Regulation of fatty acid oxidation and glucose metabolism in rat soleus muscle: effects of AICAR

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Regulation of fatty acid oxidation and glucose metabolism in rat soleus muscle: effects of AICAR. Am J Physiol Endocrinol Metab 281: E335–E340, 2001.—Previous studies have shown that 5-aminooimidazole-4-carboxamide ribonucleoside (AICAR), a cell-permeable activator of AMP-activated protein kinase, increases the rate of fatty acid oxidation in skeletal muscle of fed rats. The present study investigated the mechanism by which this occurs and, in particular, whether changes in the activity of malonyl-CoA decarboxylase (MCD) and the β-isofrom of acetyl-CoA carboxylase (ACCβ) are involved. In addition, the relationship between changes in fatty acid oxidation induced by AICAR and its effects on glucose uptake and metabolism was examined.

In incubated soleus muscles isolated from fed rats, AICAR (2 mM) increased fatty acid oxidation (90%) and decreased ACCβ activity (40%) and malonyl-CoA concentration (50%); however, MCD activity was not significantly altered. In soleus muscles from overnight-fasted rats, AICAR decreased ACCβ activity (40%), as did in fed rats; however, it had no effect on the already high rate of fatty acid oxidation or the low malonyl-CoA concentration. In keeping with its effect on fatty acid oxidation, AICAR decreased glucose oxidation by 44% in fed rats but did not decrease glucose oxidation in fasted rats. It had no effect on glucose oxidation when fatty acid oxidation was inhibited by 2-bromopalmitate. Surprisingly, AICAR did not significantly increase glucose uptake or assayable AMP-activated protein kinase activity in incubated soleus muscles from fed or fasted rats. These results indicate that, in incubated rat soleus muscle, 1) AICAR does not activate MCD or stimulate glucose uptake as it does in extensor digitorum longus and epitrochlearis muscles, 2) the ability of AICAR to increase fatty acid oxidation and diminish glucose oxidation and malonyl-CoA concentration is dependent on the nutritional status of the rat, and 3) the ability of AICAR to diminish assayable ACC activity is independent of nutritional state.

malonyl-coenzyme A; malonyl-coenzyme A decarboxylase; acetyl-coenzyme A carboxylase; AMP-activated protein kinase; nutritional state

MALONYL-CoA is an intermediate in the de novo synthesis of fatty acids and an allosteric inhibitor of carnitine palmitoyltransferase I (CPT-I), the enzyme that regulates the rate at which long-chain fatty acyl CoAs (LCFA-CoAs) enter the mitochondria, where they are oxidized (30, 41). In tissues such as skeletal and cardiac muscle, in which fatty acid synthesis is minimal (3), the primary role of malonyl-CoA is presumably the regulation of CPT-I. Malonyl-CoA levels in these muscles and possibly in other tissues may be regulated by acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in its synthesis (35, 44), and malonyl-CoA decarboxylase (MCD), which may control its degradation (2, 14, 17, 40).

ACCβ, the major isofrom expressed in skeletal muscle (1, 6, 18, 28), is thought to be regulated via two mechanisms: 1) allosteric activation by cytosolic citrate and inhibition by LCFA-CoAs, which appear to regulate alterations in its activity in response to changes in nutritional state and plasma insulin and glucose (9, 32, 35, 39, 45), and 2) covalent modification due to phosphorylation by AMP-activated protein kinase (AMPK). AMPK-mediated changes in assayable ACCβ activity have been demonstrated in response to “stressful” stimuli such as ischemia/hypoxia, inhibition of oxidative phosphorylation and glucose metabolism, and exercise (13, 20, 24–26, 29). Less is known about MCD regulation in skeletal muscle, although recent studies have shown that activation of AMPK during muscle contraction phosphorylates and activates MCD, suggesting that it also participates in the control of malonyl-CoA concentration (40).

In addition to exercise, AMPK in muscle and other tissues can be activated by perfusion or incubation with 5-aminooimidazole-4-carboxamide ribonucleoside (AICAR). When taken up into cells, AICAR is phosphorylated to form ZMP, an AMP analog that activates AMPK. In perfused rat hindquarter (31) and isolated epitrochlearis muscle preparations from fed animals (21), AICAR mimics many acute effects of exercise and electrically induced contractions, including increased fatty acid oxidation and decreased ACCβ activity and malonyl-CoA concentration (2, 31). In addition, in an incubated extensor digitorum longus (EDL) muscle preparation, it increases MCD activity (40). Yet an-
other effect of AICAR is to increase glucose uptake by enhancing glucose transporter translocation, which it does in cardiac myocytes (36) and perfused gastrocnemius muscle (27). Finally, chronic AICAR administration in vivo has been shown to mimic a number of effects of physical training, including increases in GLUT-4 protein, hexokinase activity, and muscle glycogen (23).

Although the effects of AICAR on fatty acid oxidation and its link to changes in malonyl-CoA concentration are well documented, the relative roles of ACCβ and MCD are unclear. In addition, it is not known how such factors as nutritional status and the type of muscle studied affect its action. To examine these questions, the effects of AICAR were compared in soleus muscle strips isolated from fed and overnight-fasted rats. The present report describes its effects on glucose and fatty acid oxidation, glucose uptake, and the activities of ACCβ, MCD, and AMPK.

METHODS

Animals. Male Sprague-Dawley rats (140–151 g; Charles River Breeding Laboratories, Wilmington, MA) were kept in the departmental animal house with a 12:12-h light-dark cycle (lights on from 0600 to 1800) at room temperature. They were fed standard Purina rat chow ad libitum, or food was removed at 1600 during the day before they were killed.

In vitro muscle incubation. On the experimental day, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and soleus muscle strips were prepared and tied to stainless steel clips as described previously (38). Muscles were preincubated for 20 min at 37°C in open 12-mm test tubes containing 3.0 ml of Krebs-Henseleit buffer (10 mM glucose, 10 mM U/ml insulin (67 μM), 2% fatty acid-free bovine serum albumin, and 2 mM AICAR; Sigma). The media were gassed continuously with 95% O2-5% CO2. The test tubes were then transferred to a new set of identical test tubes and incubated with fresh medium containing 0.2 mM palmitate complexed to 2% bovine serum albumin and AICAR or 2 mM 2-bromopalmitate (2-BPA). The muscles were incubated for 60 min. Gassing was terminated after the initial 10 min, and the test tubes were covered. At the end of the incubation, muscles were removed, blotted on gauze pads, and frozen in liquid nitrogen.

Glucose and fatty acid oxidation. For oxidation studies, incubation media contained 0.2 μCi/ml [U-14C]glucose or 0.2 μCi/ml [1-14C]palmitate (New England Nuclear). At the end of 60 min, the medium was transferred to a 25-m1 Erlenmeyer flask fitted with a center well containing filter paper at 37°C for 60 min, and the center wells were removed and transferred to vials for liquid scintillation counting.

\[^{14}C\text{lactate release, net lactate release, and glycogen synthesis. The release of }^{14}C\text{lactate from muscle incubated with media containing [U-14C]glucose was determined as previously described using Dowex ion-exchange chromatography (19). Net lactate release was assessed spectrophotometrically (15). Glycogen synthesis was determined by measuring the rate of [U-14C]glucose incorporated into glycogen (11).}

AMPK, ACCβ, MCD, and malonyl-CoA. AMPK and ACCβ tissue extracts were prepared as previously described (43), with two simple modifications that did not alter activity: 1) buffers A and B did not contain disopropyl fluorophosphate, and 2) the high-speed spin was eliminated, and homogenates were spun only at 13,500 g for 12 min. AMPK homogenates were immunoprecipitated with nonimmune sera or with specific antisera directed against the α2-catalytic subunit of the AMPK heterotrimer (42). Immunoprecipitates were collected on protein A/G beads and washed extensively, and the immobilized enzyme was assayed as previously described (46), with the following modifications: 50 μl of reaction mixture were added to the immunoprecipitates, and 25 μl of the resultant mixture were spotted on p81 filter paper. Also, 5% trichloroacetic acid-1% sodium pyrophosphate was used to wash the filter papers. ACCβ was assayed as previously described (43). The ACCβ antibody was kindly provided by Lee A. Witters, Dartmouth Medical School, Hanover, NH. The initial muscle homogenate was diluted 1:1 with buffer B and incubated for 2.5 h with 5.0 μl of 7AD3 monoclonal antibody and 25 μl of agarose-protein A/G plus beads. The beads were washed twice with buffer B and once with Tris-acetate buffer (pH 7.4). Beads were then assayed for ACC activity at 0.2 mM citrate by the ⁴CO₂ fixation assay (47).

MCD extracts were generated using the following method. Briefly, frozen muscles were pooled, weighed (40–70 mg total), powdered in liquid nitrogen, and then homogenized in a glass homogenizer in 30 volumes of a buffer composed of 0.1 M Tris-HCl (pH 8.0), 2 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 5 μM leupeptin, and 5 μM pepstatin A with the addition of 40 mM β-glycerophosphate, 40 mM NaF, 4 mM sodium pyrophosphate, and 1 mM Na₃VO₄ to inhibit phosphatase activity. The homogenized muscles were then centrifuged at 500 g for 10 min. Partial purification of MCD from ammonium sulfate precipitation was carried out by the method of Saha et al. (40). The amount of protein from control and AICAR-incubated muscles was essentially the same after ammonium sulfate fractionation. MCD activity was measured spectrophotometrically using a Hewlett-Packard model 8450A diode array spectrophotometer, as described previously (40). Malonyl-CoA was determined radioenzymatically in neutralized 10% perchloric acid extracts of whole muscle, as described elsewhere (38). This assay measures malonyl-CoA-dependent incorporation of [³H]acetyl-CoA into fatty acids.

Protein content. Protein amounts were determined spectrophotometrically using the Bio-Rad DC protein assay system and a Hewlett-Packard model 8450A diode array spectrophotometer.

Statistics. Values are means ± SE for the indicated number of muscles. Statistical significance was assessed by group comparison with the use of Student’s t-test or by one-way analysis of variance followed by Student-Newman-Keuls post hoc analysis.

RESULTS

Effects of AICAR on fatty acid oxidation by soleus muscles of fed rats. In soleus muscles isolated from fed rats, incubation with AICAR resulted in a 90% increase in fatty acid oxidation (Fig. 1A), which was accompanied by 50% decreases in malonyl-CoA concentration (Fig. 1B) and ACCβ activity (Fig. 1C). AICAR tended to increase MCD activity (Fig. 1D); however, the effect was not statistically significant.

Effects of AICAR on fatty acid oxidation by soleus muscles in overnight-fasted rats. In muscle from overnight-fasted rats, the rate of fatty acid oxidation was already high, and incubation with AICAR did not increase it further (Fig. 2A), nor did it decrease the
concentration of malonyl-CoA (Fig. 2B). As in muscles from fed rats, AICAR decreased the activity of ACCβ (40%) and had no effect on MCD activity (Fig. 2, C and D).

Effects of AICAR on glucose uptake and metabolism. Incubation with AICAR decreased glucose oxidation (44%) in soleus muscles isolated from fed rats but had no effect on muscles from fasted rats (Table 1). In neither group did it affect glucose uptake, as estimated by [U-14C]glucose (7), nor did it affect glycogenolysis, as assessed from net lactate release minus [14C]lactate release (48), or glycogen synthesis (Table 1).

Effect of 2-BPA on AICAR-induced changes in fatty acid and glucose oxidation. A recent report suggests that AICAR may increase glucose oxidation in human umbilical vein endothelial cells independent of its effects on fatty acid oxidation (12). In contrast, its different effects on incubated soleus muscle preparations from fed and fasted rats suggest that AICAR inhibits

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Fig. 1. Fatty acid oxidation (A), malonyl-CoA (B), and activities of β-isoform of acetyl-CoA carboxylase (ACCβ; C) and malonyl-CoA decarboxylase (MCD; D) in isolated soleus muscles of fed rats incubated with (solid bars) and without (open bars) 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Values are means ± SE; n = 5–10. Significant difference from muscles incubated with AICAR: *P < 0.05; **P < 0.001.

Fig. 2. Fatty acid oxidation (A), malonyl-CoA (B), and activities of ACCβ (C) and MCD (D) in isolated soleus muscles of fasted rats incubated with (solid bars) and without (open bars) AICAR. Values are means ± SE; n = 5–10. Significant difference from muscles incubated without AICAR: *P < 0.05.
Table 1. Effects of AICAR on glucose oxidation, glycogen synthesis, and lactate release in isolated soleus muscles of fed and fasted rats

<table>
<thead>
<tr>
<th>Glucose Disposition, μmol glucose·h⁻¹·g wet wt⁻¹</th>
<th>Fed</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidation</td>
<td>Glycogen synthesis</td>
<td>[¹⁴C]lactate release</td>
</tr>
<tr>
<td>−AICAR</td>
<td>0.9 ± 0.06</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>+AICAR</td>
<td>0.5 ± 0.04*</td>
<td>3.0 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. Soleus muscles were isolated and incubated with (+) or without (−) 2 mM 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), as described in METHODS. *Significantly different (P < 0.05) from muscles incubated without AICAR.

glucose oxidation only when it concurrently increases fatty acid oxidation. To examine this question further, muscles were incubated with 2-BPA, an inhibitor of CPT-I and other enzymes involved in fatty acid metabolism (10). As shown in Table 2, 2-BPA completely suppressed the AICAR-induced increase in fatty acid oxidation, and no decrease in glucose oxidation was observed.

Effects of AICAR on AMPK activity in soleus muscle from fed and fasted rats. Data showing the effect of AICAR on assayable AMPK activity are presented in Table 3. Surprisingly, incubation with AICAR had no effect on assayable activity of the α₂-isofrom of AMPK in soleus muscles from fed or fasted rats.

DISCUSSION

Effects of AICAR on fatty acid oxidation and ACC and MCD activities. Previous studies have shown that AICAR increases fatty acid oxidation in skeletal muscle (2, 31, 33). This effect has been attributed to the activation of AMPK, which phosphorylates and inhibits ACCβ, leading to a decrease in the concentration of malonyl-CoA (31). Recent work has shown that, in an

Table 2. Effects of AICAR on changes in fatty acid and glucose oxidation induced by 2-BPA in isolated soleus muscles of fed rats

<table>
<thead>
<tr>
<th>Addition to Incubation Media</th>
<th>Rate of Oxidation</th>
</tr>
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<tbody>
<tr>
<td>AICAR (2 mM)</td>
<td>2-BPA (2 mM)</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

Values are means ± SE; n = 5. Soleus muscles were isolated and incubated with (+) or without (−) AICAR and/or 2-bromopalmitate (2-BPA), as described in METHODS. Significant difference from muscles incubated without AICAR and 2-BPA: *P < 0.05; †P < 0.01; ‡P < 0.001.

In contrast to previous findings in which MCD was studied in the incubated EDL, the results presented here suggest that AICAR increases fatty acid oxidation in the soleus primarily by its effect on ACCβ. Thus AICAR concurrently decreased assayable ACCβ activity and the concentration of malonyl-CoA in incubated soleus muscles but had no effect on MCD activity. Why incubation with AICAR results in inhibition of ACCβ in the soleus and EDL but only increases assayable MCD activity in the latter remains to be determined.

MCD activity was measured in whole tissue homogenates, and the enzyme may exist in mitochondrial, peroxisomal, and cytosolic compartments (37). Whether changes in the activity of MCD in one of these compartments has the greatest impact on whole tissue malonyl-CoA levels and whether activity in these compartments differs in predominantly red and white muscles, to our knowledge, has not been studied.

In soleus muscles from fed rats, AICAR increased fatty acid oxidation and decreased malonyl-CoA concentration, whereas in muscles from overnight-fasted rats it did neither. Presumably, this occurred because malonyl-CoA levels were already very low and the rate of fatty acid oxidation was near maximal for a resting muscle in the fasted group. Interestingly, this lack of an effect on malonyl-CoA concentration and fatty acid oxidation occurred despite the fact that AICAR caused the same decrease in assayable ACCβ activity that it did in fed rats. The most plausible explanation for these findings is that ACCβ activity in vivo was already depressed in the soleus muscles of fasted rats because of allosteric inhibition via LCFA-CoA, which would not have been evident when the enzyme was assayed in a cell-free system. In support of this possibility, the concentration of LCFA-CoA in skeletal muscle has been shown to increase during starvation (39) and to decrease on refeeding (9).

Glucose disposition and uptake. The results also indicate that ACCβ activity does not enhance glucose oxidation, as it has been reported to do in cultured endothelium (12). Rather, incubation with AICAR leads to inhibition of glucose oxidation. This appeared secondary to its ability to activate fatty acid oxidation, since it was

Table 3. Effects of AICAR on AMPK activity in isolated soleus muscles of fed and fasted rats

<table>
<thead>
<tr>
<th>AMPK Activity, pmol·min⁻¹·mg Supernatant protein⁻¹</th>
<th>Fed</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>−AICAR</td>
<td>2.64 ± 0.72</td>
<td>3.36 ± 0.49</td>
</tr>
<tr>
<td>+AICAR</td>
<td>3.54 ± 0.38</td>
<td>4.21 ± 0.67</td>
</tr>
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</table>

Values are means ± SE; n = 5–10. Soleus muscles were isolated and incubated with (+) or without (−) AICAR, as described in METHODS. AMPK, AMP-activated protein kinase.

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observed in incubated soleus muscles from fed but not fasted rats. Furthermore, the inhibition of glucose oxidation did not occur in soleus muscles from fed rats when 2-BPA was used to suppress the increase in fatty acid oxidation caused by AICAR.

One could assume, on the basis of these data, that glucose is the major fuel in the incubated rat soleus. In fact, the major fuel appears to be endogenous lipid. As shown previously in the incubated soleus (34) and perfused hindquarter (4), glucose oxidation accounts for at most 20–30% of the ATP generated in these muscle preparations. It has also been shown that the major fuel of these muscle preparations is endogenous lipid and that measurement of $^{14}$CO$_2$ generated from medium $^{14}$C-labeled fatty acids underestimates the true rate of fatty acid oxidation by 60–90% (4, 34). This is probably attributable to the need for exogenous fatty acids to equilibrate with an intracellular pool(s) before their use for oxidation.

That glucose oxidation is decreased in the fed state after incubation with AICAR, in one respect, was surprising, since it has been proposed that the AMPK cascade acts as a "fuel gauge" that signals the cell to increase the oxidation of fatty acids and the uptake of glucose (in muscle) when there is a paucity of fuel (20). Instead, AICAR activated one oxidative pathway while inhibiting another. If the AMPK cascade responded solely to a perceived fuel deficit, one would hypothesize that an increase in AMPK activity would signal a more generalized oxidation of fuels. This, together with the observation that AICAR does not increase oxygen consumption as does muscle contraction in a perfused hindquarter preparation (31), raises the possibility that the role of AMPK in the muscle cell may extend beyond the maintenance of its ATP and creatine phosphate content.

Different effects of AICAR in the soleus and other muscles. The lack of an effect of AICAR on glucose uptake and AMPK activation is at variance with previous studies in which it was shown to activate AMPK and stimulate glucose uptake, in the presence and absence of insulin, in rat epitrochlears (22) and EDL (unpublished observations) muscles and the perfused rat hindquarter (31). This could be related to differences in muscle fiber type, since these preparations are much richer in white fibers, in which the AMPK $\alpha_2$-catalytic subunit associates with the $\beta_1$- and $\beta_2$-subunits, than in the soleus, in which the AMPK $\alpha_2$-catalytic subunit only associates with the $\beta_1$-subunit (8). Arguing against this notion, two recent studies found that AMPK activity and glucose transport in the soleus were increased in response to AICAR in vivo (5) and in vitro (16). The former study (5) showed increased glucose uptake in soleus muscles of 100- to 130-g rats infused with AICAR. The latter study (16) used isolated split soleus muscles from younger, starved (24 h) rats and different incubation media and conditions. The reason for the differences between these studies and the present study remains to be determined.

Incubation with AICAR led to a decrease in the assayable activity of ACC$\beta$, suggesting phosphorylation of ACC$\beta$ by an activated AMPK. AMPK activation can result from covalent modification because of its phosphorylation by an AMPK kinase and/or by allosteric modification, which would not be reflected when the enzyme is assayed in a cell-free system (20). AMP, and ZMP derived from AICAR metabolism, can activate AMPK by both mechanisms. Because AICAR did not increase assayable AMPK activity in the present study, one must assume that the inhibition of assayable ACC$\beta$ activity observed here was due to allosteric activation of AMPK. The possibility that a transient increase in AMPK activity due to phosphorylation was missed, however, cannot be ruled out.

In conclusion, the results indicate that the ability of AICAR to increase fatty acid oxidation and to decrease the concentration of malonyl-CoA and glucose oxidation in the incubated soleus muscle is dependent on the nutritional status of the rat. They also indicate that its ability to diminish assayable ACC activity is independent of nutritional state. Finally, they suggest that the ability of AICAR to activate MCD and stimulate glucose uptake may be fiber type specific.

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