Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise

W. W. WINDER AND D. G. HARDIE
Zoology Department, Brigham Young University, Provo, Utah 84602; and
Department of Biochemistry, The University, Dundee DD1 4HN, Scotland

Winder, W. W., and D. G. Hardie. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E299–E304, 1996.—Malonyl-CoA, an inhibitor of fatty acid oxidation in skeletal muscle mitochondria, decreases in rat skeletal muscle during exercise or in response to electrical stimulation. Regulation of rat skeletal muscle acetyl-CoA carboxylase (ACC), the enzyme that synthesizes malonyl-CoA, was studied in vitro and in vivo. Avidin-Sepharose affinity-purified ACC from hindlimb skeletal muscle was phosphorylated by purified liver AMP-activated protein kinase with a concurrent decrease in ACC activity. AMP-activated protein kinase was quantitated in resuspended ammonium sulfate precipitates of the fast-twitch red (type IIa fibers) region of the quadriceps muscle. Rats running on a treadmill at 21 m/min up a 15% grade show a 2.4-fold activation of AMP-activated protein kinase concurrently with a marked decrease in ACC activity in the resuspended ammonium sulfate precipitates at all citrate concentrations ranging from 0 to 20 mM. Malonyl-CoA decreased from a resting value of 1.85 ± 0.29 to 0.50 ± 0.09 nmol/g in red quadriceps muscle after 30 min of treadmill running. The activation of the AMP-activated protein kinase with consequent phosphorylation and inactivation of ACC may be one of the primary events in the control of malonyl-CoA and hence fatty acid oxidation during exercise.

carnitine palmitoyl transferase; fatty acid oxidation; skeletal muscle; phosphorylation of acetyl-coenzyme A carboxylase; muscle malonyl-coenzyme A; adenosine 5′-monophosphate

OXIDATION OF FREE FATTY acids becomes a source of energy for working muscles during prolonged submaximal exercise (25). In this study, one possible mechanism for regulating fatty acid oxidation during exercise has been investigated. Carnitine palmitoyltransferase (CPT) 1 is the enzyme responsible for transferring fatty acids into the mitochondria where β-oxidation occurs (18, 23). Previous studies have demonstrated that CPT 1 in skeletal muscle is inhibited by malonyl-CoA and is much more sensitive to malonyl-CoA than the liver enzyme, apparently due to the existence of a muscle-specific isoform (18, 23, 31). The concentration of malonyl-CoA decreases in the working muscle during exercise (32) and in response to electrical stimulation (11). This decrease in malonyl-CoA has been postulated to relieve inhibition of CPT 1 and therefore may serve as a signal for increasing fatty acid oxidation in the muscle during prolonged exercise. A close negative correlation has been observed to occur between malonyl-CoA concentration and the rate of fatty acid oxidation in perfused heart and isolated cardiac myocytes (1, 22).

Acetyl-CoA carboxylase (ACC), the enzyme that synthesizes malonyl-CoA, has been demonstrated to be present in skeletal muscle (3, 27, 28, 35). Unlike liver ACC, the muscle ACC does not respond to dietary changes such as fasting and refeeding and appears to be regulated differently (33). Two isoforms of ACC exist in liver (265 and 280 kDa). A unique isoform appears to exist in human and rat skeletal muscle in the range of 272–275 kDa (28, 35). There is also a minor band at 265 kDa. The previously reported contraction-induced decrease in malonyl-CoA in the muscle was postulated to involve a calcium-triggered decrease in ACC activity (11, 32). The rise in free calcium accompanying muscle contraction was hypothesized to activate a protein kinase, eventually resulting in phosphorylation and inactivation of ACC. The 265-kDa isoform of ACC has been reported to be phosphorylated and inactivated by an AMP-activated protein kinase (AMPK; see Refs. 5, 7, 9, 14, 16, 30). In this study, regulation of muscle ACC has been studied in vitro and in vivo. The effect of phosphorylation on activity of isolated muscle ACC was studied. Also, the effect of exercise on muscle ACC, AMPK activity, and malonyl-CoA level was investigated.

MATERIALS AND METHODS

In vitro phosphorylation. ACC was isolated from quadriceps and gastrocnemius muscles of the rat hindlimb. Rats were anesthetized (pentobarbital sodium, intraperitoneally) for at least 30 min before removing muscles. The muscles were clamped between aluminum blocks near 0°C. Muscle groups were separated, and fat and connective tissue were removed. Muscle was minced with scissors and suspended in cold *buffer A* [225 mM mannitol, 75 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, 0.05 mM EDTA, 5 mM potassium citrate, 2.5 mM $MnCl_2$, pH 7.5] with 10 mg/l leupeptin and antitrypsin and 10 ml/l aprotinin (Sigma, St. Louis, MO) in a ratio of 9 ml buffer/g muscle. The muscle was then homogenized with a Brinkmann PT1000 tissue homogenizer using a PT-DA 3030/2 generator. After centrifuging at 17,000 *g* for 40 min, the supernatant was collected. The ACC was precipitated by adding 200 g ammonium sulfate/l and stirring at 4°C for 1 h. The precipitate was collected by centrifuging at 17,000 *g* for 30 min and then resuspended in a minimal volume of buffer containing 100 mM Tris·HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol, pH 7.5, to which was added 5 mg anti-trypsin, 5 mg leupeptin, and 0.5 ml aprotinin/l. After centrifuging to remove insoluble material, the resuspended precipitate was dialyzed for at least 3 h at 4°C against column buffer (100 mM Tris·HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol, pH 7.5) and then purified by avidin-Sepharose affinity chromatography using Promega SoftLink Soft Release Avidin Resin (Fisher Scientific, Pittsburgh, PA). After adding the resuspended precipitate to the column, the column was washed with 30 vol column buffer to remove non-biotin-containing proteins. The ACC was then eluted with column buffer containing 5 mM biotin.

For in vitro phosphorylation studies, globulin-free albumin (Sigma) was added (500 μg/ml) to the purified enzyme (1–2 μg protein in 0.2 ml) followed by an equal volume of cold saturated ammonium sulfate. Poor yields were noted in the absence of albumin. The mixture was allowed to stand 15 min on ice and then was centrifuged at 48,000 *g* for 15 min to collect the precipitate. After discarding the supernatant, the precipitate was resuspended and used for phosphorylation studies. Final concentrations in the assay tubes were 34 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 68 mM NaCl, 0.68 mM EDTA, 0.68 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.68 mM dithiothreitol, 6.8% mM glycerol, and 0.12 mM ATP, pH 7.0. To this mixture was added 20 μCi [γ-^{32}P]ATP with or without 0.2 mM AMP and AMPK (5 U/ml). The final volume in the assay mixture was 60 μl. The AMPK was isolated from rat liver as far as the gel filtration step (4). After incubating at 30°C for 1 h, the reaction was terminated by addition of 60 μl saturated ammonium sulfate. The mixture was allowed to stand 15 min and then was centrifuged at 48,000 *g* for 15 min. The supernatant was discarded, and the precipitate was washed with 1 ml 50% saturated ammonium sulfate. For electrophoresis, the final precipitate was resuspended in 30 μl HEPES buffer to which was added 60 μl electrophoresis sample buffer [62.5 mM Tris, pH 6.8, 10% glycerol, 2.5% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 0.025% bromphenol blue]. After heating at 95°C for 4 min, the samples were subjected to polyacrylamide gel electrophoresis (PAGE) with the Bio-Rad Mini-Protean II Dual Slab Vertical Electrophoresis System using Mini-Protean II 4–15% gradient precast gels (Bio-Rad, Richmond, CA). Gels were run in the presence of 0.1% SDS, 25 mM Tris, and 192 mM glycine, pH 8.3, at 200 V for 60 min. Gels were stained using a silver stain kit (Sigma) and then dried for autoradiography (X-OMAT AR Scientific Imaging Film, Kodak).

For determination of functional effects of phosphorylation, ACC was incubated with or without AMPK in the same reaction mixture in the absence of radiolabeled ATP. ACC activity was determined at the end of the phosphorylation with no ammonium sulfate precipitation. ACC activity was determined by measuring the rate of incorporation of [^{14}C]bicarbonate into acid-stable compounds (malonyl-CoA) at 37°C for 10 min. Final concentrations of reagents were 50 mM HEPES buffer, pH 7.5, 1.5 mM $MgSO_4$, 2 mM dithiothreitol, 0.125 mM acetyl-CoA, 4 mM ATP, 12.5 mM $KHCO_3$, 2 μCi [^{14}C]bicarbonate, and 0.75 mg/ml fatty acid-free bovine serum albumin. Citrate plus magnesium acetate were added in equimolar concentrations ranging from 0 to 10 mM. The reaction was started by addition of ACC. The final reaction volume was 200 μl. The reaction was stopped by addition of 50 μl 5 N HCl. After centrifugation, 150 μl was transferred to a scintillation vial and evaporated to dryness at 80°C. The residue was dissolved in 0.4 ml water and then mixed with 5.5 ml scintiverse (Fisher Scientific) for determination of radioactivity. Preliminary experiments indicated linearity with time and enzyme concentration in this range. The citrate data were fitted to the Hill equation using the Grafit program (Sigma). This program allows determination of the activation constant (K_a) for citrate, the maximal activity as a function of citrate concentration (V_{max}), and the citrate concentration required for half-maximal activation of ACC ($K_{0.5}$).

Animal care. Male Sprague-Dawley rats (Sasco, Omaha, NE) were housed in individual cages at a temperature of 19–21°C in a light-controlled (12:12-h light-dark cycle) room and were fed a normal diet (Harlan Teklad rodent diet, Madison, WI). Rats were run on a rodent treadmill at 21 m/min up a 15% grade (10 min/day) for at least 2 wk to accustom them to running on the treadmill and to the handling procedures. Jugular catheters were implanted (ether anesthesia) 3 days before the final exercise test. These catheters were used for rapid induction of anesthesia at the end of the exercise. Rats weighed 371 ± 8 g at the time of death.

Exercise test. Rats were anesthetized via the catheter at rest or after running for 5 or 30 min on the treadmill at 21 m/min up a 15% grade. Approximately 0.5 g of the red region of the quadriceps nearest the bone was removed rapidly (within 90–120 s after anesthetization) and frozen with aluminum block tongs at liquid nitrogen temperature. Blood was removed via the abdominal aorta. An aliquot was heparinized for collection of plasma for measurement of free fatty acids (FFA; see Ref. 20). A perchloric acid extract of blood was also made for measurement of glucose (2) and lactate (13).

Muscle assays. The muscle samples were kept at liquid nitrogen temperature until analyzed. Muscles were ground to powder under liquid nitrogen. For ACC and AMPK assays, the frozen powder was weighed and then homogenized in a buffer containing 100 mM mannitol, 50 mM NaF, 10 mM Tris, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.5, and proteolytic enzyme inhibitors (5.0 ml/l aprotinin, 5.0 mg/l leupeptin, and 5.0 mg/l anti-trypsin). The homogenate was immediately centrifuged at 48,000 *g* for 30 min. The ACC was precipitated from the supernatant by addition of 144 mg ammonium sulfate/ml and by stirring for 30 min on ice. The precipitate was collected by centrifugation at 48,000 *g* for 20 min. The pellet was dissolved in 10% of the original volume of the homogenate buffer and was centrifuged again to remove insoluble protein.

The supernatant was used for determination of ACC and AMPK activity. ACC was determined as described above with citrate/magnesium acetate concentrations ranging from 0 to 20 mM. Citrate activation data were analyzed as described for the in vitro studies. AMPK activity was determined on the same supernatant by minor modifications of a method described previously (7). Final concentrations in the reaction medium for AMPK were 40 mM HEPES, 0.2 mM SAMS (peptide substrate for AMPK), 0.2 mM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, and 0.2 mM ATP (+2 μCi [^{32}P]ATP), pH 7.0, in a final volume of 25 μl for 10 min at 37°C. The SAMS peptide was synthesized as described previously (7). The reaction was linear with time and varying homogenate concentrations in this range. At the end of the incubation, an aliquot was removed and spotted on Whatman P81 filter paper. The [^{32}P]ATP was removed with six washes in 1% phosphoric acid and one wash with acetone. The papers were air-dried and radioactivity quantitated after immersing in 3 ml scintiverse (Fisher Scientific) in a scintillation vial.

Neutralized perchloric acid extracts of the red quadriceps were used for determination of ATP (17), ADP (15), AMP (15), citrate (21), and malonyl-CoA (19). Fatty acid synthetase for malonyl-CoA assays was isolated from livers of fasted/refed rats by the procedure described by Linn (cf, Ref. 32).

Results are expressed as means ± SE. Analysis of variance and Fisher's least-significant difference (as a post hoc test) were used to determine statistical differences ($P < 0.05$) between treatment groups.

RESULTS

In vitro phosphorylation studies. Figure 1 shows the silver-stained SDS-PAGE gels (Fig. 1, *lanes A-C, left*) and autoradiographs (Fig. 1, *lanes A-C, right*) of corresponding lanes after the phosphorylation reaction.

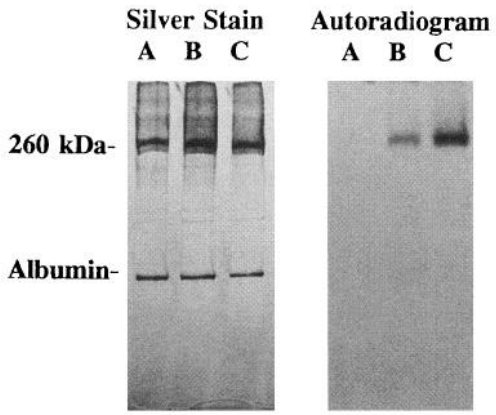

Fig. 1. Silver-stained gel and autoradiograph of dried gel from SDS-polyacrylamide gel electrophoresis of acetyl-CoA carboxylase (ACC) isolated from hindlimb skeletal muscle by avidin-Sepharose affinity chromatography and subjected to phosphorylation for 60 min as described in MATERIALS AND METHODS. ACC in *lane A* was exposed to all reagents in phosphorylation mixture except AMP-activated protein kinase (AMPK). ACC in *lane B* was exposed to all reagents in presence of AMPK but without AMP. ACC in *lane C* was exposed to all reagents + AMPK and AMP. Similar results were observed on 2 different preparations of ACC.

Little phosphorylation was noted in the absence of AMPK (Fig. 1, *lane A*). AMPK catalyzed a small amount of phosphorylation in the absence of AMP (Fig. 1, *lane B*), but the phosphorylation reaction was markedly enhanced by AMP (Fig. 1, *lane C*).

Figure 2 demonstrates a decrease in catalytic activity of ACC in response to phosphorylation, particularly at the lower citrate concentrations. At a citrate concentration of 0.5 mM, ACC activity decreased from 28.6 ± 3.9 to 9.4 ± 1.8 pmol/min (67% decrease) as a consequence of phosphorylation. The V_{max} of the enzyme is also significantly decreased in response to phosphorylation (307 ± 36 pmol/min before phosphorylation vs. 212 ± 6 pmol/min after phosphorylation). The $K_{0.5}$ for citrate activation increased from 2.2 ± 0.2 to 3.0 ± 0.0 mM. The K_a for citrate activation increased from 3.6 ± 0.2 to 6.8 ± 0.5 mM as a consequence of phosphorylation. These differences are all statistically significant ($P < 0.05$). In a separate experiment, purified muscle ACC was treated with AMPK with or without AMP ($n = 3$). In the absence of AMP, AMPK caused a 42% decrease in ACC activity at 0.5 mM citrate. Addition of AMP caused a 83% reduction in ACC activity at the same citrate concentration. Thus both incorporation of labeled phosphate into ACC and reduction of ACC activity in response to AMPK treatment were accelerated when AMP was added to the phosphorylation medium.

Treadmill exercise study. Figure 3 shows the effect of treadmill exercise on citrate dependence of ACC (precipitated from homogenates with ammonium sulfate) in skeletal muscle. Both the V_{max} and the $K_{0.5}$ for citrate activation of ACC are influenced in the muscle during exercise. The V_{max} decreased from 25.0 ± 1.8 nmol·g^{-1}·min^{-1} at rest to 15.1 ± 1.3 nmol·g^{-1}·min^{-1} after 5 min and to 14.2 ± 1.0 nmol·g^{-1}·min^{-1} after 30 min of exercise. The $K_{0.5}$ for citrate activation was 2.36 ± 0.11 mM at rest, 3.14 ± 0.13 mM after 5 min, and 3.00 ± 0.13 mM after 30 min of exercise. The calculated activation constants using the Hill equation were 4.76 ± 0.49 mM for resting rats, 8.71 ± 1.18 mM after 5 min, and 8.18 ± 0.81 mM after 30 min of running. The differences between resting and running rats were all statistically significant ($P < 0.05$). The decrease in skeletal muscle ACC activity was particularly marked at physiological concentrations of citrate. At 0.2 mM citrate, ACC activity was 0.64 ± 0.02 nmol·g^{-1}·min^{-1} at rest vs. 0.23 ± 0.02 nmol·g^{-1}·min^{-1} after 5 min and 0.22 ± 0.02 nmol·g^{-1}·min^{-1} after 30 min of exercise.

The skeletal muscle AMPK activity was significantly increased after 5 min of exercise and continued to be

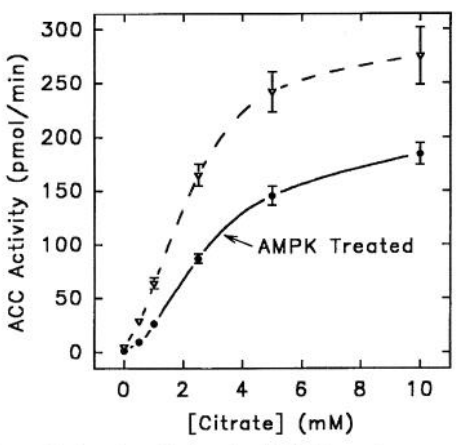

Fig. 2. Effect of phosphorylation by AMPK on citrate activation of ACC purified by avidin-Sepharose affinity chromatography. Values represent means ± SE of 3 determinations on 3 separate isolations of ACC. ACC activity of AMPK-treated enzyme is significantly lower than that of nontreated enzyme at all concentrations of citrate ($P < 0.05$). Citrate concentration required for half-maximal activation of ACC ($K_{0.5}$) for citrate was 2.0 ± 0.1 mM for nontreated vs. 2.6 ± 0.0 mM for AMPK-treated ACC ($P < 0.05$). [Citrate], citrate concentration.

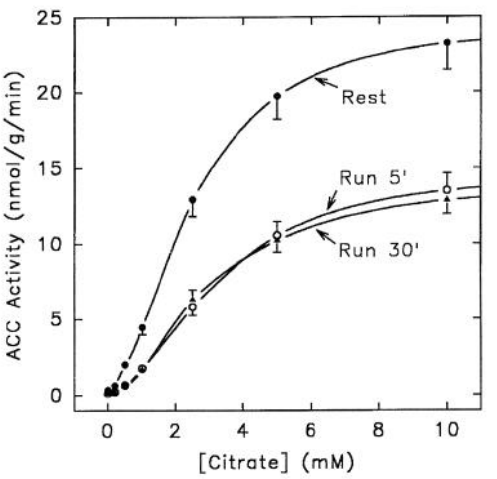

Fig. 3. Effect of treadmill exercise on citrate dependence of red quadriceps muscle ACC partially purified by ammonium sulfate precipitation. Values represent means ± SE of 8–10 rats for each treatment group. ACC activity of muscle from exercised rats (both 5 and 30 min exercised) is significantly lower than for resting rats at all citrate concentrations ($P < 0.05$). $K_{0.5}$ for citrate activation was 2.34 ± 0.11 mM at rest, 2.88 ± 0.17 mM ($P < 0.05$) after 5 min, and 3.14 ± 0.12 mM ($P < 0.05$) after 30 min of exercise.

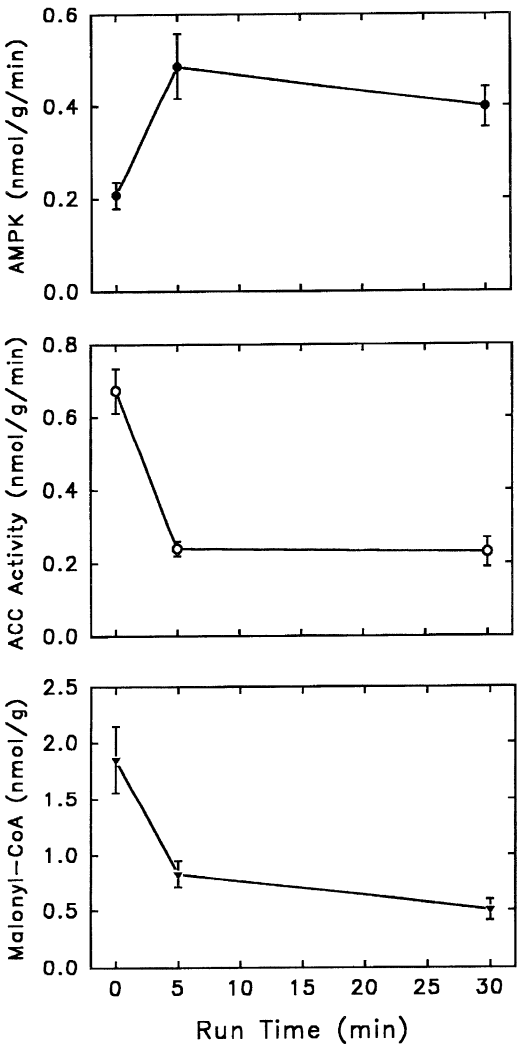

Fig. 4. Effect of treadmill exercise on time course of AMPK activity, ACC activity in presence of 0.2 mM citrate, and malonyl-CoA in red quadriceps muscle. Values are means ± SE from 8–10 rats at each time point. All values from exercising rats are significantly different from those of resting rats ($P < 0.05$).

significantly elevated after 30 min of exercise (Fig. 4, *top*). The increase in AMPK was accompanied by a decrease in muscle ACC activity (at 0.2 mM citrate; Fig. 4, *middle*) and a decrease in muscle malonyl-CoA (Fig. 4, *bottom*).

The decrease in malonyl-CoA occurred before an increase in plasma FFA (Table 1). Blood glucose was not significantly changed during the exercise bout. Blood lactate was elevated above resting values after 5 and 30 min of exercise.

Table 1. *Effect of exercise on blood glucose, blood lactate, and plasma free fatty acid concentrations*

	Rest	Run 5 min	Run 30 min
Glucose	7.0 ± 0.2	8.0 ± 0.5	7.9 ± 0.3
Lactate	1.1 ± 0.1	2.4 ± 0.6*	3.4 ± 0.4*
Plasma FFA	0.10 ± 0.01	0.10 ± 0.01	0.24 ± 0.02*

Values are means ± SE in mM; n = 7–9 rats. FFA, free fatty acid. *Significantly different from rest values, $P < 0.05$.

Table 2. *Effect of exercise on citrate, AMP, ADP, and ATP contents of fast-twitch red region of quadriceps*

	Rest	Run 5 min	Run 30 min
Citrate	0.16 ± 0.01	0.26 ± 0.02*	0.28 ± 0.01*
AMP	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
ADP	1.3 ± 0.02	1.2 ± 0.02	1.3 ± 0.04
ATP	6.0 ± 0.2	5.8 ± 0.1	5.8 ± 0.1

Values are means ± SE in μmol/g; n = 6–9 rats. *Significantly different from rest, $P < 0.05$.

The adenine nucleotides (AMP, ADP, ATP) were unchanged during exercise (Table 2). Citrate was significantly increased in response to exercise (Table 2).

DISCUSSION

Previous studies have demonstrated that the liver ACC can be phosphorylated by AMPK and protein kinase A (PKA) and that the phosphorylation is accompanied by decreases in V_{max} and increases in $K_{0.5}$ for the activator citrate (14, 16). Phosphorylation by AMPK at *serines 79, 1200*, and *1215* produced a much more marked inactivation than phosphorylation by PKA at *serines 77* and *1200* (10), and since phosphorylation in intact hepatocytes occurs at *serine 79* but not *serine 77*, AMPK appears to be the protein kinase that regulates ACC under physiological conditions in liver (24). However, skeletal and cardiac muscle contain distinct 270- to 280-kDa forms of ACC (3, 26, 28, 35). Cardiac muscle ACC is activated by a liver protein phosphatase with a concurrent decrease in citrate dependence indicating that, like the liver form, it is regulated by reversible phosphorylation (26). A minor 280-kDa form also exists in rat liver and appears to be a distinct gene product from the 265-kDa form (34). The 270–280 isoforms from liver, skeletal muscle, and cardiac muscle cross-react using a monoclonal antibody that does not recognize the 265 isoform (3). Recent data from two laboratories (28, 35) indicate the muscle isoform to be of a slightly lower molecular weight than the liver 280 isoform (272–275 kDa). Whether the 270–280 isoforms from heart, liver, and skeletal muscle all represent the same gene product remains to be determined. This paper represents the first report demonstrating that the principle muscle isoform of ACC is phosphorylated by AMPK and that phosphorylation causes a decrease in enzyme activity. The decrease in activity was particularly marked at concentrations of citrate expected to be in the physiological range in the muscle fiber.

The AMPK used in the in vitro studies was isolated from rat liver (4). This kinase has been well characterized in previous studies (4–10, 30). The purified kinase is activated up to fivefold by AMP, and this is antagonized by ATP so that the kinase responds to the AMP-to-ATP ratio rather than simply to the AMP concentration (4, 6). In addition, the kinase is phosphorylated and activated by an upstream protein kinase (AMPK kinase), and this reaction is completely

dependent on AMP. The combination of the two effects result in >100-fold activation overall when AMP rises (6, 30). Tissue distribution studies have demonstrated that AMPK activity can be detected in many tissues, including skeletal muscle (7). The kinase activity was apparently much higher in liver than in muscle (7), but this may be an artifact caused by kinase activation during isolation of liver tissue (8). Intriguingly, it has recently been found that the 63-kDa catalytic subunit of AMPK, and its mRNA, is more highly expressed in skeletal muscle than in any other tissue (29).

Previous studies have demonstrated a decline in malonyl-CoA in skeletal muscle in exercising rats and in electrically stimulated muscle (11, 32). In the exercising rats, significant declines in malonyl-CoA were observed in the type IIa (fast-twitch red) fibers of the quadriceps after 5 min of exercise, with maximal decreases occurring after ~30 min of exercise (32). A slower rate of decline was observed in the superficial white region of the quadriceps (type IIb fibers), which is likely recruited to a lesser extent during exercise of this intensity. The present study demonstrates clearly that the decrease in malonyl-CoA in the active fast-twitch red region of the quadriceps could be caused by a decrease in muscle ACC activity. The in vitro studies demonstrate that citrate dependence of ACC is regulated by phosphorylation. The decrease in citrate-dependent activity may be used as indirect evidence that phosphorylation of the muscle ACC has occurred in the in vivo study. The observation of rapid activation of the AMPK activity after the beginning of exercise supports this hypothesis.

The changes in AMPK activity observed in this study persisted during partial purification by ammonium sulfate precipitation, and the kinase assays were conducted in the presence of a saturating concentration (200 μM) of AMP. These effects were therefore not due to allosteric activation by AMP and are likely to be due to increased phosphorylation by a skeletal muscle AMPK kinase. We have recently shown that rat liver AMPK is essentially inactive after complete dephosphorylation and that the dephosphorylated form can be activated at least 50-fold by rat liver AMPK kinase (S. Hawley, unpublished observations). Because the catalytic subunits of rat liver and rat muscle AMPK are indistinguishable by the criteria of Northern and Western blotting (29), this implies that an AMPK kinase must exist in skeletal muscle. The obvious hypothesis to explain the effect of exercise on AMPK activity was therefore that an increase in the cellular AMP-to-ATP ratio stimulated the phosphorylation and activation of AMPK by AMPK kinase. We could not detect a change in the AMP-to-ATP ratio in response to exercise in this study. It has been previously reported that relatively high contraction rates are required to produce a change in the free AMP concentration in the muscle (12). An important caveat was that a delay of 90–120 s between the termination of exercise and freezing of the tissue was unavoidable. We believe it is possible that the AMP-to-ATP ratio did change during exercise but that these nucleotides returned to resting levels during application of anesthesia and dissection before freezing. Even if the nucleotides did return to their resting levels, the effect on AMPK activity would persist until the kinase was dephosphorylated. In isolated rat hepatocytes, where metabolism can be instantly quenched by adding perchloric acid to the cell suspension, there is an excellent correlation between the AMP-to-ATP ratio and AMPK activity in response to stress treatments such as heat shock and the mitochondrial inhibitor arsenite (5). The possibility must also be considered that another contraction-coupled event, such as an increase in sarcoplasmic free calcium concentration, could activate the putative AMPK kinase.

It is clear from this and a previous study (32) that the activation of the AMPK, inactivation of ACC, and decline in malonyl-CoA occur in the muscle before a rise in plasma FFA during exercise of this intensity. It is possible that intramuscular FFA become available from hydrolysis of intramuscular triglyceride stores before the rise in FFA (cf, Ref. 25). In either case, the decline in malonyl-CoA would be anticipated to relieve inhibition of CPT 1, allowing increased fatty acid oxidation as fatty acids become available to the muscle mitochondria.

The significant increase in muscle citrate content seen during exercise could conceivably offset the decrease in ACC activity. It is unclear what proportion of the total muscle citrate is in the sarcoplasmic (vs. mitochondrial) compartment. In addition, as can be noted from the citrate activation curves for ACC, the ACC activity increases to only a small extent in the range of 0–0.2 mM citrate, the range likely to be seen in the sarcoplasmic compartment where the ACC is located. Regardless of the possible increase in sarcoplasmic citrate, a definite decrease was observed in muscle malonyl-CoA, the product of the ACC reaction.

In summary, the principle muscle isoform of ACC can be phosphorylated and inactivated by AMPK in vitro. The V_{max} decreases, and K_a for citrate activation increases as a consequence of phosphorylation. The AMPK is also activated in the contracting muscle within 5 min of the beginning of exercise. There is a concurrent decline in V_{max} and increase in K_a for citrate activation of muscle ACC during treadmill exercise. This decrease in ACC activity is accompanied by a decrease in muscle malonyl-CoA. From these findings, the following sequence of events is proposed. *1*) A rise in AMP (and/or a rise in sarcoplasmic free calcium) accompanying muscle contraction activates a putative AMPK kinase. *2*) This stimulates phosphorylation of AMPK by AMPK kinase. *3*) The activated AMPK phosphorylates ACC. *4*) The consequent decrease in ACC activity results in a decline in malonyl-CoA. *5*) The decrease in malonyl-CoA relieves inhibition of CPT 1, allowing increased fatty acid oxidation as fatty acids become available during the course of the exercise.

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

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