE (n = 7-10 per group). *P < .05 compared with basal levels.

2.11. Blood lactate, insulin, and glucose concentrations

Blood lactate concentration was measured by the lactate oxidase method using an automated analyzer (Lactate Pro; Arcray, Kyoto, Japan). Serum insulin concentration was determined using an Insulin ELISA kit (Morinaga Institute of Biological Sciences, Yokohama, Japan). Blood glucose concentration was measured by the glucose oxidase method with an automated blood glucose analyzer (Glutest Ace, Sanwa Kagaku, Nagoya, Japan).

2.12. Statistical analysis

Results are presented as means ± SE. Two means were compared by the unpaired Student t test. Multiple means were compared by analysis of variance followed by post hoc comparison using the Fisher protected least-significant difference method. P < .05 was considered statistically significant.

3. Results

3.1. Moderate-intensity exercise and AICAR activated predominantly α2 AMPK to a similar extent

After 30 minutes of moderate-intensity swimming exercise, α2 AMPK activity increased by 80%, but α1

AMPK activity did not change significantly in the hind limb muscles (Fig. 1). Similarly, subcutaneous and intraperitoneal injection of AICAR (250 mg/kg) activated α2 AMPK by 110% and 100%, respectively, but did not activate α1 AMPK (Fig. 1). The stimulation of α2 AMPK activity by exercise did not differ significantly from that induced by AICAR injection. The exercise-stimulated activation of α2 was abolished within 2 hours after exercise.

3.2. AICAR increased α2 AMPK activity, AMPK phosphorylation, and ACCβ phosphorylation in skeletal muscle

α2 AMPK activity was significantly higher 30, 60, and 90 minutes after subcutaneous injection of AICAR and returned to baseline within 2 hours after injection in the hind limb muscles (Fig. 2A). α1 AMPK activity did not change at any time point examined (Fig. 2A). Phosphorylation of Thr^{172}, an essential residue for full kinase activity [27], increased significantly in parallel with α2 AMPK activation (Fig. 2B). Phosphorylation of ACCβ, a known substrate of AMPK [28], also displayed a similar

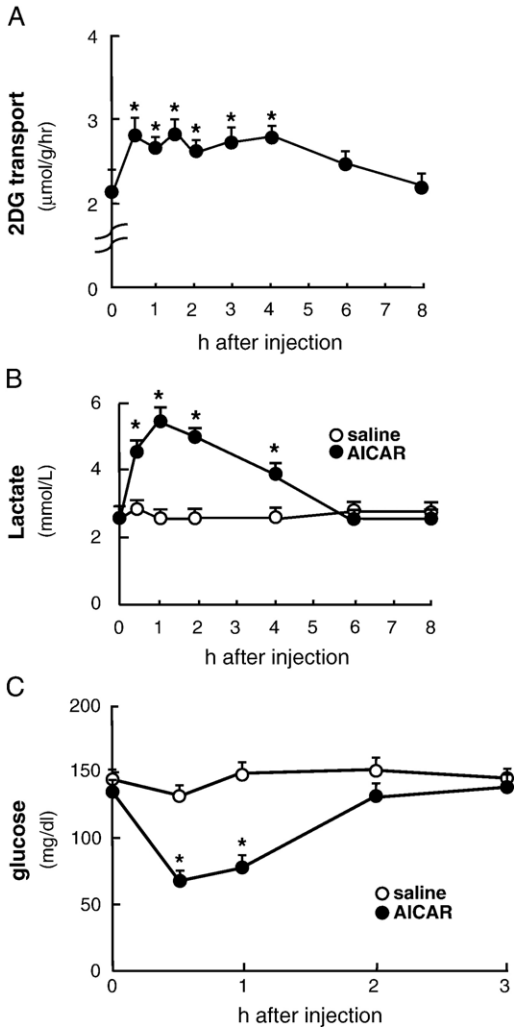

Fig. 3. Time course of 2DG transport activity in soleus muscle (A), blood lactate concentration (B), and blood glucose concentration (C) after subcutaneous injection of AICAR (250 mg/kg). Results are means ± SE (n = 7 to 10 per group). *P < .05 compared with basal levels.

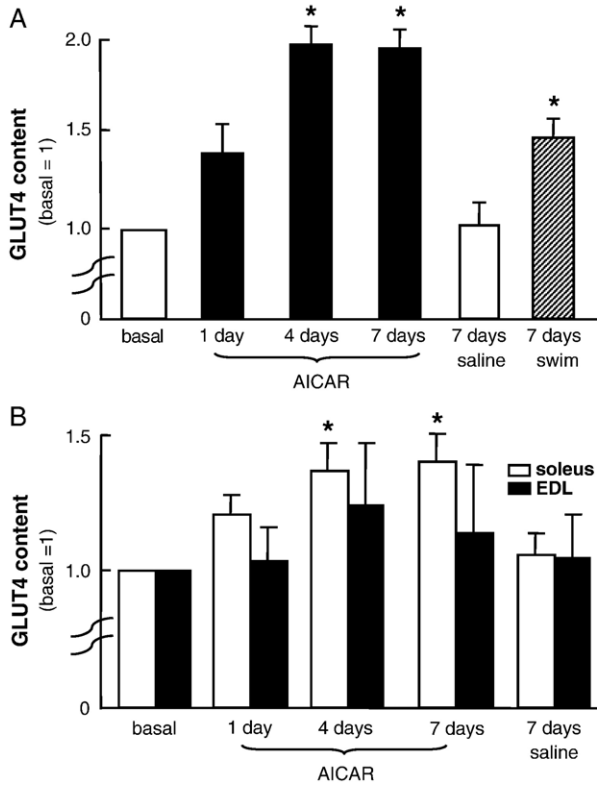

Fig. 4. Glucose transporter 4 protein content in hind limb muscles (A), and soleus and EDL muscles (B). After repeated intraperitoneal injection of AICAR (250 mg/kg) or saline 3 times a day for up to 7 days, or exercise swim training for 7 days, either hind limb or soleus and EDL muscles were isolated, and GLUT4 content was determined with immunoblotting. Results are means ± SE (n = 10 per group). *P < .05 compared with basal levels.

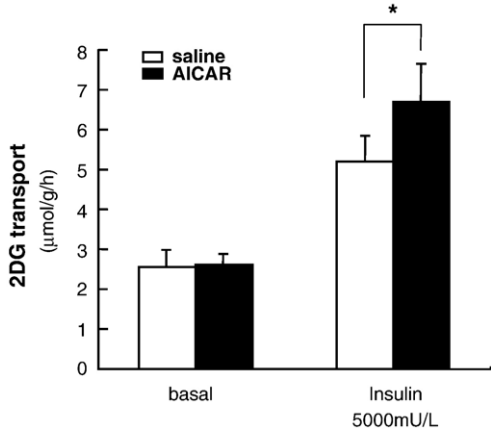

Fig. 5. Basal and insulin-stimulated 2DG transport activity in isolated soleus muscle after repeated intraperitoneal injection of AICAR or saline for 7 days. Soleus muscle was isolated 12 to 16 hours after the last injection, and 2DG transport activity was determined in the absence or presence of 5000 mU/L insulin. Results are means ± SE (n = 7-10 per group). *P < .05 compared with the saline group.

pattern as α2 AMPK activity (Fig. 2C). The α2 AMPK activity was significantly elevated for 4 hours after a single intraperitoneal injection of AICAR, whereas the α1 AMPK activity did not change.

3.3. The AICAR-induced increase in 2DG transport activity into skeletal muscle was accompanied by an increase in blood lactate concentration and decrease in blood glucose concentration

In the soleus muscle, a single subcutaneous injection of AICAR increased the rate of 2DG transport by 30%, and this elevated activity was maintained for 4 hours (Fig. 3A). Neither glycogen concentration nor glycogen synthase activity was altered (glycogen: baseline, 36.3 ± 1.6 μmol/g; 0.5 hour, 36.6 ± 0.6 μmol/g; 1.0 hour, 37.9 ± 1.9 μmol/g; 2.0 hours, 38.4 ± 1.3 μmol/g; n = 7-8 per group; glycogen synthase: baseline, 14.9% ± 0.2%; 0.5 hour, 15.2% ± 0.3%; 1.0 hour, 14.4% ± 0.4%; 2.0 hours, 14.6% ± 0.3%; n = 8 per group). In the EDL muscle, 2DG transport did not increase significantly (baseline, 2.0 ± 0.1 μmol/g per hour; 0.5 hour, 2.2 ± 0.1 μmol/g per hour; n = 8 per group, $P < .10$). Blood lactate concentration, an indicator of nonoxidative glucose utilization, was also elevated for 4 hours after injection (Fig. 3B). Blood glucose concentration decreased after a single AICAR injection, and this reduction was abolished within 2 hours after injection (Fig. 3C). Plasma insulin concentration was unchanged at the time points studied (baseline, 1.3 ± 0.3 μg/L; 0.5 hour, 1.3 ± 0.1 μg/L; 1.0 hour, 1.3 ± 0.3 μg/L; 2.0 hours, 1.3 ± 0.3 μg/L; n = 7 per group).

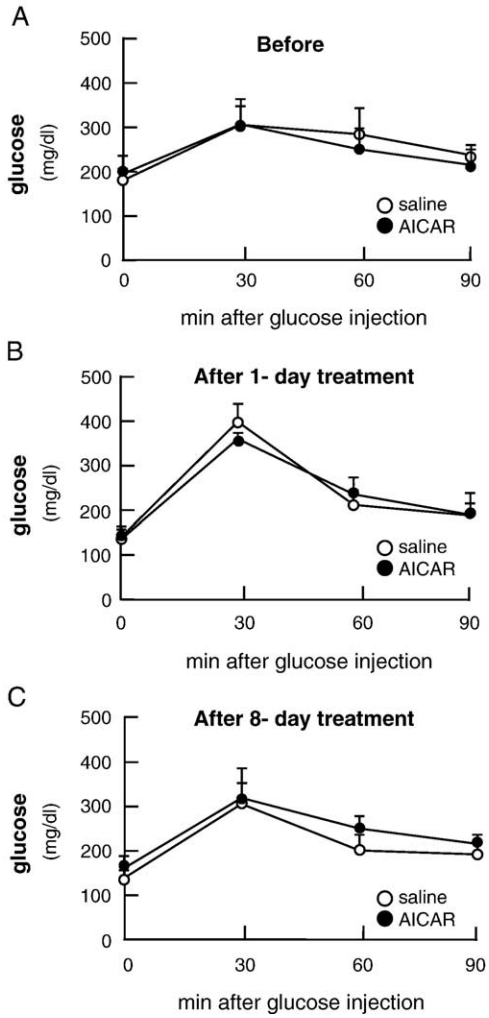

Fig. 6. Glucose tolerance in AICAR- and saline-treated mice. The intraperitoneal GTT was performed before (A) and after repeated intraperitoneal injection of AICAR (250 mg/kg) or saline 3 times a day for 1 day (B) and 8 days (C). Glucose (2.0 g/kg body weight) was administered by intraperitoneal injection 12 to 16 hours after the last injection of AICAR. Results are means ± SE (n = 7-10 per group).

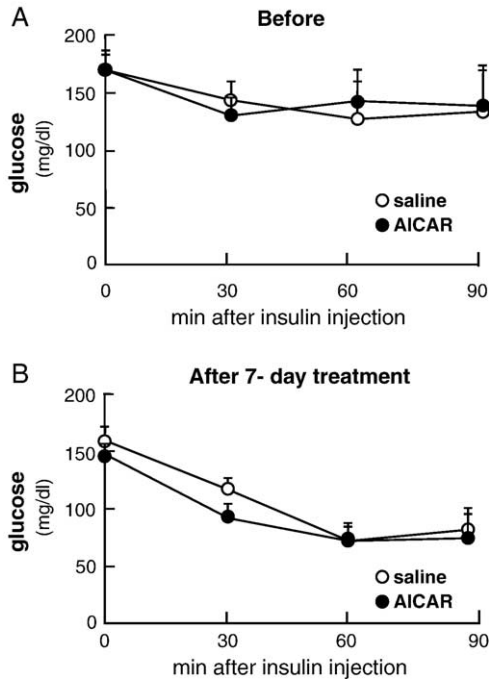

Fig. 7. Insulin tolerance in AICAR- and saline-treated mice. The intraperitoneal ITT was performed before (A) and after (B) repeated intraperitoneal injections of AICAR (250 mg/kg) or saline 3 times a day for 7 days. Glucose (1.2 U/kg body weight) was administered by intraperitoneal injection 12 to 16 hours after the last injection of AICAR. Results are means ± SE (n = 7-10 per group).

3.4. Repeated AICAR injection increased GLUT4 content and insulin-stimulated glucose transport in skeletal muscle

Glucose transporter 4 content in the hind limb muscles increased by 50% after 7 consecutive days of swimming (Fig. 4A). Similarly, repeated intraperitoneal AICAR injection 3 times a day for 4 to 7 days increased GLUT4 content in the hind limb muscles by 90% (Fig. 4A) and in soleus muscle by 40% (Fig. 4B). The increase in GLUT4 in EDL muscle was not significant (Fig. 4B). To determine whether the increased GLUT4 content was associated with enhanced insulin-stimulated glucose transport, we measured 2DG transport activity in soleus muscle treated with AICAR and saline for 7 days. As shown in Fig. 5, the baseline rate of 2DG transport was not affected by AICAR, whereas the

insulin-stimulated rate of 2DG transport activity was 20% higher in AICAR-treated than in saline-treated soleus muscle. In soleus muscle, glycogen synthase activity (% I-form) was not affected (baseline, 14.1% ± 0.3%; 7 days, 14.8% ± 0.8%; n = 8), and glycogen content did not change in response to AICAR treatment (baseline, 39.2 ± 3.1 μmol/g; 7 days, 44.0 ± 3.5 μmol/g; n = 10).

3.5. Whole-body glucose tolerance and insulin tolerance were not affected by repeated AICAR injection

The intraperitoneal GTT (Fig. 6) and ITT (Fig. 7) were performed to determine the effects of repeated AICAR injection on whole-body glucose metabolism. The GTT was performed before (Fig. 6A), after 1 day (Fig. 6B), and after 8 days (Fig. 6C) of administration of AICAR or saline. Glucose concentration did not differ between the AICAR- and saline-treated groups at any time point. Fasting insulin concentration was not affected by the AICAR treatment (baseline, 1.3 ± 0.1; 4 days, 1.2 ± 0.2; 7 days, 1.2 ± 0.2; n = 7). The ITT was performed before (Fig. 7A) and after 7 days (Fig. 7B) of repeated intraperitoneal injection of AICAR or saline. Similar to the results of the GTT, the response to the ITT did not differ significantly between the AICAR- and saline-treated groups. The responses to the GTT (Fig. 8A) and ITT (Fig. 8B) did not differ between sedentary animals and those exercised for 7 days. Body weight was unchanged after AICAR administration (baseline, 24.2 ± 0.6 g; 7 days, 24.4 ± 0.4 g; n = 10).

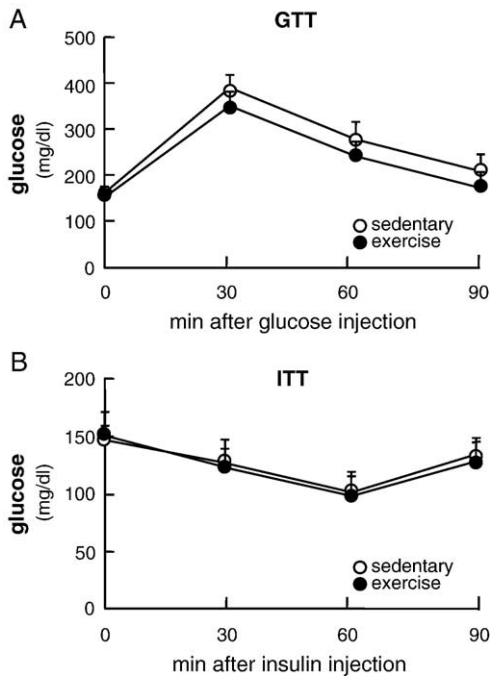

Fig. 8. Glucose tolerance and insulin tolerance in sedentary and exercise-trained mice. The exercise group swam for 30 minutes twice a day for 7 days. The intraperitoneal GTT (A) and intraperitoneal ITT (B) were performed 16 hours after the last bout of exercise. Results are means ± SE (n = 7-10 per group).

4. Discussion

Endurance exercise activates predominantly α2 AMPK in human and animal skeletal muscles. Fujii et al [17] reported a 2-fold increase in α2 AMPK activity in human vastus lateralis muscle after 60-minute cycle exercise at 70% $\dot{V}O_2$max, but no change in α1 AMPK after exercise. Similarly, Wojtaszewski et al [18] reported a 3-fold activation of α2 AMPK in human vastus lateralis after cycle exercise at 75% $\dot{V}O_2$max for 55 minutes followed by 90% $\dot{V}O_2$max for 5 minutes. Musi et al [8] showed that α2 AMPK is activated by 50% to 100% after treadmill running (18-32 m/min at 10% grade for 1 hour) and that this activation is accompanied by a significant increase in 3-*O*-methylglucose transport in rat epitrochlearis muscle. Our observations of a 2-fold increase in α2 AMPK activity after 30-minute swimming at ~60% $\dot{V}O_2$max in mouse skeletal muscle, but no increase in α1 AMPK activity, are consistent with these previous findings.

We administered 250 mg/kg AICAR subcutaneously or intraperitoneally to activate α2 AMPK to the same extent as the activation observed in skeletal muscle after exercise. AICAR is taken up into skeletal muscle and metabolized by adenosine kinase to form ZMP, a monophosphorylated derivative that mimics the effects of AMP on AMPK without changing the intracellular levels of AMP or ATP [29]. The concentration of intracellular AMP and the AMP/ATP ratio are both important determinants of AMPK activity; α2 AMPK has greater dependence on AMP than the α1 isoform in both the allosteric activation by AMP and the covalent activation by upstream kinase [27,30]. In our study, both types of injections activated α2 AMPK in skeletal muscle, but the intraperitoneal injection produced a longer-lasting activation than the subcutaneous injection. α2 AMPK activity increased for at least 4 hours after the intraperitoneal injection, but the enzyme activity returned to baseline within 2 hours after subcutaneous injection. Although the precise mechanism by which AICAR activates α2 AMPK is unknown, the site of injection may have a substantial influence on the rate of absorption, and the time course and intensity of activation.

Although AICAR is not strictly specific for AMPK [31-33], recent studies with AICAR have provided important information about the function of AMPK in muscle glucose transport and GLUT4 expression. Mu et al [7] selectively blocked AMPK in mouse skeletal muscle with muscle-specific expression of a dominant-negative, kinase-dead form of α2 AMPK. In this mouse, the stimulatory effects of AICAR on glucose transport [7] and GLUT4 expression [15] were blocked completely. In addition, the AICAR-stimulated glucose transport was abolished in skeletal muscles from whole-body α2 knockout mouse, but not in muscles from whole-body α1 knockout mouse [9]. Furthermore, incubating isolated animal muscles in the presence of AICAR increased glucose transport [4,34] and GLUT4 protein expression [11]. These results strongly

indicate that the metabolic effects of AICAR on skeletal muscle involve α2 AMPK-dependent signaling events, which can occur independent of changes in systemic factors. Interestingly, in mice with muscle-specific expression of a dominant-negative, kinase-dead AMPK, glucose transport is only partially reduced in response to electrically stimulated contractions of hind limb muscles [7], and muscle GLUT4 messenger RNA increases after endurance exercise (two 3-hour bouts of treadmill running) [15]. Similarly, the rate of glucose transport increases after electrical stimulation of isolated muscles from the transgenic mouse expressing the dominant-negative α2 AMPK [9]. Although the previous studies did not examine the effects of moderate-intensity exercise, there may be additional signaling mechanisms, other than α2 AMPK, leading to exercise-induced metabolic events in skeletal muscle.

We found that the α2 AMPK activity decreased within 2 hours after subcutaneous injection of AICAR (Fig. 2A), whereas 2DG transport remained elevated for at least 4 hours (Fig. 3A). Musi et al [8] previously demonstrated that the time course of AMPK is dissociated from the glucose transport activities in isolated rat epitrochlearis muscle during in vitro electrical stimulation of muscle contractions. AMPK activity decreased rapidly after the cessation of tetanic contractions ($t_{1/2}$ = 8 minutes), whereas the rate of decrease in 3-*O*-methylglucose transport was much slower and had decreased by only 48% after 60 minutes. This previous report and our findings suggest that, although AMPK may be involved in stimulating glucose transport, sustained AMPK activity is not required to maintain transport activity.

Activation of AMPK and its effects vary by muscle fiber type. In the studies using rat skeletal muscle, long-term AICAR administration has the greatest effects on GLUT4 and glycogen content in fast-twitch muscles [10,12,35]. Daily subcutaneous injections of AICAR at a dose of 1 g/kg body weight for 4 weeks increased GLUT4 and glycogen content in the red and white quadriceps, but not in the soleus muscle in rats [35]. Moreover, acute AICAR exposure stimulates glucose transport in white muscles, but has no effect in rat soleus muscle [34,36]. In contrast, incubation with AICAR markedly increases glucose transport in both soleus and EDL muscles in the mouse [34]. We also found significant increases in 2DG transport activity and GLUT4 content ($P < .05$) in mouse soleus muscle. This discrepancy in the effects of AICAR between rat and mouse soleus muscles may be due to a greater percentage of fast-twitch muscle fibers within mouse soleus (ie, the mouse soleus has proportionately more fast-twitch fibers than the rat soleus) [34]. Because of the nonspecific stimulation by AICAR in mouse skeletal muscle, we believe that most skeletal muscles responded to the subcutaneous and intraperitoneal AICAR administration in our study.

The concept that a large number of skeletal muscles are stimulated by AICAR in mouse is indirectly supported by our observation that AICAR treatment caused a marked increase in blood lactate concentration and reduction in blood glucose concentration, with a corresponding increase in glucose transport activity (Fig. 3). The hypoglycemic effect of AICAR is blunted in mice with muscle-specific expression of the dominant negative AMPK [7], emphasizing the pivotal role of muscle AMPK in AICAR-induced hypoglycemia. However, it has also been reported that, after the conversion into ZMP, AICAR exerts a dose-dependent inhibition of fructose-1,6-bisphosphatase, which inhibits gluconeogenesis and enhances lactate production in the liver [32,33]. Thus, the effects on both skeletal muscle and other tissues, including liver, may contribute to the hypoglycemia and elevated lactate concentrations caused by AICAR.

The contribution of increased muscle GLUT4 to glucose tolerance and insulin sensitivity has been clearly documented in studies using transgenic mice with muscle-specific overexpression of GLUT4 [37,38]. However, we found that AICAR treatment had no effect on blood glucose excursions during the GTT and ITT compared with the saline treatment (Figs. 6 and 7). Similarly, swimming exercise for 7 days did not affect glucose excursions compared with sedentary mice (Fig. 8). Although the underlying mechanism is unclear, the effects of long-term AICAR treatment and swimming exercise may be below the detectable limit of the GTT or ITT because we used the metabolically normal mouse (C57/B6). This concept is consistent with the observation that metabolic improvements occurred after 7 days of treatment with AICAR and were detected by the oral GTT (3 mg/kg) and ITT (10 U/kg) in KKA^y-CETP mice, a model of insulin-resistant type 2 diabetes mellitus [39]. Because we performed ITTs on fed animals, the food consumption of the animals coming into the test could affect the response, and there may have been a different response at 15-minute time point that returned to control at 30 minutes in GTTs.

In summary, we found that pharmacological activation of α2AMPK by AICAR at a physiological level led to a short-term increase in glucose transport and that long-term activation of the isoform increased GLUT4 protein and enhanced insulin-stimulated glucose transport in mouse skeletal muscle. These results strongly suggest that activation of α2 AMPK during moderate exercise plays pivotal roles in exercise-stimulated glucose uptake and utilization in skeletal muscle. Our data also support the hypothesis that α2 AMPK can be a target of pharmacological manipulation aiming to improve glucose metabolism in skeletal muscle.

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