AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle

G. F. MERRILL,1 E. J. KURTH,2 D. G. HARDIE,3 AND W. W. WINDER2
1Department of Biological Sciences, Rutgers University, New Brunswick, New Jersey 08903; 2Zoology Department, Brigham Young University, Provo, Utah 84602; and 3Department of Biochemistry, The University, Dundee DD1 4HN, Scotland, United Kingdom

Merrill, G. F., E. J. Kurth, D. G. Hardie, and W. W. Winder. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E1107–E1112, 1997.—5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) has previously been reported to be taken up into cells and phosphorylated to form ZMP, an analog of 5'-AMP. This study was designed to determine whether AICAR can activate AMP-activated protein kinase (AMPK) in skeletal muscle with consequent phosphorylation of acetyl-CoA carboxylase (ACC), decrease in malonyl-CoA, and increase in fatty acid oxidation. Rat hindlimbs were perfused with Krebs-Henseleit bicarbonate containing 4% bovine serum albumin, washed bovine red blood cells, 200 µU/ml insulin, and 10 mM glucose with or without AICAR (0.5–2.0 mM). Perfusion with medium containing AICAR was found to activate AMPK in skeletal muscle, inactivate ACC, and decrease malonyl-CoA. Hindlimbs perfused with 2 mM AICAR for 45 min exhibited a 2.8-fold increase in fatty acid oxidation and a significant increase in glucose uptake. No difference was observed in oxygen uptake in AICAR vs. control hindlimb. These results provide evidence that decreases in muscle content of malonyl-CoA can increase the rate of fatty acid oxidation.

acetyl-CoA carboxylase; malonyl-CoA; palmitate oxidation; 5-aminoimidazole-4-carboxamide riboside; ZMP

FATTY ACID OXIDATION in skeletal muscle has been postulated to be regulated in part by malonyl-CoA, an inhibitor of carnitine palmitoyltransferase (CPT 1) (21, 22, 25, 27, 30–32). Marked decreases in skeletal muscle malonyl-CoA occur in response to fasting and muscle contraction, two conditions expected to result in an increased rate of fatty acid oxidation (14, 16, 27, 30–32). The decrease in malonyl-CoA that occurs in muscle during exercise or in response to electrical stimulation is accompanied by a decrease in activity of acetyl-CoA carboxylase (ACC), the enzyme that synthesizes malonyl-CoA (14, 27, 31). This enzyme is regulated by both allosteric mechanisms and by phosphorylation (21, 25, 31, 33). Citrate is an activator and palmitoyl-CoA is an inhibitor of skeletal muscle ACC (21, 25, 31, 33). Phosphorylation of purified ACC, catalyzed by AMP-activated protein kinase (AMPK), causes a decrease in the maximal velocity of the reaction (Vmax), an increase in the activation constant (Kd) for citrate, and increases in Michaelis constants (Km) for each of the substrates, ATP, bicarbonate, and acetyl-CoA (31, 33). AMPK activity also has been reported to increase in response to muscle contraction, both in exercising rats and in electrically stimulated gastrocnemius muscle (14, 27, 31). The postulated sequence of events in muscle during exercise includes activation of AMPK kinase by 5'-AMP, phosphorylation and activation of AMPK, phosphorylation and inactivation of ACC, and a decrease in malonyl-CoA. The decrease in malonyl-CoA relieves inhibition of CPT 1 and allows fatty acid oxidation to increase as exercise continues.

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) has previously been reported to be taken up into cells and phosphorylated to form ZMP, the monophosphorylated derivative of AICAR. ZMP mimics the multiple effects of AMP on AMPK, not only causing allosteric activation but also promoting phosphorylation and activation of the upstream kinase, AMPK kinase (4, 11, 24). This in turn has been demonstrated to inactivate target enzymes in these lipogenic tissues (4, 11, 24). This study was designed to determine whether AICAR can activate AMPK in skeletal muscle in the isolated perfused hindlimb. If AMPK is activated, is there a subsequent decrease in ACC activity and a decrease in malonyl-CoA? If there is a decrease in malonyl-CoA, will this change the rate of fatty acid oxidation by the hindlimb muscles?

METHODS

Animals. Male Sprague-Dawley rats (Sasco, Madison, WI) weighing 388 ± 5 g were housed in individual cages in a temperature (20–22°C) and light-controlled (12:12-h light-dark cycle) room. Rats were fed ad libitum with Harlan Teklad rodent diet (Madison, WI).

Hindlimb perfusion. The protocol for hindlimb perfusion was approved by the Institutional Animal Care and Use Committee. Rats were anesthetized with pentobarbital sodium (60 mg/kg), intraperitoneal catheters were inserted into the abdominal aorta and inferior vena cava, and tips were advanced to the level of the left iliac artery and vein, as described previously by Gorski et al. (7). Vessels to the tail and the right hindlimb were ligated. Skin was removed from the left hindlimb. The exposed muscles were kept moist with gauze wetted with warm Krebs-Henseleit bicarbonate (KHB). After vessels were flushed with KHB plus heparin (80 USP units ia), the left hindlimb was perfused with medium (37°C) composed of washed bovine erythrocytes and KHB containing 4 g/100 ml bovine serum albumin (Sigma, St. Louis, MO), 200 µU/ml insulin (Humulin, Eli Lilly), and 10 mM glucose with or without AICAR. The bovine serum albumin had been treated with charcoal and dialyzed extensively to remove low molecular weight substances. The KHB-albumin was filtered sequentially through 5-, 1-, and 0.45-µm filters on the day of perfusion just before combination with the erythrocytes. The hematocrit after addition of all components to the medium was ~41.

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The perfusions were carried out in a Plexiglas perfusion chamber (37°C) similar to that described by Gorski et al. (7). The medium was oxygenated as it traversed a Silastic tubing coil (6 m, 0.16 cm ID, 0.24 cm OD) in a 1-liter chamber equilibrated with 95% O2-5% CO2. Rats were killed with an overdose of pentobarbital sodium once the perfusion started. Hindlimbs were initially perfused at a rate of 3 ml/min for ~5 min. The rate was then increased to 6 ml/min and sustained throughout the experiment.

Dose response. Hindlimbs were perfused without AICAR or with 0.5, 1.0, or 2 mM AICAR. Different rats were used for each AICAR concentration. After a 25-min perfusion with the basic medium described above, AICAR or vehicle (4% albumin in KHB) was added to the perfusate reservoir. The gastrocnemius-plantaris muscle was excised and immediately frozen with stainless steel block tongs at liquid nitrogen temperature 15 min after AICAR entered the muscle.

Time course and palmitate oxidation. Hindlimbs were perfused without AICAR or with 2 mM AICAR for 7.5, 15, and 45 min. Gastrocnemius-plantaris muscles were frozen at the end of each interval. For the hindlimbs perfused for 45 min, 1.6 µCi of 1-[14C]palmitic acid (in KHB-albumin) were added per 100 ml perfusate medium at the beginning of the perfusion. Samples of medium were collected from a port between the oxygenation chamber and the arterial catheter (referred to as the arterial sample) and from the venous catheter (referred to as the venous sample). 14CO2 was quantitated in arterial and venous samples at 7.5, 15, 30, and 45 min after exposure to AICAR or vehicle by the method described by Turcotte et al. (26). Briefly, a filter paper wick (1 × 2 cm) was glued to a cap from a 20-ml glass liquid scintillation vial. The wick was saturated with 2-aminoethanol (0.075 ml) immediately before sample collection. Samples (5 ml) of media were added to the wicks containing 1 ml of 4.5 M lactic acid. Vials were immediately capped and incubated 20–24 h to allow the 14CO2 to be absorbed on the wick. The wicks were then quickly transferred to another scintillation vial. EcoLite (ICN, Irvine, CA) was added immediately, and the wicks were kept in the dark 2 days before quantitation of radioactivity. Arterial and venous samples were also taken at the same intervals for determination of oxygen consumption, glucose uptake, and lactate production. A 0.5-ml sample of medium was added to 2.0 ml of 10% perchloric acid for glucose and lactate determinations.

Analytic methods. Glucose (2) and lactate (8) were determined on neutralized (using 2 M KOH, 0.4 M KCl, 0.4% imidazole) perchloric acid extracts of the medium. Cell-free perfusate was collected by centrifugation of arterial and venous samples for measurement of free fatty acid (FFA) concentration (18) and for fractional extraction of labeled palmitate in the oxidation study. Perfusate samples were kept at −20°C until analyzed. Oxygen hemoglobin saturation was determined on arterial and venous samples with an OSM 2 Hemoximeter (Radiometer, Copenhagen, Denmark) for calculation of oxygen consumption.

Muscles were kept under liquid nitrogen until they were analyzed. They were first ground to powder under liquid nitrogen. Perchloric acid extracts (400 mg of tissue powder added to 2.0 ml of 6% perchloric acid) were prepared and neutralized for measurement of malonyl-CoA (17). For determination of AICAR, ATP, ADP, AMP, ZTP, and ZMP, a 1-ml aliquot of the 6% perchloric acid extract was vortexed vigorously with 1 ml of 1,1,2-trichlorotrifluoroethane-tri-n-octylamine (1:1) to remove the perchloric acid. After centrifugation, the supernatant was stored at −70°C until analysis. AICAR, ATP, ADP, ZTP, and ZMP were determined using a Beckman high-performance liquid chromatography (HPLC) system (supported by System Gold software) by a modification of the method described by Sabina et al. (19). Briefly, we used a Hitachi P10SAX column (0.45 × 25 cm) (iDyChrom, Santa Clara, CA) with a P10SAX-10C5 guard column. At a flow rate of 2 ml/min, the elution began with 100% buffer A (5 mM NH4H2PO4, pH 2.8) and 0% buffer B (750 mM NH4H2PO4, pH 3.9). Buffer B was increased linearly to 9.3% over a 14-min period. Then over the next 36 min buffer B was increased to 100% in a linear gradient. Under these conditions, 5′-AMP was not always well separated from an unknown peak. Therefore, AMP was determined by an enzymatic method (cf. Ref. 14) or by a second HPLC method. In the latter a 40-µl aliquot of the extract was eluted from the same column with 10 mM KH2PO4, pH 4.6, for 10 min. The concentration of the buffer was then increased to 500 mM KH2PO4 using a concave gradient for 40 min. AMPK activity and citrate dependence curves for ACC activity were determined using ammonium sulfate precipitates from homogenates prepared from the powdered (under liquid nitrogen) muscles as described previously (31). Curves were analyzed and fitted to the Hill equation using Grafit (Sigma). Muscle glycogen was determined on the freeze-dried samples by the anthrone method (10).

Results are expressed as means ± SE. Statistically significant differences (P < 0.05) between groups were determined using Student’s t-test (when control perfused hindlimbs were compared with AICAR perfused hindlimbs at a single time point) or one-way analysis of variance and Fisher’s least significant difference test (for the dose-response data).

RESULTS

Control rats weighed 389 ± 7 g, and AICAR-perfused rats weighed 387 ± 6 g on the day of the experiment.

Values for muscle content of adenine nucleotides for hindlimbs perfused with no AICAR or with 0.5, 1.0, and 2.0 mM AICAR for 15 min are shown in Fig. 1. Note that ATP, ADP, and AMP are unaffected by the AICAR
treatment. ZMP concentration in the muscle increases as a function of concentration of AICAR in the perfusion medium.

Figure 2 shows the effect of different concentrations of AICAR on AMPK, ACC activity at 0.2 mM citrate, and malonyl-CoA in gastrocnemius-plantaris muscles perfused with medium containing different concentrations of AICAR for 15 min. Values are means ± SE (n = 4–7 for each concentration). *Significantly different from muscles perfused with no AICAR (P < 0.05).

Table 1. Kinetic constants of ACC extracted from control muscles and from muscles perfused with 0.5, 1.0, and 2.0 mM AICAR for 15 min

<table>
<thead>
<tr>
<th>AICAR (mM)</th>
<th>ACC $V_{\text{max}}$, mmol·g⁻¹·min⁻¹</th>
<th>$K_a$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>39.9 ± 1.1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>26.7 ± 0.8*</td>
<td>10.4 ± 0.4*</td>
</tr>
<tr>
<td>1.0</td>
<td>28.3 ± 0.6*</td>
<td>11.2 ± 1.1*</td>
</tr>
<tr>
<td>2.0</td>
<td>27.7 ± 1.9*</td>
<td>11.8 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 4–7. AICAR, 5-aminimidazole-4-carboxamide ribonucleoside; ACC, acetyl-CoA carboxylase; $V_{\text{max}}$, maximal velocity; $K_a$, activation constant. *Significantly different from 0 mM AICAR, P < 0.05.

of AICAR. Malonyl-CoA was significantly lower at 2.0 mM AICAR than at 0.5 mM AICAR.

Table 1 shows activation constants for citrate activation of ACC as well as $V_{\text{max}}$ for ACC partially purified from muscle extracts. Note the marked inactivation at all concentrations of AICAR.

Figure 3 shows citrate activation curves for ACC for control muscles and for muscles exposed to 2 mM AICAR for 15 and 45 min. Note that ACC becomes much less sensitive to citrate activation in the physiological concentration range. After 45 min of exposure to 2 mM AICAR, the $K_a$ for citrate activation of muscle ACC was 16.1 ± 0.5 mM compared with a value of 3.3 ± 0.09 mM in control muscles (P < 0.05). At the same time, the ACC $V_{\text{max}}$ was 21.6 ± 0.9 µmol·g⁻¹·min⁻¹ for AICAR-infused vs. 35.6 ± 1.8 µmol·g⁻¹·min⁻¹ for control muscles (P < 0.05).

Figure 4 shows the increase in gastrocnemius-plantaris content of ZMP during the course of 45 min of perfusion with 2 mM AICAR. Consistent detection of ZTP was possible only after 45 min of perfusion with 2 mM AICAR, when ZTP levels averaged 0.93 ± 0.08 µmol/g. The IMP content of muscle was significantly increased by AICAR (0.013 ± 0.008 in controls vs. 0.037 ± 0.009 in AICAR-perfused). ATP, ADP, and AMP were not significantly influenced by the AICAR treatment in muscles frozen at 45 min.
Figure 5 shows values for AMPK, ACC at 0.2 mM citrate, malonyl-CoA, and fatty acid oxidation for muscles perfused with medium containing no AICAR or with 2.0 mM AICAR for periods up to 45 min. With the exception of fatty acid oxidation after 7.5 min of exposure to AICAR or vehicle, all values for AICAR-treated muscles were significantly different ($P < 0.05$) from controls perfused for the same period.

DISCUSSION

Previous studies have demonstrated that malonyl-CoA in muscle is decreased by fasting and exercise (14, 16, 30–32), two conditions that are expected to increase the rate of fatty acid oxidation. Others have demonstrated a negative correlation between malonyl-CoA and the rate of fatty acid oxidation in heart and isolated myocytes (1, 15, 20). To our knowledge, this is the first report of an increase in fatty acid oxidation by skeletal muscle in response to a decrease in malonyl-CoA.

That malonyl-CoA inhibits CPT 1 in isolated skeletal muscle mitochondrial preparations is well documented (16, 22). In these in vitro studies, the concentration of malonyl-CoA that results in 50% inhibition is in the range of 0.015 µM. This means that, if all the muscle malonyl-CoA were exposed to the enzyme, CPT 1 would be completely inhibited and little fatty acid oxidation would occur even after prolonged fasting or exercise, because malonyl-CoA content does not decrease below ~0.2 µmol/kg in those conditions. This is assuming, of course, that the conditions in the CPT 1 in vitro assay duplicate those inside the intact muscle fiber, which is

in the case of the AICAR-perfused hindlimbs. Oxygen consumption was not significantly influenced by AICAR treatment (18.1 ± 1.5 for controls vs. 16.3 ± 1.7 µmol·min$^{-1}$·hindlimb$^{-1}$ for AICAR).

Figure 6 shows the effect of perfusion with 2 mM AICAR on uptake of glucose by the rat hindlimb. Significantly different increases in glucose uptake were observed after 15 min of exposure of the muscles to AICAR. Lactate production was 8.0 ± 0.9 in AICAR-perfused hindlimbs vs. 5.2 ± 0.9 µmol·min$^{-1}$·hindlimb$^{-1}$ in controls ($P = 0.051$) after 45 min of perfusion with AICAR or vehicle, respectively. Glycogen content was 51 ± 4 µmol glucose units/g in AICAR-perfused muscle compared with 42 ± 3 µmol glucose units/g in control muscle ($P = 0.11, n = 6$) after the 45 min of perfusion with AICAR or vehicle.

Figure 6 shows the effect of perfusion with 2 mM AICAR on uptake of glucose by the rat hindlimb. Significantly different increases in glucose uptake were observed after 15 min of exposure of the muscles to AICAR. Lactate production was 8.0 ± 0.9 in AICAR-perfused hindlimbs vs. 5.2 ± 0.9 µmol·min$^{-1}$·hindlimb$^{-1}$ in controls ($P = 0.051$) after 45 min of perfusion with AICAR or vehicle, respectively. Glycogen content was 51 ± 4 µmol glucose units/g in AICAR-perfused muscle compared with 42 ± 3 µmol glucose units/g in control muscle ($P = 0.11, n = 6$) after the 45 min of perfusion with AICAR or vehicle.
not likely the case. The present study clearly demonstrates that, when malonyl-CoA is artificially reduced by treating the perfused muscle with AICAR, the rate of fatty acid oxidation is stimulated markedly. The reduction in malonyl-CoA levels seen after AICAR are similar to those produced by exercise or fasting (30, 32).

Previous studies demonstrated that muscle ACC can be phosphorylated by both AMPK and adenosine 3',5'-cyclic monophosphate-dependent protein kinase (31, 33). Only phosphorylation by AMPK results in a change in activity. Phosphorylated ACC was observed to have an increase in $K_v$ for citrate, a decrease in $V_{\text{max}}$ and increases in $K_m$ for all substrates compared with ACC not phosphorylated by AMPK (31, 33). The net effect of these changes would be a marked decrease in activity of the enzyme and consequently a decrease in synthesis of malonyl-CoA in vivo. The changes in ACC $K_v$ and $V_{\text{max}}$ that occur in response to AICAR clearly mirror those seen when the enzyme is phosphorylated in vitro by AMPK and hence provide evidence that AICAR triggers phosphorylation of ACC by AMPK.

This extrapolation becomes even more tenable, considering the increase in AMPK activity in muscles treated with AICAR.

Liver AMPK activity can be increased by both allosteric and covalent mechanisms (3, 5, 9, 23, 28). AMP is the natural allosteric activator. AMP also activates an AMPK kinase that phosphorylates and activates AMPK (29). The fact that our measured increase in AMPK activity survives ammonium sulfate precipitation implies that a phosphorylation has occurred, because most allosteric activators would be discarded in the supernatant after precipitation and centrifugation of the enzyme. This provides additional evidence, along with previous reports on activation of AMPK in response to exercise and muscle stimulation (14, 27, 31), that skeletal muscle also has an AMPK kinase. In the present study, ZMP is likely responsible for activation of the putative AMPK kinase and AMPK, because AMP levels in the muscle did not change during the course of the AICAR perfusion.

Previous studies have demonstrated that AICAR activates phosphorylase but only at basal levels of insulin (34). In this study, we saw no evidence of activation of phosphorylase, because muscle glycogen was not significantly altered during the course of the perfusion with 2 mM AICAR. In fact, we noted a tendency for higher glycogen in the muscles from hindlimbs perfused with 2 mM AICAR for 45 min.

Glucose and insulin values in this study were on the high side of the physiological range. These concentrations were selected to ensure that values for malonyl-CoA in muscles of control hindlimbs would be high. It is significant that, in spite of insulin levels of 200 µU/ml and arterial glucose levels near 10 mM, AICAR caused a decrease in malonyl-CoA and an increase in fatty acid oxidation. This occurred in the face of a relatively low fatty acid concentration in the perfusion medium (0.15 mM). These fatty acid levels are close to plasma levels observed in nonfasted resting rats (31, 32). This implies that, in resting muscle under these conditions, CPT 1 is rate limiting for fatty acid oxidation and that CPT 1 activity is governed by prevailing malonyl-CoA concentrations.

It should be clear that, even in the AICAR-perfused hindlimbs, fatty acid oxidation was responsible for a relatively small proportion (2.2%) of the total oxygen uptake. This is likely a consequence of the high glucose and insulin concentrations and the low fatty acid concentrations. Under these conditions, much of the fatty acid taken up by the hindlimb likely enters the muscle or adipose tissue triglyceride pools or is used for phospholipid synthesis (cf. Ref. 6). We anticipate that, with higher fatty acid concentrations, a much larger proportion of the oxygen utilization would be required to oxidize fatty acids supplied to the muscle.

The increase in glucose uptake by the AICAR-perfused hindlimbs was surprising, in view of the concurrent increase in fatty acid oxidation. It is unclear at this time whether the increase is due to an effect on glucose transporters or is secondary to metabolic changes in the cell. The increase in AMPK activity, which is normally activated secondary to muscle contraction, may not only result in increased oxidation of fatty acids to provide energy for muscle contraction, but also may play a role in increasing uptake of glucose to meet increased energy requirements. The insulin-like effect of muscle contraction and of hypoxia (both of which increase AMP in the muscle) in increasing glucose uptake is well documented, but the mechanisms are not well understood (13). There appears to be an increased incorporation of GLUT-4 transporters into the sarcolemma, but the signals coupling contraction with GLUT-4 incorporation have not been elucidated (13). We propose that the muscle AMPK, when activated by contraction, serves the dual role of increasing oxidation of fatty acids (via the phosphorylation-induced decrease in ACC and malonyl-CoA) and of increasing uptake of glucose (by phosphorylation of undefined target proteins) to meet the increased energy needs of the contracting muscle.

In summary, AICAR stimulates an increase in skeletal muscle AMPK, a decrease in muscle ACC activity, a decrease in muscle malonyl-CoA, an increase in fatty acid oxidation, and an increase in glucose uptake. All these changes are consistent with the hypothesis that the activation of AMPK during muscle contraction increases the ability of the muscle to meet energy needs by increasing both fatty acid oxidation and glucose uptake.

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Address for reprint requests: W. W. Winder, 545 WIDB, Zoology Dept., Brigham Young Univ., Provo, UT 84602.

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