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## Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle

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**Winder, W. W., B. F. Holmes, D. S. Rubink, E. B. Jensen, M. Chen, and J. O. Holloszy.** Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* 88: 2219–2226, 2000.—Muscle contraction causes an increase in activity of 5'-AMP-activated protein kinase (AMPK). This study was designed to determine whether chronic chemical activation of AMPK will increase mitochondrial enzymes, GLUT-4, and hexokinase in different types of skeletal muscle of resting rats. In acute studies, rats were subcutaneously injected with either 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR; 1 mg/g body wt) in 0.9% NaCl or with 0.9% NaCl alone and were then anesthetized for collection and freezing of tissues. AMPK activity increased in the superficial, white region of the quadriceps and in soleus muscles but not in the deep, red region of the quadriceps muscle. Acetyl-CoA carboxylase (ACC) activity, a target for AMPK, decreased in all three muscle types in response to AICAR injection but was lowest in the white quadriceps. In rats given daily, 1 mg/g body wt, subcutaneous injections of AICAR for 4 wk, activities of citrate synthase, succinate dehydrogenase, and malate dehydrogenase were increased in white quadriceps and soleus but not in red quadriceps. Cytochrome *c* and  $\delta$ -aminolevulinic acid synthase levels were increased in white, but not red, quadriceps. Carnitine palmitoyl-transferase and hydroxy-acyl-CoA dehydrogenase were not significantly increased. Hexokinase was markedly increased in all three muscles, and GLUT-4 was increased in red and white quadriceps. These results suggest that chronic AMPK activation may mediate the effects of muscle contraction on some, but not all, biochemical adaptations of muscle to endurance exercise training.

$\delta$ -aminolevulinic acid synthase; carnitine palmitoyl transferase; citrate synthase; citric acid cycle enzymes; endurance training; GLUT-4; muscle mitochondria

BIOCHEMICAL ADAPTATIONS OF skeletal muscle to endurance exercise have been extensively studied, beginning with a report in 1967 that showed an increase in mitochondrial oxidative enzyme activities in response to 3 mo of endurance training in rats (3, 4, 14–16). However, the mechanisms that couple chronic muscle contractions with an increase in mitochondrial enzymes have been elusive (3). Previous studies (3) have provided some evidence that the energy charge of the

cell is somehow involved. When creatine and creatine phosphate (CP) are depleted in the muscle by giving rats  $\beta$ -guanidinopropionic acid in their food for 6 wk, CP is replaced by  $\beta$ -guanidinopropionic acid phosphate, ATP concentration is decreased, and levels of mitochondrial enzymes, GLUT-4, and hexokinase are increased (31, 34). Chronic exposure of muscle cells to hypoxia results in an increase in mitochondrial enzyme content (cf. Ref. 3), and thyroid hormone treatment of rats also produces an increase in muscle mitochondria (39).

5'-AMP activated protein kinase (AMPK) has recently been implicated as being important as a metabolic master switch in the muscle, controlling both fat metabolism and glucose uptake (38). This enzyme is controlled by both allosteric and covalent mechanisms. It is activated allosterically by an increase in 5'-AMP and inhibited by ATP and CP (8, 9, 38). Phosphorylation of AMPK by an upstream kinase (AMPKK), also activated by 5'-AMP, results in activation of AMPK. A large amplification of activity can result from maximal stimulation by both mechanisms (8, 9, 38). AMPK activity increases in the muscles of rats running on a treadmill and in response to electrical stimulation (32, 37, 38).

5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) is an analog of adenosine that is taken up by muscle and phosphorylated to form 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranosyl-5'-monophosphate (ZMP) that also activates AMPK (9, 25, 38). Therefore, AICAR treatment can be used to mimic the effects of exercise on AMPK activity. This approach has been used previously to determine the effects of AMPK activation in perfused muscle, incubated muscle, and intact, sedentary rats (2, 11, 18, 23, 25). Evidence has been reported for AMPK involvement in the control of fatty acid oxidation and in the insulin-like effect of muscle contraction on glucose transport (11, 23, 25, 38).

More recently, evidence was obtained for the involvement of AMPK in coupling the effect of muscle contraction with some of the adaptations to exercise training. Daily injections of AICAR in resting rats were found to increase GLUT-4 and hexokinase activity in gastrocnemius and epitrochlearis muscles, similar to the activity found after a few days of endurance exercise training (18). Incubation of epitrochlearis muscle with AICAR resulted in significant increases in GLUT-4 and hexokinase during an 18-h period (28). The present study was designed to determine whether chronic activation of AMPK with AICAR would produce mitochondrial adap-

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tations similar to those induced by exercise training. Because of the longer half-lives of the mitochondrial enzymes, a prolonged treatment was deemed necessary. We were also interested in determining whether the adaptations in GLUT-4 and hexokinase seen after 5 days of treatment (18) would persist or be enhanced by a prolonged AICAR treatment. Fiber type-specific responses were also studied.

## MATERIALS 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 (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 Harlan Teklad rodent diet (Madison, WI) and water ad libitum. All rats were handled daily for at least 5 days before the beginning of treatment to accustom them to the experimental procedures.

**Acute studies.** The purpose of these studies was to determine whether AMPK activity was acutely increased in muscle by AICAR treatment. Three days before the experiment, jugular catheters were installed and exteriorized on the back of the neck to allow rapid anesthesia of the rat and blood and tissue collection. Rats were then subcutaneously injected with AICAR (1 mg/g body wt) in sterile 0.9% NaCl or were given 0.9% NaCl ( $n = 6$  animals per group). One hour after the subcutaneous injection, rats were anesthetized by intravenous injection of pentobarbital sodium (4.8 mg/100 g body wt). The superficial white and the deep red regions of the quadriceps muscles and the soleus muscles were quickly removed with stainless steel clamps and frozen at liquid nitrogen temperature.

Resuspended ammonium sulfate precipitates of tissue homogenates were analyzed for AMPK activity and acetyl-CoA carboxylase (ACC) activity, as described previously (25, 37). This measurement of AMPK only detects increases in AMPK activity that survive ammonium sulfate precipitation of the muscle homogenate (i.e., increases due to phosphorylation) and does not provide information concerning allosteric control by AMP, CP, and ATP. ACC activity at 0.2 mM citrate provided some indication of the *in vivo* activity of AMPK because ACC is a target for phosphorylation of AMPK. ACC activity was previously reported to decrease in response to phosphorylation by AMPK (37). The acute experiment was also repeated on rats treated with AICAR or saline for 4 wk to determine whether the responses of AMPK and ACC to AICAR persisted for the entire treatment period. In both experiments, ATP and CP were measured on neutralized perchloric acid extracts of muscle.

**Chronic studies.** To determine the effect of chronic injections of AICAR on muscle enzyme activities or expression and on muscle GLUT-4, rats were given daily subcutaneous injections, between 8 and 10 AM, of AICAR (1 mg/g body wt) or saline vehicle for  $28 \pm 1$  successive days. Beginning with the first injection, saline-injected controls were pair-fed with AICAR-injected rats. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium 22–25 h after the last AICAR injection, and the white and red regions of the quadriceps and the soleus muscles were removed and frozen, as described previously. Muscles were kept frozen at  $-70^\circ\text{C}$  until analyzed. Liver, heart, kidney, and fat pads were also weighed.

After the results of blunted acute response to AICAR injection on AMPK in chronically treated rats were obtained, an additional experiment was performed to determine whether

intermittent treatment of rats with AICAR would influence the extent of the increase in citrate synthase. This experiment was of shorter duration to minimize the downregulation that appeared to occur with prolonged treatment. Rats were injected (1 mg/g body wt) for 3 days, followed by 2 days of no treatment. They were then injected for 5 days, followed by another 2 days with no treatment, then injected for 3 additional days before being killed 20–24 h after the last injection. Rats were anesthetized, and tissues were collected and frozen as before.

**Analytical methods.** Muscles from rats killed 1 h after injection of AICAR or saline were analyzed for AMPK (25, 37), ACC at 0.2 mM citrate (25, 37), ATP and CP (13), and ZMP (25).

To determine the chronic effects of AICAR injection, muscles were analyzed for glycogen (10), GLUT-4, hexokinase, lactate dehydrogenase, and several mitochondrial enzymes. GLUT-4 was quantitated by Western blotting, as described previously (18), using GLUT-4 polyclonal antibody RaIRGT (Biogenesis, Sandown, NH) and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL).  $\delta$ -Aminolevulinic acid synthase was determined using Western blotting techniques as described by Li et al. (24). For determination of cytochrome *c*, muscles were homogenized in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 1 mM EDTA, and 250 mM sucrose (hydroxyethyl starch buffer). Homogenates were centrifuged at 700 *g* for 10 min, and aliquots of the supernatant containing 100  $\mu\text{g}$  of protein were solubilized in Laemmli sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (15% resolving gel), and then transferred to nitrocellulose. Cytochrome *c* was detected by incubating the nitrocellulose blots with a rabbit polyclonal antibody against rabbit heart cytochrome *c* (Alpha Diagnostics International, San Antonio, TX) followed by horseradish peroxidase-conjugated anti-rabbit IgG. Antibody-bound GLUT-4,  $\delta$ -aminolevulinic acid synthase, and cytochrome *c* were visualized using enhanced chemiluminescence. Protein bands were quantified by densitometry.

For enzyme activity measurements, 10% homogenates were made from the respective muscles in 175 mM KCl, 10 mM GSH, and 2 mM EDTA, pH 7.4. This homogenate was frozen and thawed three times and mixed thoroughly before enzymatic measurements. For succinate dehydrogenase and lactate dehydrogenase assays, an aliquot of the homogenate was centrifuged at 700 *g* for 10 min at  $4^\circ\text{C}$ . The remainder of the assays were performed on aliquots and dilutions of the mixed whole homogenate. Assays were performed by the following methods: citrate synthase (35), succinate dehydrogenase (21), the mitochondrial fraction of malate dehydrogenase (33), hexokinase (36), lactate dehydrogenase (29), carnitine palmitoyl transferase (1, 26), and hydroxyacyl-CoA dehydrogenase (22).

Differences between the saline-injected control rats and AICAR-injected rats were determined using Student's *t*-test. Values are expressed as means  $\pm$  SE.

## RESULTS

**Acute studies.** A single injection of AICAR in rats not previously treated with AICAR resulted in significant increases in AMPK activity in white quadriceps and soleus muscle but not in red quadriceps muscles (Table 1) 60 min after the injection. In these same muscle extracts, the activity of ACC at 0.2 mM citrate was markedly decreased in all three muscle types. In the AICAR-injected rats, white quadriceps exhibited the

Table 1. ATP, CP, AMPK and ACC in muscles of rats 60 min after injection of saline (control) or AICAR

	White Quadriceps		Red Quadriceps		Soleus	
	Control	AICAR	Control	AICAR	Control	AICAR
<i>Single injection</i>						
CP, $\mu\text{mol/g}$	24.6 $\pm$ 0.9	17.6 $\pm$ 0.8*	25.8 $\pm$ 2.8	22.4 $\pm$ 3.5	14.2 $\pm$ 0.8	12.2 $\pm$ 1.2
ATP, $\mu\text{mol/g}$	8.2 $\pm$ 0.5	8.0 $\pm$ 0.3	8.0 $\pm$ 0.4	9.2 $\pm$ 0.3*	6.0 $\pm$ 0.9	6.5 $\pm$ 0.8
AMPK, $\text{pmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	386 $\pm$ 32	1,102 $\pm$ 80*	283 $\pm$ 30	405 $\pm$ 62	180 $\pm$ 17	447 $\pm$ 42*
ACC, $\text{nmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ at 0.2 mM citrate	0.75 $\pm$ 0.07	0.08 $\pm$ 0.02*	0.90 $\pm$ 0.06	0.19 $\pm$ 0.02*	0.80 $\pm$ 0.06	0.19 $\pm$ 0.02*
<i>Daily injections for 4 wk</i>						
CP, $\mu\text{mol/g}$	23.8 $\pm$ 1.5	21.9 $\pm$ 0.8	21.9 $\pm$ 1.3	19.6 $\pm$ 0.4	17.7 $\pm$ 0.9	13.8 $\pm$ 0.9*
ATP, $\mu\text{mol/g}$	8.3 $\pm$ 0.2	9.6 $\pm$ 0.2*	7.6 $\pm$ 0.2	8.6 $\pm$ 0.2*	4.0 $\pm$ 0.2	5.1 $\pm$ 0.5
AMPK, $\text{pmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	300 $\pm$ 49	258 $\pm$ 32	226 $\pm$ 33	200 $\pm$ 17	138 $\pm$ 15	191 $\pm$ 22
ACC, $\text{nmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ at 0.2 mM citrate	0.67 $\pm$ 0.09	0.12 $\pm$ 0.01*	0.72 $\pm$ 0.07	0.21 $\pm$ 0.01*	0.55 $\pm$ 0.06	0.28 $\pm$ 0.01*

Values are means  $\pm$  SE;  $n = 4-8$  muscles. AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; CP, creatine phosphate; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase. \*Significantly different from control value ( $P < 0.05$ ).

lowest activity of ACC (44% and 61% of that seen in soleus and red quadriceps, respectively;  $P < 0.05$ ).

The acute response to AICAR injection was also measured in muscles of rats that had been given daily injections of AICAR for 4 wk (Table 1). No significant increase in AMPK activity ( $n = 6$ ) was detected in white or red quadriceps or in soleus muscles 1 h after injection of AICAR. The ACC activity declined to values similar to those seen in rats before chronic AICAR treatment ( $n = 4$ ). The ZMP concentration 1 h after injection was  $0.42 \pm 0.04$ ,  $0.94 \pm 0.08$ , and  $1.15 \pm 0.07$   $\mu\text{mol/g}$  in white quadriceps, red quadriceps, and soleus muscles, respectively. These values were in the same range as those seen in the gastrocnemius muscle 60 min after a single injection of AICAR (see Ref. 18). ZMP was not detectable in rats injected with saline. CP was significantly lower in the AICAR-injected rats compared with the saline-injected controls but only in the white quadriceps before and in soleus muscle after 4 wk of AICAR injections (Table 1). ATP was increased, to a small extent, by AICAR injection in red quadriceps before chronic treatment and in white quadriceps after chronic treatment (Table 1).

**Chronic studies.** Food intake and body weight of rats injected with either saline or AICAR in saline for 4 wk is shown in Fig. 1. No statistically significant differences were noted between AICAR- and saline-injected rats. Final body and organ weights are shown in Table 2. No statistically significant differences were noted in muscle, heart, or kidney weights, but the liver showed significant hypertrophy in the AICAR-treated animals. There was also a significant decrease in fat pad weight in the AICAR-treated rats.

Muscle content of  $\delta$ -aminolevulinic acid synthase, as determined by Western blot, and cytochrome *c* were significantly increased in white, but not red, quadriceps (Fig. 2).

Citrate synthase was significantly increased in white quadriceps and soleus but not in red quadriceps (Fig. 3). Two other citric acid cycle enzymes, succinate dehydrogenase and malate dehydrogenase, showed the same adaptive pattern (Fig. 4).

Lactate dehydrogenase, hydroxyacyl-CoA dehydrogenase, and carnitine palmitoyl transferase were not

significantly influenced in any of the muscle types (Table 3). An increase in glycogen was observed to occur in white and red quadriceps, but not in soleus, the day after the last injection of AICAR (Table 3).

GLUT-4 and hexokinase activity were both significantly increased in response to chronic AICAR injection in red and white regions of the quadriceps (Fig. 5). The increase in hexokinase activity in soleus was statistically significant, but the small change in GLUT-4 was not ( $P = 0.12$ ).

In the study with intermittent injections of AICAR, citrate synthase in the white quadriceps of controls was  $15.5 \pm 1.3$  vs.  $23.0 \pm 1.2$   $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$  in AICAR-

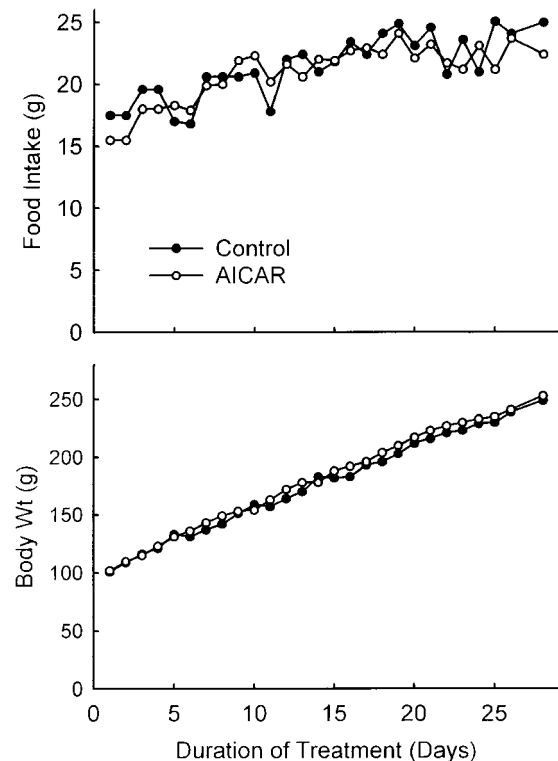


Fig. 1. Food intake (top) and body weight (bottom) of rats injected with 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR; 1 mg/g body wt) for 4 wk. The final data points represent final values the day before killing.

Table 2. Effects on body weight and organ weights of rats after 4 wk of AICAR injections

Weight, g	Saline-Injected Rats	AICAR-Injected Rats
Body weight	249 ± 5	254 ± 5
Heart	0.77 ± 0.07	0.80 ± 0.02
Gastrocnemius/plantaris muscle	1.35 ± 0.03	1.30 ± 0.04
Kidney	1.02 ± 0.07	0.99 ± 0.02
Epididymal fat pad	2.07 ± 0.13	1.21 ± 0.10*
Retroperitoneal fat pad	1.72 ± 0.18	0.90 ± 0.08*
Liver	11.0 ± 0.40	16.0 ± 0.50*

Values are means ± SE;  $n = 8$ . \* $P < 0.001$ .

injected rats. This represented a 48% increase and was highly significant ( $P < 0.001$ ). A significant increase also occurred in the red region of the quadriceps in response to AICAR [ $47.0 \pm 2.8$  (controls) vs.  $57.4 \pm 2.5 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$  (AICAR-injected); ( $P < 0.025$ )].

## DISCUSSION

One of the key adaptations to endurance exercise training is the increase in mitochondrial oxidative enzymes of the muscles involved in exercise (3, 4, 14–16). Previous studies have indicated that skeletal muscle enzymes of the citric acid cycle, of the electron transport chain, and also of fatty acid oxidation all increase in response to an endurance training program or to chronic electrical stimulation (3, 4, 14–16). The physiological consequence of this adaptation is an

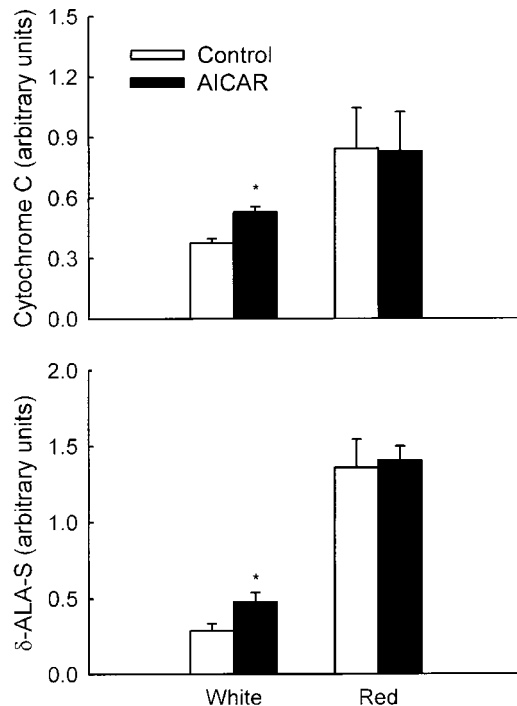


Fig. 2. Cytochrome *c* (top) and  $\delta$ -aminolevulinic synthase ( $\delta$ -ALA-S; bottom) protein expression in red and white portions of quadriceps muscles of rats injected with AICAR or with saline (controls) for 4 wk. Muscle extracts were subjected to Western blotting using rabbit anti- $\delta$ -aminolevulinic synthase or rabbit antiserum against rabbit heart cytochrome *c*. Values are means ± SE;  $n = 6$  muscles per group. \*Significantly different from saline-injected controls ( $P < 0.05$ ).

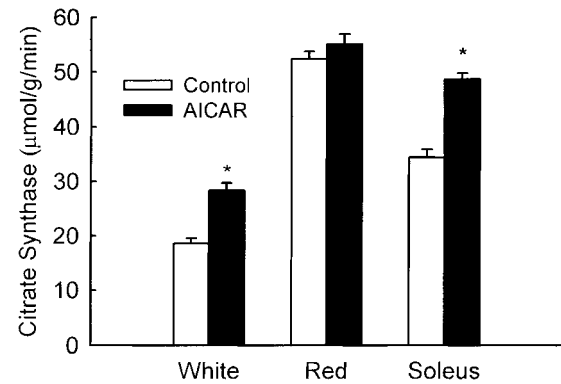


Fig. 3. Citrate synthase activity in red and white quadriceps and soleus muscles of rats injected with AICAR or saline for 4 wk. Values are means ± SE;  $n = 8$  muscles per group. \*Significantly different from saline-injected controls ( $P < 0.001$ ).

increase in the capacity to oxidize pyruvate and fatty acids and to generate ATP. The insulin-sensitive glucose transporter GLUT-4 and hexokinase tend to adapt in the same direction as the mitochondrial oxidative enzymes (3, 5, 7, 12, 17, 19, 20, 27, 30, 31).

Although these adaptations are well-characterized, little is known about the mechanisms coupling muscle contractile activity to the increase in expression of these proteins in muscle (see Refs. 3, 4). A recent study provides evidence that AMPK may be involved in some of these adaptations: activation of muscle AMPK by injecting rats with AICAR for 5 successive days resulted in significant increases in GLUT-4 and hexokinase activities of epitrochlearis and gastrocnemius

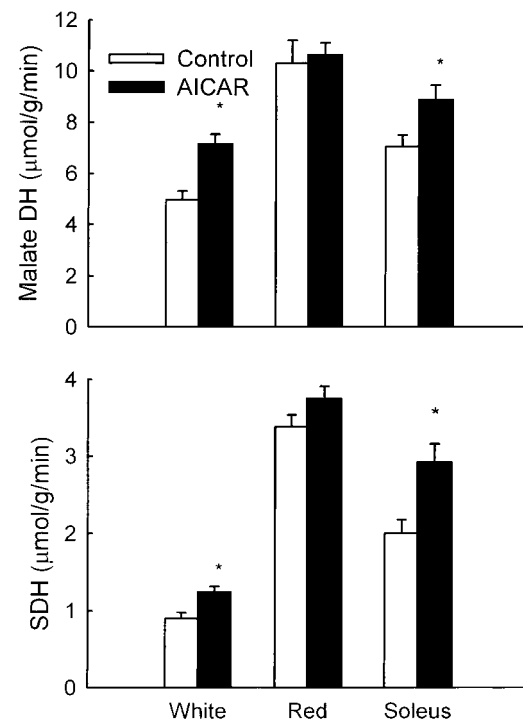


Fig. 4. Malate dehydrogenase (DH; top) and succinate dehydrogenase (SDH; bottom) activities in red and white quadriceps and soleus muscles of rats injected with AICAR or saline for 4 wk. Values are means ± SE;  $n = 8$ –14 muscles per group. \*Significantly different from controls ( $P < 0.05$ ).

Table 3. Effect of chronic AICAR injections on enzyme activities and glycogen in different skeletal muscles

	White Quadriceps		Red Quadriceps		Soleus	
	Control	AICAR	Control	AICAR	Control	AICAR
LDH, $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$	674 $\pm$ 15	685 $\pm$ 20	529 $\pm$ 14	557 $\pm$ 26	158 $\pm$ 11	162 $\pm$ 7
CPT, $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$	0.13 $\pm$ 0.01	0.16 $\pm$ 0.01	0.45 $\pm$ 0.01	0.46 $\pm$ 0.02	0.55 $\pm$ 0.02	0.62 $\pm$ 0.02
HADH, $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$	3.8 $\pm$ 0.4	4.6 $\pm$ 0.3	9.9 $\pm$ 0.3	9.0 $\pm$ 0.4	4.2 $\pm$ 0.4	4.6 $\pm$ 0.4
Glycogen, mg/g	7.2 $\pm$ 0.3	12.3 $\pm$ 0.4*	6.9 $\pm$ 0.3	9.1 $\pm$ 0.4*	6.4 $\pm$ 0.4	5.8 $\pm$ 0.4

Values are means  $\pm$  SE;  $n = 8-14$  muscles. LDH, lactate dehydrogenase; CPT, carnitine palmitoyl-transferase; HADH, hydroxyacyl-CoA dehydrogenase. \*  $P < 0.05$ .

muscles (18). The present results show that this adaptive response is maintained, but not further enhanced, during 28 days of AICAR injection. It has also been shown that incubation of epitrochlearis muscle with AICAR for 18 h results in increases in GLUT-4 and hexokinase, providing additional evidence of AMPK involvement in control of muscle gene expression (28). AMPK activity has been shown to increase in skeletal muscle of rats running on treadmills and in electrically stimulated muscle (32, 37, 38). These observations suggest that AMPK activation may be involved in mediating the effect of exercise on at least some biochemical adaptations of muscle (see Fig. 6).

The current study was undertaken to determine whether mitochondrial adaptations would also be mediated by chronic AMPK activation. We previously observed that ZMP is increased in muscle 15 min after a subcutaneous injection of AICAR and remains elevated for at least 2 h after the injection (18). In the present

study, we found that AMPK activity was increased in white quadriceps and soleus, but not in red quadriceps, 60 min after the AICAR injection. It should be emphasized that AMPK can be activated by phosphorylation by AMPKK and also by allosteric mechanisms (Fig. 6). The AMPK activity that we measured in muscle extracts provides an indirect measure of the phosphorylation state of AMPK. However, both AMPKK and AMPK are also allosterically activated by the free AMP concentration that has been shown to increase in the muscle in response to contraction (cf. Ref. 38). In addition, AMPK is inhibited allosterically by CP, making the decline in CP during muscle contraction an important signal for allowing activation of this enzyme (cf. Refs. 9, 38). These allosteric effects are lost during preparation of the AMPK extracts and, therefore, cannot be detected with the AMPK assay. However, it is possible to obtain an estimation of the in vivo activation of AMPK by

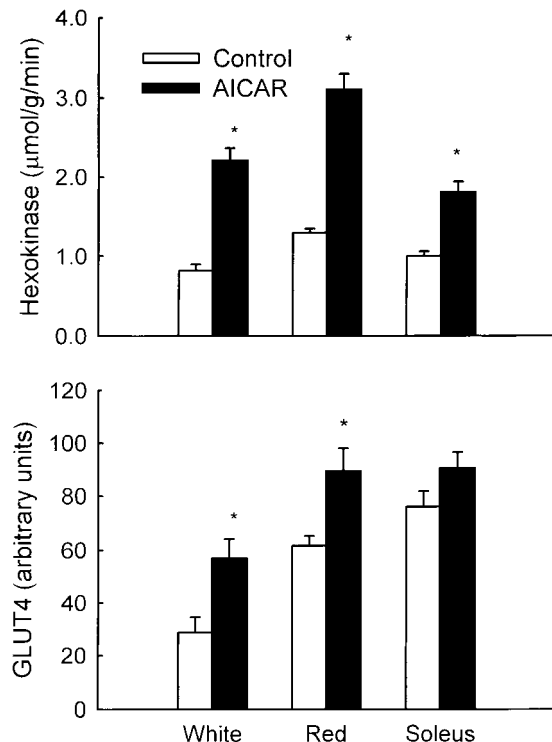


Fig. 5. Hexokinase activity (top) and GLUT-4 (measured by Western blot) activity (bottom) in the red and white regions of the quadriceps and in soleus muscles of rats injected with AICAR (1 mg/g body wt) for 4 wk. Values are mean  $\pm$  SE;  $n = 8$  muscles per group. \*Significantly different from controls ( $P < 0.05$ ).

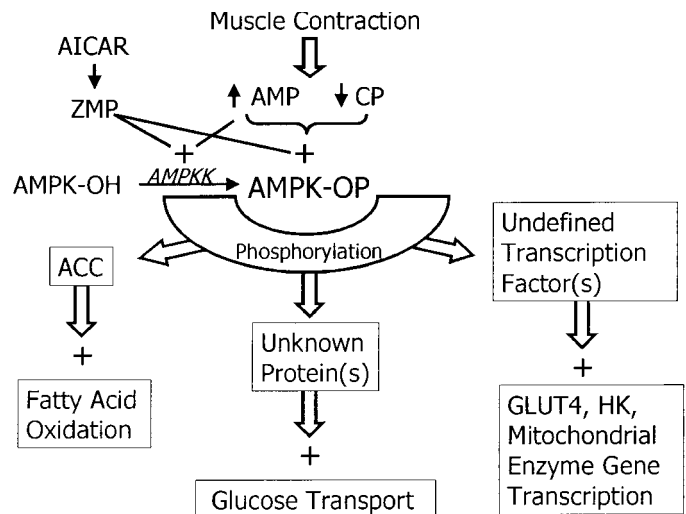


Fig. 6. Putative actions of 5'-AMP-activated protein kinase (AMPK) in skeletal muscle. Effects on fatty acid oxidation, glucose transport, and expression of GLUT-4 and hexokinase (HK) have been described previously (18, 38), and the effects on mitochondrial enzymes are presented in this report. Of these 3 actions, acetyl-CoA carboxylase (ACC) is the only phosphorylation target for AMPK that has been identified in skeletal muscle. AMPK is naturally activated during muscle contraction but may be artificially activated in rats by subcutaneous injection of AICAR or by exposing incubated or perfused muscle to AICAR. AMP may directly activate AMPK or the AMP upstream kinase (AMPKK), which, in turn, phosphorylates/activates AMPK. Creatine phosphate (CP) is an allosteric inhibitor of AMPK. The decline in CP during muscle contraction is thought to relieve inhibition of AMPK, resulting in increased activity. ZMP, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranosyl-5'-monophosphate; OH, hydroxyl group; OP, phosphate ester.

measuring the effects of the phosphorylation of one of its known target proteins.

Purified muscle ACC has been shown to be phosphorylated *in vitro* by AMPK (37). This also results in an increase in the activation constant for citrate and a decrease in the maximal velocity of the purified ACC (37). Similar changes in kinetic properties of ACC are observed in muscles of rats running on treadmills and in electrically stimulated muscle (32, 37, 38). Phosphorylation by AMPK results in marked decreases in activity of ACC at physiological concentrations of citrate of ~0.2 mM; therefore, measurement of ACC activity at 0.2 mM citrate provides information regarding the true activity of AMPK *in vivo*. In the present study, injection of AICAR resulted in decreases in ACC activity in all three muscle types, but the greatest decrease occurred in the white portion of the quadriceps. The decrease in ACC activity provides evidence that AMPK activity was stimulated in all three muscle types in response to AICAR. In the chronically treated animals and in the red quadriceps before chronic treatment, AMPK was apparently activated by predominately allosteric mechanisms (i.e., ZMP effects on AMPK) and not by phosphorylation of AMPK by AMPKK.

Citrate synthase, cytochrome *c*,  $\delta$ -aminolevulinate synthase, and malate dehydrogenase were all significantly increased in the white region of the quadriceps muscle in response to 4 wk of AICAR injections.  $\delta$ -Aminolevulinate synthase activity was previously reported to increase within 16 h of a prolonged bout of exercise (see Ref. 15); however, preliminary experiments on the effect of a single injection of AICAR 16 h before tissue collection have produced negative results (unpublished data). Hexokinase activity increased in response to AICAR in all muscle types. Interestingly, neither of the enzymes involved in fatty acid oxidation that we measured increased in response to AICAR injection. The fact that some enzymes appear to respond and others do not provides evidence that more than one signal is responsible for the concerted rise in mitochondrial oxidative enzyme activity in response to endurance training. It should be noted that AICAR injection does not precisely mimic the effects of contraction on this control system. The decline in CP accompanying contraction may be a critical component of the signal, and muscle CP was not significantly changed in red quadriceps in response to AICAR. A previous study (Winder, unpublished data) demonstrated that the time course of the decline in gastrocnemius ACC activity during electrical stimulation correlates more closely with the decline in CP than with the measured AMPK activity. The increase in cytosolic calcium during excitation-contraction coupling and the rise in plasma fatty acids that accompanies prolonged exercise bouts may also be important in inducing the increase in mitochondrial enzymes. These effects of exercise are not mimicked by AICAR injections.

Evidence has recently been presented for the role of calcium and protein kinase C in inducing increases in cytochrome *c* gene expression (6). An increase in cyto-

chrome *c* gene expression was observed in myotube culture by treatment with a calcium ionophore (6). The ionophore-induced response was increased by enhancing expression of calcium-sensitive  $\alpha$  and  $\beta_2$  protein kinase C isoforms but not of the calcium-insensitive  $\delta$ -isoform. It is unclear at this time how the putative AMPK-induced pathway is related to the calcium-triggered pathway. It is also possible that the AMPK pathway is activated with this calcium ionophore. AMPK activity was not quantitated in that study.

It is unclear, at this time, why the white quadriceps muscle responded with an increase in mitochondrial enzymes and the red quadriceps did not. One possible interpretation is that AMPK activation is not responsible for the mitochondrial adaptations. It is also possible that the phosphorylated species of AMPK is responsible for triggering increased rates of synthesis of mitochondrial enzymes. If the decline in ACC activity is a true measure of *in vivo* AMPK activity, we may conclude that AMPK is activated allosterically in red quadriceps by AICAR injection, but the phosphorylation state of AMPK is unchanged with respect to controls. It is also important to consider the total daily signal rather than the isolated effects of AICAR. The total daily contractile activity would certainly be expected to be greater in red quadriceps and in soleus fibers than in fibers of the white quadriceps.

In addition, the AMPK response to AICAR appeared to be downregulated by the end of the 4-wk treatment period. No significant increase was observed in AMPK activity in any of the muscle types 1 h after injection of AICAR in the chronically treated rats. However, the decrease in ACC activity at 0.2 mM citrate was similar to that seen in rats at the beginning of the 4-wk treatment regimen. The reason for this downregulation response is not clear. We did note significant liver hypertrophy, increasing the probability of more rapid metabolism of the AICAR after the daily injections. However, 1 h after the chronically treated rats were injected with AICAR, the ZMP concentration was elevated in all three muscle fiber types to levels in the same range as observed previously (18). There is also the possibility that expression of the AMPK or AMPKK genes changes in response to chronic activation.

When it was apparent that the response of AMPK to AICAR was diminishing over the course of the 4-wk chronic study and that the magnitude of the adaptation appeared to be less than that seen in response to 2 h/day of endurance training (a twofold increase), it seemed important to determine whether an intermittent pattern of AICAR injection would prevent downregulation, allowing the true response of a mitochondrial marker enzyme to AMPK activation to be observed. Previous studies clearly demonstrated that training adaptations occur in rats run only 5 days/wk, 2 h/day, representing an intermittent stimulus (14). The fact that a significant increase in citrate synthase was observed in red quadriceps in response to 2 wk of intermittent treatment with AICAR suggests the possibility that, in the 4-wk chronic-injection study, the

mitochondrial enzymes may have increased early in the treatment but, because of downregulation, subsided in red quadriceps as the treatment was extended to 4 wk.

The marked decrease in fat pad size is of considerable interest in terms of treatment of Type 2 diabetes and obesity using AMPK activators. The chronic AMPK activation is accompanied by ACC inactivation and a consequent decline in malonyl-CoA (see Ref. 18). The decline in malonyl-CoA would allow an increased influx of fatty acids into the mitochondria and an increase in fatty acid oxidation. AICAR-treated rats were pair-fed so that they ate the same amount of food and gained weight at the same rate as controls, but the size of the fat pads was still markedly decreased. These results provide an additional rationale for the development of more potent AMPK activators to treat Type 2 diabetes and obesity, but the large amount of AICAR required to induce these adaptations makes it an unlikely candidate for use in human patients for these purposes.

In summary, chronic AMPK activation using AICAR injections in resting rats results in significant increases in  $\delta$ -aminolevulinic synthase, cytochrome *c*, citrate synthase, and malate dehydrogenase in white, but not red, quadriceps. Hexokinase activity was significantly increased in both quadriceps muscle types and in soleus muscles. GLUT-4 was increased in both red and white quadriceps, and a trend toward an increase was noted in soleus. The extent of AMPK activation appeared to be greatest in the white quadriceps, as evidenced by AMPK activity measurements in the presence of maximally effective AMP concentrations and by the extent of reduction in ACC activity (an AMPK target). These results suggest that the activation of AMPK that accompanies muscle contraction during daily bouts of training may play a role in mediating some of the biochemical adaptations that are induced in skeletal muscle by endurance exercise. The data also suggest that not all muscular adaptations to training are mediated by activation of AMPK.

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