Coordinate Regulation of Malonyl-CoA Decarboxylase, sn-Glycerol-3-phosphate Acyltransferase, and Acetyl-CoA Carboxylase by AMP-activated Protein Kinase in Rat Tissues in Response to Exercise*

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Changes in the concentration of malonyl-CoA in many tissues have been related to alterations in the activity of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in its formation. In contrast, little is known about the physiological role of malonyl-CoA decarboxylase (MCD), an enzyme responsible for malonyl-CoA catabolism. In this study, we examined the effects of voluntary exercise on MCD activity in rat liver, skeletal muscle, and adipose tissue. In addition, the activity of sn-glycerol-3-phosphate acyltransferase (GPAT), which like MCD and ACC can be regulated by AMP-activated protein kinase (AMPK), was assayed. Thirty min after the completion of a treadmill run, MCD activity was increased ~2-fold, malonyl-CoA levels were reduced, and ACC and GPAT activities were diminished by 50% in muscle and liver. These events appeared to be mediated via activation of AMPK since: 1) AMPK activity was concurrently increased by exercise in both tissues; 2) similar findings were observed after the injection of 5-amino 4-imidazole carboxamide, an AMPK activator; 3) changes in the activity of GPAT and ACC paralleled that of MCD; and 4) the increase in MCD activity in muscle was reversed in vitro by incubating immunoprecipitated enzyme from the exercised muscle with protein phosphatase 2A, and it was reproduced by incubating immunopurified MCD from resting muscle with purified AMPK. An unexpected finding was that exercise caused similar changes in the activities of ACC, MCD, GPAT, and AMPK and the concentration of malonyl-CoA in adipose tissue. In conclusion: MCD, GPAT, and ACC are coordinately regulated by AMPK in liver and adipose tissue in response to exercise, and except for GPAT, also in muscle. The results suggest that AMPK activation plays a major role in regulating lipid metabolism in many cells following exercise. They also suggest that in each of them, it acts to increase fatty acid oxidation and decrease its esterification.

Malonyl-CoA, in addition to being an intermediate in the de novo synthesis of fatty acids, is an inhibitor of carnitine palmitoyltransferase I, the enzyme that regulates the transfer of long-chain fatty acyl-CoA into mitochondria, where they are oxidized (1). One factor governing the concentration of malonyl-CoA is acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in its synthesis. ACC is subject to both allosteric and covalent (by phosphorylation) regulation, and in some tissues, to changes in its abundance. A multitude of studies in such tissues as liver and muscle have clearly shown that increases and decreases in malonyl-CoA levels correlate closely with changes in ACC activity (2–4). It is less clear whether malonyl-CoA decarboxylase (MCD), an enzyme that degrades malonyl-CoA, also regulates its concentration under physiological conditions. Recent studies suggest that the concentration of malonyl-CoA in liver and muscle in certain circumstances correlates inversely with changes in MCD activity. Thus, increases in MCD activity have been observed in rat liver during starvation (5) and in skeletal muscle (6) in response to electrically induced contractions. In the latter situation, the increase in activity was attributable to activation of AMP-activated protein kinase (AMPK), an enzyme that also phosphorylates and inhibits ACC. Despite this, the physiological role of MCD in regulating the concentration of malonyl-CoA remains open to question as are the mechanisms by which its activity is regulated (7).

The present study explores the effect of voluntary exercise on MCD activity in rat liver, skeletal muscle, and adipose tissue and how observed changes relate temporarily to alterations in the activities of ACC, AMPK, and glycercophosphate acyltransferase (GPAT), another enzyme shown previously to be regulated by AMPK (16) and malonyl-CoA concentration. In addition, the response to exercise was compared with that following the administration in vivo of the AMPK activator 5-amino 4-imidazolecarboxamide riboside (AICAR), and in skeletal muscle, the effect of purified AMPK on MCD activity was examined in vitro. Finally, since it has recently been suggested that measurements of MCD activity may vary with the assay used (7), in some studies, results obtained with spectrophotometric and radiometric methods were compared.

EXPERIMENTAL PROCEDURES

Experimental Animals for Exercise Study

Male Sprague-Dawley rats, weighing 245–275 g, were obtained from Charles River Laboratories (Wilmington, MA). They were ran-
were sacrificed by exsanguination. On the day of experimentation, one group remained sedentary, and the other ran on a rodent treadmill (Economical Exercise Treadmill Model Exer-4, Columbus Instruments International Corporation, Columbus, OH) for 1 week to acclimate them to handling and to running on the treadmill. They were housed in individual cages in a temperature-controlled room (22 ± 1°C) on a 12:12 h light-dark cycle and provided water and Purina rat chow ad libitum.

Experimental Animals for AICAR Study

Male Sprague-Dawley rats, weighing 320–360 g, were obtained from Charles River Laboratories. They were ad libitum on a 12:12 h light-dark cycle and provided water and Purina rat chow ad libitum.

Materials and Methods

Experimental Animals for AICAR Study

Male Sprague-Dawley rats, weighing 320–360 g, were obtained from Charles River Laboratories. They were ad libitum on a 12:12 h light-dark cycle and provided water and Purina rat chow ad libitum.

Metabolite Assay

Plasma insulin was measured by radioimmunoassay with a rat insulin standard (Linco Research, St. Charles, MO), and plasma glucose was determined by the hexokinase method (8). Malonyl-CoA in muscle and liver was measured by the radioisotopic method described by Mutto et al. (5). Briefly, frozen muscle or liver tissue was 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, 5 μM pepstatin, 40 mM NaF, 4 mM Na3VO4, and 1 mM Na2VO3. Tissue homogenates were then centrifuged at 50 × g for 5 min at 4°C. A small volume of filtrate was saved for determination of protein concentration by the method of Bradford (13) with bovine serum albumin as the standard. MCD was partially purified from the remaining supernatant by adding (NH4)2SO4 until 50% saturation was achieved. The mixture was then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant from this spin was aspirated and treated with additional (NH4)2SO4 until 55% saturation was achieved. The mixture was then recentrifuged at 14,000 × g for 10 min at 4°C, and the resultant pellet was reconstituted in 0.1 M Tris-HCl (pH 8.0) and stored at 4°C until assay.

Purification of MCD by Immunoprecipitation

Supernatants (500 × g for 5 min at 4°C) from frozen muscles (300–350 μg of muscle) were purified by immunoprecipitation as described previously (6). Affinity-purified antibody from rabbits immunized with the N-terminal region of MCD, which lacks both peroxisomal and mitochondrial targeting sequences, was used (14). In one study, a C-terminal MCD antibody, the C-terminal part of the enzyme ending with a peroxisomal targeting motif, was also used.

MCD Assay

Fluorimetric Method—In most studies, MCD activity was measured fluorometrically using a Hitachi F-2500 fluorescence spectrophotometer (6). The excitation and emission wavelengths were set at 341 and 455 nm, respectively, to generate a 70–80% deflection. In addition, the photomultiplier voltage was set at 700 V to provide the greatest sensitivity. A reaction mixture was prepared in a cuvette by adding 0.1 M Tris-HCl (pH 8.0), 0.5 mM diithiothreitol, 0.6 mM NADH, 1.0 μM malate, and malate dehydrogenase (74 units). Fluorescence was then measured, and upon obtaining temperature equilibration, citrate synthase (1.7 units) was added to the reaction mixture. Once equilibrium was established, 300 μM malonyl-CoA was added, and another baseline measurement was obtained. Subsequently, the immunopurified enzyme or the enzyme purified by (NH4)2SO4 fractionation was added, and the rate of reaction was measured from the change in fluorescence. For each sample, an identical reaction mixture lacking exogenous malonyl-CoA was used as a control.

Radioimetric Method—A radiometric MCD assay (14) was also used for measurement of MCD activity. The 500 × g supernatant of the muscle homogenate was incubated for 10 min in the standard reaction mixture containing 0.5 mM malonyl-CoA. The reaction was stopped by adding perchloric acid, and the precipitated proteins were sedimented by centrifugation. The incorporation of acetyl-CoA, formed by the MCD reaction, into [14C]citrate was determined by coupled enzymatic reactions in which [14C]citrate was first converted to [14C]oxaloacetate with glutamic-oxaloacetic transaminase, and then citrate synthase was added to cause the [14C]oxaloacetate to react with the acetyl-CoA to form [14C]citrate. In the final step of the process, unaerated [14C]citrate and [14C]citrate were separated by stirring the solution into a 1:2 (w/v) suspension of Dowex 50W-X8 (100–200 mesh) and centrifuging the mixture at 44 × g for 10 min. Supernatant in, expressed as nmol of acetyl-CoA formed/mg of muscle supernatant protein, was quantified by comparison with acetyl-CoA standards.

AMPK and ACC Assays

AMPK was immunoprecipitated from a 500 × g supernatant fraction (12 min) of muscle and liver with nonimmune sera or with specific antisera directed against the α1 or α2 catalytic subunit of the AMPK heterotrimer. Immunoprecipitates were then collected on A/G beads and washed extensively. The immobilized enzyme was assayed as described previously (4). In brief, 50 μl of reaction mixture was added to the immunoprecipitates, and 25 μl of the resultant mixture was then spotted on p81 filter paper, which was washed with 5% trichloroacetic acid-1% sodium pyrophosphate. It was not possible to assay immunopurified AMPK activity in adipose tissue for reasons not determined. Therefore, AMPK activity in adipose tissue was determined in an ammonium sulfate fraction prepared as for the ACC assay. ACC activity in muscle, liver, and adipose tissue was assayed as described by Vavvas et al. (4). Tissues were ground to a powder under liquid nitrogen. The frozen powder was weighed (0.2 g) and then homogenized (4). The homogenate was immediately centrifuged at 13,500 × g for 12 min. The ACC was precipitated from the supernatant by the addition of 144 mg of ammonium sulfate/ml and stirred on ice for 30 min. The precipitate was then collected by centrifugation at 45,000 × g for 30 min, and the pellet was dissolved in 10% of the original volume of homogenizing buffer (see "MCD Assay"), and recentrifuged to remove insoluble protein. The supernatant was used for determination of ACC activity.

GPAT Assay

Glycerol 3-phosphate acyltransferase (GPAT) was assayed with 300 μM [3H]glycerol-3-P and 80 μM palmitoyl-CoA in the presence or absence of 1 mM N-ethylmaleimide to inhibit the microsomal isofrom as described by Muscio et al. (16). Results are presented as the incorporation of [3H]glycerol-3-P into lysophosphatidic acid and MCD activity in, expressed as nmol of palmitoyl-CoA formed/mg of muscle supernatant protein, was quantified by comparison with acetyl-CoA standards.

Phosphorylation of MCD by AMPK, cAMP-dependent Protein Kinase, and Casein Kinase II

Muscle supernatant was immunoprecipitated and incubated at 37°C with or without purified AMPK (kindly provided by Dr. Lee Witters) (1) micromolar, where 1 unit equals 1 μmol of phosphate transferred per minute and 1 SAMS peptide and 0.2 mM ATP. AMPK and Mg2+ for different time periods as indicated in the legend for Fig. 4. For the cAMP-dependent protein kinase (5 units of the catalytic subunit of bovine heart cAMP-dependent protein kinase, Sigma) and casein kinase II (5 units of the rat liver casein kinase II, Sigma) studies, the reaction mixture was the same as for AMPK. MCD activity was determined as described previously.

Statistics

Results are expressed as means ± S.E. Statistical differences between multiple groups were determined by the Student's t test, where p < 0.05 was considered statistically significant.
RESULTS

Exercise Study—Malonyl-CoA levels and MCD, GPAT, and ACC activities were measured in tissues of sedentary rats and rats that had run on a treadmill for 30 min. All samples were taken 30 min after the completion of the exercise. Mean plasma glucose levels were higher in exercised than in sedentary rats (8.3 ± 0.2 mM versus 6.2 ± 0.15 mM). In contrast, plasma insulin levels tended to be lower in the exercised group (0.4 ± 0.16 ng/ml versus 0.6 ± 0.14 ng/ml), although the difference was not statistically significant.

The concentration of malonyl-CoA and the activity of ACC in the gastrocnemius muscle decreased by 50% after treadmill running (Fig. 1) in agreement with previous findings by Winder et al. (17, 18). Concurrently, malonyl-CoA decarboxylase activity was increased nearly 2-fold from 5.0 ± 0.5 nmol/min/mg of muscle supernatant protein at rest to 9.7 ± 0.4 nmol/min/mg (p < 0.05) after exercise (Fig. 1). A similar pattern of events was observed in liver, in which exercise increased MCD activity 2-fold and caused the concentration of malonyl-CoA and ACC activity to decrease by 50% (Fig. 1), and in epididymal adipose tissue, in which exercise increased MCD activity by 70% and decreased the concentration of malonyl-CoA and the activity of ACC by 40–60% (Fig. 1).

The effects of exercise on AMPK activity in the three tissues are shown in Fig. 2. The activities of both the α1 and α2 AMPK isoforms increased in response to exercise in both liver and muscle with predominantly α1-AMPK increasing in liver and α2-AMPK in muscle (Fig. 2). In adipose tissue, in which we were unable to immunopurify the individual isoforms, total AMPK activity was increased by 50% (Fig. 2).

Mitochondrial GPAT activity in gastrocnemius muscle was not affected by exercise. In contrast, liver mitochondrial GPAT activity was diminished by 50% after exercise, as was mitochondrial GPAT activity in adipose tissue (Fig. 3). Exercise did not affect microsomal GPAT activity in any of the tissues examined.

AICAR Study—Previously, it was shown that AICAR administration diminishes the concentration of malonyl-CoA in liver and muscle (19, 20). In this study, we found that the subcutaneous administration of AICAR (250 mg/kg of body weight) decreased the concentration of malonyl-CoA in epididymal fat from 14 ± 1.2 nmol/g to 7.6 ± 0.5 nmol/g (Table I). In addition, it caused alteration in the activities of ACC, MCD, and GPAT in the three tissues very similar to those observed 30 min after exercise.

Relation of Changes in MCD Activity in Muscle to Alterations in its Phosphorylation, Role of AMPK—Fig. 4 shows that incu-
bation of immunopurified MCD from the gastrocnemius of a sedentary rat with purified AMPK led to a 2-fold increase in MCD activity by 60 min. Further changes did not occur after longer periods of incubation. Incubation of immunopurified MCD with cAMP-dependent protein kinase and casein kinase II also activated MCD but to a substantially lesser extent and more slowly than did AMPK. When MCD immunoprecipitates from the gastrocnemius muscle taken 30 min after the completion of the exercise bout were treated with protein phosphatase 2A (PP2A, 200 milliunits), the observed increase in enzyme activity was markedly reduced (Fig. 5). This effect of PP2A was prevented by adding the phosphatase inhibitor okadaic acid to the medium. Fig. 6 shows that the effect of incubation with AMPK on the activity of immunopurified MCD varied between muscles. Thus, MCD activity was increased by 100% in the extensor digitorum longus and by 70% in the gastrocnemius muscle but not significantly in the soleus.

We have demonstrated previously that treatment with PP2A
in vitro diminishes the increase in immunopurified MCD activity produced in rat muscle by contraction or incubation with AICAR (6). As shown in Fig. 5, PP2A had an identical effect on MCD activity after exercise.

**MCD Activity Measured by Radiometric Assay**—We also measured MCD activity by a radiometric assay in 500 × g supernatants of muscle homogenates taken after 5 min of contraction induced by electrical stimulation of the sciatic nerve. MCD activity was increased 2–3-fold in the electrically stimulated muscle (0.37 ± 0.09 versus 0.97 ± 0.25 nmol/min/mg of protein, n = 6), an increase in activity similar to that obtained previously when a spectrophotometric assay was used (see Ref. 6 and “Discussion”).

**DISCUSSION**

The principal findings of this study are as follows. 1) After exercise, malonyl-CoA decarboxylase participates with acetyl-CoA carboxylase in regulating the concentration of malonyl-CoA in liver and adipose tissue, as well as in muscle. 2) In all three tissues, the activities of ACC and MCD are coordinately regulated by AMPK. 3) GPAT activity is diminished in liver and adipose tissue after exercise, and this too appears to be regulated by AMPK. 4) The net effect of these changes should be to increase the oxidation of fatty acids and to diminish their esterification.

Previous studies have shown that the concentration of malonyl-CoA is decreased in skeletal muscle after exercise as a result of a decrease in ACC activity (17, 18). They have also suggested that the latter results from activation of AMPK, which phosphorylates and inhibits ACC. The results of the present study indicate that voluntary exercise also increases the activity of MCD and that this too is secondary to a change in AMPK activity. Thus, at the same time that decreases in ACC activity and malonyl-CoA concentration and an increase in AMPK activity were observed in muscle (30 min after a treadmill run), the activity of MCD was increased by 2-fold.

The finding that the increase in MCD activity was reproduced by administration of AICAR in vivo is consistent with this notion, as is the observation that incubation of immunopurified MCD from a sedentary muscle with purified AMPK produced a substantial increase in MCD activity.

Such a coordinate regulation of ACC and MCD attributable to AMPK has also been observed in rat muscle made to contract by electrical stimulation of the sciatic nerve in vivo and following incubation of the rat extension digitorum longus muscle with the AMPK activator AICAR (6). As with MCD isolated from these muscles, treatment of immunopurified enzyme from an exercised muscle with PP2A reversed the increase in MCD activity.

Interestingly, increases in MCD and AMPK activity and decreases in ACC activity and malonyl-CoA concentration were also observed in liver and adipose tissue at 30 min after exercise. That AMPK activity was increased in liver and fat at 30 min after exercise was surprising since one would not expect changes in ATP and AMP levels at this time in these tissues, if they occurred at all. Decreases in ACC activity and malonyl-CoA concentration associated with an increase in AMPK have been observed by Carlson and Winder (21) in rat liver immediately after the completion of a 20-min treadmill run; however, they did not observe a similar change after 2 h of intense exercise (21). MCD was not measured nor were studies carried out well after the termination of the exercise as was done here. Another possibility is that these changes in liver and adipose tissue, and possibly also in muscle, are mediated by increases in catecholamines, which have long been known to occur during exercise (22). In this context, Minokoshi et al. (23) have recently demonstrated that a leptin infusion in vivo causes an increase in AMPK activity in mouse skeletal muscle that is inhibited by the α-adrenergic antagonist phenolamine. In addition, Moule and Denton (24) have shown that incubation with isoproterenol causes an increase in AMPK in incubated adipocytes, and we have found a similar effect of isoproterenol in skeletal muscle.2 Whatever the precise mechanism, the findings raise the interesting possibility that exercise via its action on AMPK could also exert long term effects on metabolism, signal transduction, and gene expression in adipose tissue and liver as well as muscle (19, 25, 26). In this context, AMPK has been implicated in the regulation of the expression of various genes including those encoding gluconeogenic enzymes in liver (27) and the GLUT-4 glucose transporter in muscle (28).

A novel finding in this study was that prior exercise also

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1. V. Kaushik, A. Saha, and N. Ruderman, unpublished data.

2. V. Kaushik, A. Saha, and N. Ruderman, unpublished data.
resulted in a significant decrease in mitochondrial-associated GPAT activity in liver and adipose tissue. An earlier study by Muoio et al. (16) showing inhibition of hepatic GPAT activity by AMPK is consistent with this finding. Interestingly, GPAT activity in muscle was not diminished after exercise. Whether this was a technical problem related to the fact that its activity was much lower than that in either liver or adipose tissue remains to be determined. A very low activity of GPAT in muscle was also found by Muoio et al. (16), who suggested that this accounted for their inability to demonstrate an effect of AICAR on GPAT activity in this tissue although it substantially inhibited triglyceride synthesis.

Overall the results suggest that MCD, ACC, and GPAT are coordinately regulated by AMPK in liver, adipose tissue, and possibly in muscle after exercise (Fig. 7). The net effect of these events would be to enhance fatty acid oxidation and diminish its esterification. It has long been known that both fatty acid oxidation and esterification are altered in muscle during moderate intensity exercise (29) and that fatty acid oxidation in muscle is increased after exercise, when the muscle cell uses glucose predominantly to replete its glycogen stores. AMPK activation would presumably enhance glycogen repletion after exercise since it also increases glycogen synthesis (30, 31). The precise effects of AMPK activation in liver and adipose tissue after exercise are less clear, although one would predict that it increases fatty acid oxidation and inhibits triglyceride synthesis in both tissues (29, 32). In adipose tissue, the latter effect would make more fatty acid, derived from lipolysis, available for release into the circulation, where it would provide for the fuel needs of muscle and liver.

In an earlier study, we found that contractions induced by electrical stimulation of the sciatic nerve caused a 2–3-fold increase in MCD activity in rat gastrocnemius muscle (6). Recently Habinowski et al. (7) carried out a similar study in which they were unable to reproduce this finding. They attributed this to the fact that they utilized a radiometric assay for MCD that was more specific than the spectrophotometric assay used by us (5, 33). As reported here, we also observed 2–3-fold increases in MCD activity following electrical stimulation of the sciatic nerve when the radiometric method was used to assay MCD. The reason for the different results in the two studies is unclear, but it could reflect the fact that we assayed MCD in a 500 × g cell supernatant, whereas Habinowski et al. (7) used a whole homogenate that may contain factor(s) interfering with the assay. In keeping with this notion, Goodwin and Taegtmeyer (33), who showed increased MCD activity in hypoxic myocardium, and Dycket et al. (5), who observed changes in MCD activity in liver during starvation and refeeding, both utilized tissue supernatant fractions for their assays.

In conclusion, MCD, ACC, and GPAT appear to be coordinately regulated by AMPK in liver, epididymal fat, and possibly in muscle. Such changes would both increase the oxidation of long-chain fatty acids and decrease their use for the synthesis of triglycerides and other glycerolipids. Still to be determined are the factors responsible for the increase in AMPK activity in the three tissues and whether the prolonged increase in AMPK activity following exercise leads to changes in the expression of genes encoding key enzymes of lipid partitioning and other proteins.

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REFERENCES