Exercise Diminishes the Activity of Acetyl-CoA Carboxylase in Human Muscle

David Dean, Jens R. Daugaard, Martin E. Young, Asish Saha, Demetrios Vavvas, Sven Asp, Bente Kiens, Ki-Han Kim, Lee Witters, Erik A. Richter, and Neil Ruderman

Studies in rats suggest that increases in fatty acid oxidation in skeletal muscle during exercise are related to the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC), and secondary to this, a decrease in the concentration of malonyl-CoA. Studies in human muscle have not revealed a consistent decrease in the concentration of malonyl-CoA during exercise; however, measurements of ACC activity have not been reported. Thus, whether the same mechanism operates in human muscle in response to physical activity remains uncertain. To investigate this question, ACC was immunoprecipitated from muscle of human volunteers and its activity assayed in the same individual at rest and after one-legged knee-extensor exercise at 60, 85, and 100% of knee extensor V02max. ACC activity was diminished by 50–75% during exercise with the magnitude of the decrease generally paralleling exercise intensity. Treatment of the immunoprecipitated enzyme with protein phosphatase 2A restored activity to resting values, suggesting the decrease in activity was due to phosphorylation. The measurement of malonyl-CoA in the muscles revealed that its concentration is 1/10 of that in rats, and that it is diminished (12–17%) during the higher-intensity exercises. The respiratory exchange ratio increased with increasing exercise intensity from 0.84 ± 0.02 at 60% to 0.99 ± 0.04 at 100% V02max. Calculated rates of whole-body fatty acid oxidation were 121 mg/min at rest and 258 ± 35, 264 ± 63, and 174 ± 76 mg/min at 60, 85, and 100% V02max, respectively. The results show that ACC activity, and to a lesser extent malonyl-CoA concentration, in human skeletal muscle decrease during exercise. Although these changes may contribute to the increases in fat oxidation from rest to exercise, they do not appear to explain the shift from mixed fuel to predominantly carbohydrate utilization when exercise intensity is increased. Diabetes 199:1295–1300, 2000

Physical activity is associated with substantial increases in both fatty acid and carbohydrate oxidation in skeletal muscle, with the relative use of the 2 fuels varying with exercise intensity (1). Thus, in overnight-fasted humans, during low-intensity exercise (30–40% V02max), fatty acids are the principal oxidative substrate, whereas during somewhat more intense exercise (60–70% V02max), the absolute rates of both fatty acid and carbohydrate oxidation are higher, but the oxidation of fatty acids relative to carbohydrate is decreased. Furthermore, during very intense (>90% V02max) versus moderate-intensity exercise, carbohydrate oxidation is still further increased, and even the rate of fatty acid oxidation may be diminished. Studies in both rats (2–6) and humans (7,8) indicate that the rate of carbohydrate oxidation in muscle is elevated during exercise by a coordinated series of events that lead to increases in glucose transport, glycogenolysis, glycolysis, and pyruvate dehydrogenase activity. In contrast, the mechanism by which fatty acid oxidation is increased is less clear. In rats, a reasonably compelling body of evidence suggests that it involves activation of the α-2 isoform of AMP-activated protein kinase (AMPK) (9,10), which phosphorylates and inhibits the muscle isoform of acetyl-CoA carboxylase (ACC)-β leading to a decrease in the concentration of malonyl-CoA. Malonyl-CoA is an inhibitor of carnitine palmitoyltransferase 1 (CPT1), the enzyme that controls the rate of long-chain fatty acyl-CoA transfer into mitochondria. For this reason, a decrease in its concentration should increase fatty acid oxidation, and a close correlation between the change in malonyl-CoA concentration and the rate of fatty-acid oxidation in resting rat skeletal muscle has been described (11–14). Whether changes in ACC activity decrease the concentration of malonyl-CoA leading to an increase in fatty acid oxidation in human muscle during exercise is less clear. The work from Spriets laboratory (15,16) generally failed to observe decreases in malonyl-CoA in human muscle during exercise, even when rates of fatty-acid oxidation were clearly increased. In addition, measurements of ACC-β, whose activity can be altered to a much greater extent than is the concentration of malonyl-CoA during exercise (contraction) in rats (10,17), have not been reported in human muscle.

With these considerations in mind, a method was developed for studying ACC-β in human muscle after its immunoprecipitation. The present report describes the changes in ACC-β activity and phosphorylation in normal male volunteers after exercise at various intensities and the relationship...
RESEARCH DESIGN AND METHODS

Human subjects and exercise protocol. Thirteen normal male volunteers aged 24 ± 1 years, with a mean weight of 76.3 ± 4.5 kg and BMI 23.3 ± 1.0 kg/m², were studied. None of them was on medication. After an overnight fast, subjects exercised on a one-leg dynamic knee-extensor apparatus (18). During the exercise, they sat in an upright position with an ankle cuff attached to a rod that pedals a cycle ergometer on one leg. Expired air was collected in Douglas bags and analysed using an infrared CO₂ analyzer (Beckman LB-2) and a paramagnetic O₂ analyzer (Servomex, UK). The standard exercise protocol is depicted in Fig. 1. VO₂max was determined at least 2 days before the actual experiment, as described by Andersen and Saltin (18). The subjects exercised for 45 min at 60% VO₂max, 10 min at 85% VO₂max, and until exhaustion (100% VO₂max) with 10-min rest periods after each period of exercise. Muscle biopsies were taken from the thigh muscle immediately after each exercise period with a Bergstrom needle and immediately frozen in liquid N₂ within the needle. For the protein phosphatase 2A (PP2A) studies, the other 4 subjects underwent 100% VO₂max, and until exhaustion (100% VO₂max) with 10-min rest periods after each period of exercise. Muscle biopsies were taken from the thigh muscle immediately after each exercise period with a Bergstrom needle and immediately frozen in liquid N₂ within the needle. For the protein phosphatase 2A (PP2A) studies, the other 4 subjects underwent 100% VO₂max, and until exhaustion (100% VO₂max) with 10-min rest periods after each period of exercise. Muscle biopsies were taken from the thigh muscle immediately after each exercise period with a Bergstrom needle and immediately frozen in liquid N₂ within the needle. For the protein phosphatase 2A (PP2A) studies, the other 4 subjects underwent 100% VO₂max, and until exhaustion (100% VO₂max) with 10-min rest periods after each period of exercise. Muscle biopsies were taken from the thigh muscle immediately after each exercise period with a Bergstrom needle and immediately frozen in liquid N₂ within the needle. For the protein phosphatase 2A (PP2A) studies, the other 4 subjects underwent 100% VO₂max, and until exhaustion (100% VO₂max) with 10-min rest periods after each period of exercise.

Preparation of muscle for ACC studies. The tissue processing was as described previously for rat muscle (10) with minor modifications. In brief, 30–50 mg frozen muscle was homogenized in 10× (vol/wt) of an ice-cold medium (buffer A) containing 30 mmol/l HEPES, pH 7.4, 2.5 mmol/l EGTA, 3 mmol/l EDTA, 32% glycerol, 20 mmol/l KCl, 40 mmol/l NaF, 4 mmol/l NaPPi, 1 mmol/l Na3VO4, 0.1% (g/gal) Ca-630, 40 mmol/l β-glycerophosphate, 5 mmol/l aprotonin, 5 mmol/l leupeptin, 5 µmol/l pepstatin, 1 mmol/l dithiothreitol (DTT), and 2 mmol/l phenylmethylsulfonyl fluoride (PMSF) using Kontes glass-on-glass homogenization tubes. Homogenates were centrifuged at 15,000g for 12 min to remove debris and the resultant supernatants were diluted in an equal volume of a medium (buffer B) containing 30 mmol/l HEPES, pH 7.4, 2.5 mmol/l EGTA, 3 mmol/l EDTA, 30 mmol/l KCl, 20 mmol/l β-glycerophosphate, 20 mmol/l NaF, 2 mol/l NaPPi, 1 mmol/l Na3VO4, 0.1% (g/gal) Ca-630, 5 mmol/l aprotonin, 5 µmol/l leupeptin, 5 µmol/l pepstatin, 1 mmol/l DTT, and 2 mmol/l PMSF after centrifugation at 15,000g for 12 min. The protein concentration in each supernatant was determined by the Bio-Rad detergent compatible protein assay. The supernatants were then diluted to equivalent protein concentrations and a final glycerol concentration of 1% with a 1:1 mixture of buffers A and B. The resultant supernatants were either aspirated or saved for additional analyses. The beads were washed 3 times with buffer B, twice with the ACC reaction buffer (70 mmol/l Tris-Acetate, pH = 7.5), and ACC activity was determined by the 14CO₂ fixation method (10,22). Because of the limited amount of tissue, immunoprecipitated enzyme was initially assayed for ACC activity at 0 mmol/l citrate and then, after rewashing with buffer B and the ACC reaction buffer, at higher citrate concentrations. Studies done using the muscle obtained from the open biopsy established that rewashing the immunopellets in this way did not result in a measurable change in ACC activity (results not shown).

For experiments in which the effect of PP2A was determined, immunoprecipitated enzymes from sedentary and 100% VO₂max samples were incubated for 2.5 h with PP2A (500 µM/ml; Upstate Biotechnology) and washed with buffer B and the ACC reaction buffer before the assay of ACC activity. Immunoprecipitates, treated in an identical manner but without PP2A added, served as controls for these experiments. For Western blotting, samples denatured with Laemmli buffer (23) were subjected to SDS-PAGE and biotin-containing proteins were identified with streptavidin-horseradish peroxidase (Amersham 1:5,000) (10). Determination of malonyl-CoA. Malonyl-CoA was measured radioenzymatically in perchloric acid filtrates of the whole-muscle homogenate by the method of McGarry et al. (24) as modified by Saha et al. (25). Statistics. A paired 1-way analysis of variance was used to assess if exercise led to differences between groups. Dunnett’s posthoc test was used to determine the level of significance between each of the various exercise intensities and the sedentary group. In studies in which PP2A was used, a paired Student’s t test was performed.

RESULTS

Presence of carboxylases in human muscle. A streptavidin blot of a 15,000g supernatant revealed 3–4 bands, depending on the duration of exposure of the blot to the film (Fig. 2). Based on their estimated molecular weight and comparison with rat muscle (10), the bands were identified as ACC, pyruvate carboxylase (PC), methyl-CoA carboxylase (MCC), pyruvate carboxylase (PCC), and pyruvate carboxylase (PCC). These bands were also present in human muscle homogenates (Fig. 2). The presence of these bands was confirmed by immunoblotting with antibodies specific for ACC, PC, MCC, and PCC. The identity of these bands was further confirmed by immunoprecipitation with antibodies specific for ACC, PC, MCC, and PCC.

Fig. 1. Exercise protocol. Muscle biopsy was taken prior to exercise (SED). Subjects then exercised (see Research Design and Methods) for 45 min at 60% VO₂max, then 85% VO₂max for 10 min, and finally at 100% VO₂max until exhaustion. Muscle samples were obtained during 10-min rest periods and at the end of the study as indicated by the arrow and box.

Fig. 2. Streptavidin blot of muscle supernatants at various levels of exercise. Proteins in the supernatant from 1 subject were separated on a 7% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and probed with streptavidin-horseradish peroxidase. Bands were visualized using enhanced chemiluminescence (ECL) and exposed to film for detection. Similar results were obtained when muscle supernatants from 2 other subjects were studied. A, sedentary; B, 60% VO₂max; C, 85% VO₂max; D, 100% VO₂max. The identification of carboxylases on the membrane is based on molecular weight.
crotonyl carboxylase (MCC), and propionyl-CoA carboxylase (PCC). Their relative abundance was MCC = PCC > ACC > PC. Importantly, although no attempt was made to remove any gross fat from these biopsies to immediately freeze and maintain the phosphorylation status, the lack of any detectable human ACC-H9251 on any of the blots strongly suggests that any contamination from adipose tissue was negligible and likely does not confound the present results. As shown in Fig. 2, after exercise of different intensities, neither the abundance of ACC nor the other biotin-containing enzymes were altered.

Selective immunoprecipitation of ACC-H9252 and measurement of its activity. A polyclonal antibody against human ACC-H9252 selectively and quantitatively immunoprecipitated ACC with other carboxylases remaining in the supernatant (Fig. 3). As shown in Fig. 4, the immunoprecipitated ACC was active and its activity was increased by citrate. In a sample of muscle obtained from a surgically amputated limb of an anesthetized patient during surgery (RESEARCH DESIGN AND METHODS), the V$_{\text{max}}$ of ACC was $\sim$145 pmol · min$^{-1}$ · mg$^{-1}$ supernatant protein and its A$_{0.5}$ for citrate was 0.3 mmol/l.

Effect of exercise on ACC activity. ACC activity decreased by 50–75% during exercise. The effect was observed at all citrate concentrations and tended to be greater with increasing exercise intensity (Fig. 5).

Effect of phosphatase treatment. To assess whether the decrease in ACC activity caused by physical activity was related to phosphorylation, ACC-H9252 immunoprecipitated from the muscle of 4 different subjects, studied at rest and after exercise at 100% V$_{\text{O2max}}$, was incubated for 2.5 h with PP2A. Such treatment increased ACC-H9252 activity in postexercise muscle by 2- to 3-fold and eliminated the difference between control and exercised muscle (Fig. 6). In contrast, PP2A had no effect on ACC-H9252 activity in control muscles at 0.2 or 1.0 mmol/l. The absolute values of ACC activity measured at 0.2 and 1 mmol/l citrate (Fig. 6) were somewhat

FIG. 3. The ACC-β-specific polyclonal antibody precipitates ACC-β that is free of contaminating carboxylases. Extracts of human muscle were incubated with the Ab and Protein A/G-agarose beads. Proteins in the pre-immunoprecipitation (Pre-IP) and post-immunoprecipitation (Post-IP) extracts and immunopellets (IP) were separated using 7% SDS-PAGE, transferred to polyvinylidine difluoride membranes, and probed with streptavidin-horseradish peroxidase. The identification of carboxylases on the membrane is based on molecular weight. See RESEARCH DESIGN AND METHODS and Figure 2 legend for details.

FIG. 4. The human ACC-β antibody allows for ACC activity determination. The citrate activation curve was generated using a sample of muscle obtained by an open biopsy during surgery on one individual (RESEARCH DESIGN AND METHODS). Each point represents an individual immunoprecipitation.

FIG. 5. Exercise inhibits human skeletal muscle ACC activity at various citrate concentrations. Immunoprecipitated ACC activity was initially assayed at 0 mmol/l citrate. Thereafter, the immunopellet was washed with buffer B and ACC reaction buffer as described in RESEARCH DESIGN AND METHODS and reassayed at 1.0 mmol/l citrate and 10 mmol/l citrate. Results are means ± SE for n = 5. *P < 0.05, **P < 0.01 vs. sedentary values.
EXERCISE DIMINISHES HUMAN MUSCLE ACC ACTIVITY

The results indicate that the activity of ACC-β diminishes in human muscle during exercise and that this decrease is likely to be related to an increase in its phosphorylation. Previous studies in rats have demonstrated that 30–50% decreases in malonyl-CoA are accompanied by similar or greater decreases in ACC-β activity during both voluntary exercise (17) and contractions induced by electrical stimulation of the sciatic nerve.

CONCENTRATION OF MALONYL-COA AND RESPIRATORY EXCHANGE RATIO AT REST AND AFTER EXERCISE OF DIFFERENT INTENSITIES.

Table 1

<table>
<thead>
<tr>
<th>Intensity</th>
<th>RER</th>
<th>V̇O₂ (l/min)</th>
<th>Malonyl-CoA (nmol/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>0.77 ± 0.01 (5)</td>
<td>0.28 ± 0.01 (5)</td>
<td>0.204 ± 0.015 (5)</td>
</tr>
<tr>
<td>60% V̇O₂max</td>
<td>0.84 ± 0.02 (9)</td>
<td>0.98 ± 0.09 (9)*</td>
<td>0.206 ± 0.009 (9)</td>
</tr>
<tr>
<td>85% V̇O₂max</td>
<td>0.88 ± 0.03 (9)*</td>
<td>1.26 ± 0.07 (9)*</td>
<td>0.179 ± 0.013 (9)*</td>
</tr>
<tr>
<td>100% V̇O₂max</td>
<td>0.99 ± 0.04 (7)*</td>
<td>2.09 ± 0.21 (7)*</td>
<td>0.169 ± 0.011 (9)*</td>
</tr>
</tbody>
</table>

Data are means ± SE (n). Sedentary RER and V̇O₂ values are taken from an earlier study (Richter et al. [20]). *P < 0.01 vs. sedentary values; †P < 0.05 vs. sedentary values.
greater than the concentration at which CPT1 is inhibited. While whole-tissue concentrations of malonyl-CoA are much less than the concentration at which CPT1 is inhibited, they are still relatively high. Because the subjects in the latter study had eaten before exercise, increases in insulin and glucose (24,26) could have contributed to a decrease in malonyl-CoA caused by physical activity. For this reason, we examined the effects of exercise in overnight-fasted subjects. Small but significant decreases in malonyl-CoA (12–17%) were observed after knee-extensor exercise at 85 and 100% \( V_{\text{O2max}} \). However, the changes were substantially smaller than those observed in rat muscle and no change was observed after exercise at 60% \( V_{\text{O2max}} \), when fatty acid oxidation was near its maximum. This led us to examine the effects of exercise on the activity of ACC-\( \beta \), which is more dramatically diminished by contraction in rat muscle than is the concentration of malonyl-CoA (10,17).

ACC-\( \beta \) activity in muscle was measured after immunopurification of the ACC-\( \beta \) isoform. Antibodies previously used to immunoprecipitate active ACC from rat muscle (10) did not bring down human ACC-\( \beta \) levels (data not shown); however, a polyclonal antibody against human ACC-\( \beta \) produced by Ha et al. (21) quantitatively immunoprecipitated ACC-\( \beta \) that was both active and citrate-dependent. Assays of the immunoprecipitated enzyme revealed that its activity diminished by 50–75% during exercise and that the magnitude of the decrease generally paralleled the intensity of the physical activity. In keeping with earlier findings in rats (10), these decreases in activity were reversed by treatment with PP2A (Fig. 6) and they were not associated with a decrease in ACC-\( \beta \) abundance (Fig. 2), suggesting that they were due to phosphorylation. ACC is a known target for AMPK (9). Thus, the current data suggest that AMPK was activated during muscle contractions. However, we were unable to measure the \( \alpha \)-2 AMPK activity in our human muscle lysates using an antibody against the rat muscle \( \alpha \)-2 AMPK. Using a different antibody, such an increase in \( \alpha \)-2 AMPK activity has, in fact, recently been reported (N. Fujii, T. Hayashi, M.F. Hirshman, J. Mu, S.A. Habinowski, L.A. Witters, M.J. Birnbaum, A. Thorell, L.J. Goodyear, unpublished data). Thus, the AMPK-ACC system appears to be operative in human muscle during exercise.

In spite of the 50–75% decrease in ACC activity in human muscle during exercise, only a small (12–17%) decrease in malonyl-CoA concentration, if any, was observed. This could be related to the fact that the concentration of malonyl-CoA in human muscle is 1/10 of that in rats, which may be at the limit of sensitivity for our assay. Alternatively, measurement of malonyl-CoA concentration in whole muscle may be a less sensitive indicator of its concentration in the cytosol (i.e., the compartment in which it would come in contact with CPT1) than in rats. In support of this possibility, a recent study has demonstrated that a 19% increase in the whole-muscle concentration of malonyl-CoA in normal human volunteers during a euglycemic-hyperinsulinemic clamp is associated with a substantial decrease in whole-body and presumably muscle fatty acid oxidation (27). In contrast, during a similar clamp in rats, increases of malonyl-CoA concentration in excess of 100% have been reported (26).

In both humans (0.1–0.4 nmol/l) and rats (1–4 µmol/l), whole-tissue concentrations of malonyl-CoA are much greater than the concentration at which CPT1 is inhibited (K\(_i\) = 0.024–0.04 µmol/l). Despite this, in whole-tissue malonyl-CoA correlate closely with changes in fatty acid oxidation in vitro (11,12) and in vivo in resting muscle (13) in rats. This finding has led to the suggestion that either a binding protein alters the effective concentration of malonyl-CoA in the vicinity of CPT1 or that malonyl-CoA is compartmented with the muscle cell (13,28,29).

Despite the relatively strong evidence that changes in ACC activity and malonyl-CoA concentration regulate fatty acid oxidation in resting muscle, a critical question is whether they do so during exercise. In fact, the evidence is inconclusive. Thus, in going from rest to 60% one-legged \( V_{\text{O2max}} \), in the present study, an ~50% reduction in ACC activity was accompanied by more than a 2-fold increase in the rate of fatty acid oxidation, but no detectable change in malonyl-CoA concentration was observed. With more intense exercise, malonyl-CoA concentration was modestly decreased and ACC activity tended to be further diminished; however, rates of fatty acid oxidation were unchanged, and at the highest intensity of exercise, they were diminished. At higher intensities, however, the acute rise in blood lactate can contribute to a bicarbonate shift, increasing the amount of expired CO\(_2\) independent of altered fuel utilization. This artifact will raise the measured RER, thus the true rate of fatty acid oxidation during intense exercise is likely to be greater than that calculated using RER. However, given that during high-intensity exercise malonyl-CoA concentration and ACC activity are at their nadir, it seems likely that factors other than changes in ACC activity and malonyl-CoA concentration must be involved for the shift in fuel utilization during intense exercise. These factors could include the following: 1) increased activation of glucose transport (30,31) and key enzymes of glycolysis (31), glycogenolysis (31,32), and glucose oxidation (e.g., pyruvate dehydrogenase) (32,33); 2) a reduction in the concentrations of carnitine, CoASH, or palmitoyl-CoA (33,34) and substrates that are necessary for fatty acid oxidation; and 3) inhibition of 3-ketoacyl-CoA thiolase, an enzyme involved in the \( \beta \)-oxidation of fatty acids (34). Another factor could be the shift from the use of type 1 to type 2b muscle fibers, which are more glycolytic and have a lower capacity for fatty acid oxidation during very intense exercise.

In conclusion, the results indicate that ACC activity is diminished because of phosphorylation in human muscle during exercise at all levels of intensity. This should lead to decreases in the concentration of malonyl-CoA that in turn diminish CPT1 and enhance fatty acid oxidation. However, the only detectable reductions in whole-muscle malonyl-CoA concentration occurred at the 2 highest exercise intensities, at which the rates of fatty acid oxidation were either similar or somewhat lower than during exercise of more modest intensity. Whether this indicates that whole-muscle concentration of malonyl-CoA in humans is not a sensitive indicator of its cytosolic concentration remains to be determined.

**ACKNOWLEDGMENTS**

This study was supported in part by USPHS Grants DK49147, DK19514, and T32DK07201, and an American Diabetes Mentor-based fellowship (N.R.), National Institutes of Health Grant DK35712 (L.W.), and the Danish National Research Foundation Grant 504-14 (E.A.R., B.K.). D.D. is the recipient of a post-doctoral fellowship from the Juvenile Diabetes Foundation International. J.R.D. is a recipient of a post-doctoral fellowship from the Carlsberg Foundation.
The authors thank Dr. Keith Tornheim for his advice and constructive criticism.

REFERENCES