Effect of acute exercise on citrate synthase activity in untrained and trained human skeletal muscle

BRYAN T. LEEK, SUNDAR R. D. MUDALIAR, ROBERT HENRY, ODILE MATHIEU-COSTELLO, and RUSSELL S. RICHARDSON

Department of Medicine, University of California San Diego, La Jolla, California 92093–0623

Received 29 March 2000; accepted in final form 26 September 2000


Maximal citrate synthase activity (CS) is routinely used as a marker of aerobic capacity and mitochondrial density in skeletal muscle. However, reported CS has been notoriously variable, even with similar experimental protocols and sampling from the same muscles. Exercise training has resulted in increases in CS ranging from 0 to 100%. Previously, it has been reported that acute exercise may significantly affect CS. To investigate the hypothesis that the large variation in CS that occurs with training is influenced by alterations during the exercise itself, we studied CS in human vastus lateralis both in the rested and acutely exercised state while trained and untrained (n = 6). Tissues obtained from four biopsies (untrained rested, untrained acutely exercised, trained rested, and trained acutely exercised) were analyzed spectrophotometrically for maximal CS. Exercise training measured in a rested state resulted in an 18.2% increase in CS (12.3 ± 0.3 to 14.5 ± 0.3 μmol·min⁻¹·g tissue⁻¹, P < 0.05). However, even greater increases were recorded 1 h after acute exercise: 49.4% in the untrained state (12.3 ± 0.3 to 18.3 ± 0.5 μmol·min⁻¹·g tissue⁻¹, P < 0.05) and 50.8% in the trained state (14.5 ± 0.3 to 21.8 ± 0.4 μmol·min⁻¹·g tissue⁻¹, P < 0.05). Ultrastructural analysis, by electron microscopy, supported an effect of acute exercise with the finding of numerous swollen mitochondria 1 h after exercise that may result in greater access to the CS itself in the CS assay. In conclusion, although unexplained, the increased CS with acute exercise can clearly confound training responses and artificially elevate CS values. Therefore, the timing of muscle sampling relative to the last exercise session is critical when measuring CS and offers an explanation for the large variation in CS previously reported.

Methods

Subjects

Six sedentary males [(means ± SE) 80.2 ± 2.7 kg, 26.67 ± 0.7 yr, height 175.3 ± 1.2 cm] volunteered to participate in this study after health histories and physical examinations were completed and informed consent was obtained according to the University of California, San Diego Human Subjects Committee requirements. None of the subjects had performed endurance exercise on a regular basis before the study and, as indicated by the mean screening peak oxygen

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consumption (Vo2) measured during conventional cycle ergometer exercise (31.8 ± 3.4 ml·kg⁻¹·min⁻¹), they were appropriately classified as sedentary. Maximum heart rate achieved during this screening cycle test was 193 ± 7 beats/min.

**Exercise Apparatus**

The knee-extensor ergometer used in training and testing was designed to isolate the quadriceps muscle of the left leg (21, 22). Dynamic exercise of the quadriceps muscle was maintained at 60 contractions/min.

**Acute Exercise**

Initially, subjects underwent an acute knee-extensor exercise bout with their left leg that consisted of a 5-min warm-up with negligible resistance followed by a graded maximal-exercise test (to determine a maximum work rate) followed by 30 min of knee-extensor exercise at 50% of the maximum work rate. Although this exercise was designed to be physically taxing, local muscle fatigue was reported as 5–7 (on a scale of 0–10 for rate of perceived exertion) and was not exhaustive. The graded exercise test required subjects to maintain each work rate at 60 rpm for 60 s, and each work rate was incremented by 5 W starting from 0.

**Exercise Training Program**

On the basis of the initial maximum power output during knee-extensor exercise, an individualized 8-wk training regime was designed using the knee-extensor ergometer. Subjects trained under supervision, for 1 h, 3 times/wk for a total of 8 wk. Every sixth training session, a reassessment of maximum power output was performed that determined the training intensity for the following 2 wk. Training involved both low-intensity/long-duration and repeated high-intensity/short-duration sessions.

**Muscle Biopsies**

Approximately 1 h (50–70 min) after the completion of this knee-extensor exercise, muscle biopsies of the vastus lateralis were taken using a 5-mm diameter biopsy needle (Bergstrom) attached to sterile tubing and a syringe to apply a negative pressure and assist in the muscle sample collection. At the same time, a pretrained biopsy was taken from the rested right leg. All biopsies were taken at an approximate depth of 3.5 cm, 15 cm proximal to the knee and slightly distal to the ventral midline of the muscle. Lidocaine (2%) was used as local anesthetic and was infiltrated beyond the depth of the biopsy several minutes before the procedure. The muscle samples from each biopsy were either immediately frozen in liquid nitrogen and stored at −80°C for future CS analysis or immersion fixed in glutaraldehyde solution in 0.1 M cacodylate buffer (total osmolarity 1,100 mosM) for morphometric analysis. At the conclusion of 8 wk of left leg knee-extensor training, two more biopsies were collected following an identical protocol: a trained rested biopsy was taken from the left leg after 72 h of rest; on a separate occasion, a trained and acutely exercised muscle biopsy sample was taken from the left leg on average 1 h (50–70 min) after the acute exercise stimulus described above. In summary, muscle biopsies were collected in four different states: 1) untrained rested, 2) untrained acutely exercised, 3) trained rested, and 4) trained acutely exercised.

**CS Activity**

The CS was determined spectrophotometrically at 30°C in a Milton Roy spectrophotometer (model 21D) by the standard and unmodified method of Srere (27). Briefly, muscle samples were diluted by a factor of 20 in a solution of 100 mM KPO4 + 5 mM EDTA and 5 mM EGTA (pH 7.4) and homogenized while removed of connective tissue using a Brinkman homogenizer (model PCU-11, Kinematica) and kept on ice. After initial homogenization, the homogenate was vortexed for 20 s to eliminate any foam that had formed during homogenization. The homogenate was sonicated for 1 min to further disrupt the mitochondrial membrane using a 4710 series Ultrasonics homogenizer (Cole-Parmer Instrument, Chicago, IL), again on ice. After sonic oscillation, the homogenate was frozen at −20°C, allowing ice crystals to form and penetrate any intact membrane. The above procedure was repeated after the initial freezing in a similar fashion. The homogenates were homogenized for 80 s, vortexed for 20 s, and then sonicated for 1 min. The homogenates were frozen for a second time at −20°C. After the second freezing, the samples were thawed and diluted further by a factor of 20 using the solution of 100 mM KPO4 + 5 mM EDTA and 5 mM EGTA (pH 7.4).

CS was determined spectrophotometrically at 30°C by measuring the appearance of the CoA-SH [acetyl-CoA + oxaloacetate + H2O→citrate + CoA-SH + H+] (side reaction: CoA-SH + DTNB → mercaptide ion). Aliquots (0.100 ml) of the diluted homogenate were used for the assay. In a water bath, maintained at 30°C to increase reaction kinetics, samples were prepared in triplicate by adding the following reagents: 0.650 ml of 100 mM Tris (pH 8.1), used as a buffer, 0.05 ml of 3 mM acetyl-CoA, 0.1 ml of 1 mM DTNB, and 5 mM oxaloacetate. The samples were poured, after waiting −1 min to eliminate false readings, from the side reaction (CoA-SH + DTNB → mercaptide ion) into a 1-ml quartz cuvette and inserted into a Beckman DU 640B spectrophotometer maintained at 30°C. Readings were taken at 412 nm, the ideal wavelength for mercaptide ion absorption, at 20-s intervals for 2–2.5 min to measure acetyl-CoA decarboxylase activity. The repeated (20 s) absorbance values were then plotted against time and determined to be linear (indicating adequate substrate). This absorbance rate is then multiplied by a factor (17,674.1) that accounts for the dilutions employed, converts moles to micromoles and seconds to minutes, and results in a value in micromoles per minute per gram tissue. Within each subject, all samples were analyzed in a random order. The assay was performed in six individual sessions, one subject per session.

**Mitochondrial Density**

After excision, the muscle biopsies were immersed in glutaraldehyde fixative and processed for electron microscopy (EM), as described previously (17). One-micrometer sections of the plastic-embedded samples were cut on an LKB Ultratome III and stained with 0.1% toluidine blue aqueous solution. The angles of sectioning used to provide the transverse sections were determined as described previously (17). Ultrathin sections (50–70 nm) were cut transversely to the muscle fiber axis in each sample and were contrasted with uranyl acetate and bismuth oxinitrate. The electron micrographs for morphometry were taken on 70-mm films with a Zeiss 10 electron microscope, and contact prints of the EM films were projected on a 144-point square grid using a microfilm reader (Documentar DL-2, Jenoptic, Jena, Germany). The volume density of mitochondria per volume of muscle fiber was estimated by standard point counting at a final magnification of ×49,000 (17). All measurements were performed blind, and the order of samples was randomized.
Statistical Analyses

Repeated-measures two-way ANOVA was used with a Tukey’s post hoc (Instat, San Diego, CA). Variables were considered significantly different when the P value was ≤ 0.05.

RESULTS

Functional Response to Exercise Training

All subjects demonstrated an improvement in maximal knee-extensor performance (33%) after the 8 wk of training (64 ± 5 W untrained; 85 ± 6 W trained, P < 0.05).

Coefficient of Variation for the CS Assay

In our hands, the coefficient of variation for the CS assay was 5.5% (same sample repeated measurements), indicating the relatively low variance in the measurement itself and supportive of the authenticity of the much greater changes seen as the result both of acute exercise and training on muscle CS.

CS Response to Exercise Training

The CS increased significantly in response to the exercise training regime when studied in the rested state (Table 1 and Fig. 1).

CS Response to Acute Exercise

CS increased in all six subjects after acute exercise both before and after 8 wk of training. Thus a similar magnitude of CS increase after acute exercise was superimposed on the rested value both in the untrained and trained states (Table 1 and Fig. 2).

Comparisons Between Acute and Chronic Exercise

It is interesting to note that the pretrained acutely exercised muscle had a CS that was 26.4% greater than the posttrained rested state (P ≤ 0.05). Thus muscle CS was significantly greater after 1 h of acute exercise than after 8 wk of training. The comparison of the pretrained rested state and the trained acutely exercised state revealed a 79.3% increase in CS (P ≤ 0.05), a clear overestimation of the effect of chronic training on CS.

EM

In the pretrained rested state, EM analysis determined mitochondrial volume density to be 4.9 ± 0.5% (n = 5), a typical value for untrained humans (Fig. 3A) (6). Exercise-trained mitochondrial volume density appeared to increase to higher values (6–7%), but we are reluctant to report these changes due to the presence of variable numbers of swollen mitochondria in all but a few biopsies of the acutely exercised and trained muscles (Fig. 3B). Such artifacts will certainly confound the interpretation of such mitochondrial volume data that would usually be indicative of a change in volume of normal mitochondria. In this case, it is impossible to determine which portion of the increased volume is due to growth and which is due to mitochondrial distortion. Consequently, further quantitative results are not presented. However, the qualitative assessments of the tissue do offer further insight into the possible reason for the variability in CS after exercise (Fig. 3B). Specifically, as described above, EM analysis revealed typical mitochondrial density and normal ultrastructure in the pretrained rested state. In only one

Table 1. Effect of acute exercise and training on citrate synthase activity

<table>
<thead>
<tr>
<th></th>
<th>Untrained</th>
<th>Trained</th>
<th>%Change</th>
</tr>
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<tbody>
<tr>
<td>Rested muscle</td>
<td>12.3 ± 0.3</td>
<td>14.5 ± 0.3†</td>
<td>18.2</td>
</tr>
<tr>
<td>Exercised muscle</td>
<td>18.3 ± 0.5*</td>
<td>21.8 ± 0.4*</td>
<td>19.3</td>
</tr>
<tr>
<td>Percentage change</td>
<td>49.4</td>
<td>50.8</td>
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Values are means ± SE (in μmol·min⁻¹·g tissue⁻¹). †Significantly elevated from the rested to exercised state P ≤ 0.05. *Significantly elevated from the untrained to trained state P ≤ 0.05. There were no significant interaction effects.

Fig. 1. The effect of acute exercise on muscle citrate synthase (CS) when studied in the untrained (A) and trained state (B). *Significantly increased CS from rested to acutely exercised muscle (P < 0.05).
subject did the mitochondria appear swollen, and this sample was excluded from the untrained group \( (n = 5) \). In the other conditions, variable degrees of mitochondrial swelling and, in some cases, supercondensation were apparent in the majority of biopsies (Fig. 3B). Interestingly, this finding was even more prevalent in the samples that were taken after acute exercise (in both the untrained and trained states), with five of six subjects showing swollen mitochondria in each group under these conditions. This is suggestive of intrinsic mitochondrial changes associated with the acute exercise that may have an influence on the CS assay.

**DISCUSSION**

In agreement with our hypotheses, the major finding of this study is that CS, a routinely used marker of muscle metabolic capacity (26), is significantly elevated by acute exercise in human skeletal muscle. In fact, in both exercise-trained and untrained muscle, a single bout of exercise resulted in an identical 50% increase in CS that overshadowed the elevation in CS observed after 2 mo of exercise training (18%). Both of these CS changes fall within the wide range of exercise-related increases in CS in the literature (2, 9, 28). These data are in agreement with the previous observation of Tonkonogi et al. (30), who reported a 20% increase in CS immediately after acute cycle exercise in humans, but extend these findings by demonstrating that this phenomenon is reproducible, independent of training status, and is still measurable 50–70 min after acute exercise. It is possible that the observed changes are an immediate response to exercise intended to increase oxidative function in human skeletal muscle and thus offers a new perspective on exercise adaptation. These findings also have important ramifications both for data interpretation and experimental design, because the assessment of muscle metabolic adaptation using CS can be confounded if muscle samples are harvested at rest versus 1 h after acute exercise. Additionally, it should be noted that the exercise protocol used to produce this response was...
submaximal and lasted only 30 min. This contrasts with the prolonged exercise protocol employed by Tonkonongi et al. (30) and thus leaves the nature of the exercise stimulus necessary to induce an increase in CS uncertain.

Time Course for CS Changes

The time course of changes in CS as the result of chronic exercise training is not clearly understood. In fact, controversy currently exists as to how rapidly mitochondrial enzymes adaptively increase in human skeletal muscle with short-term cycle exercise training (2, 7, 26). It has been demonstrated that 5–12 days of cycle exercise training induce increased exercise capacity, as seen by increased \( \dot{V}_O_2 \), decreased carbohydrate utilization in favor of increased fat utilization, and lesser increases in P, and lactate levels (2, 7, 26). However, with the use of CS as a marker of exercise training-induced mitochondrial adaptation, both increased and unaltered mitochondrial capacity have been reported (2, 26). Unaltered CS suggests that the increased exercise capacity occurred due to peripheral factors (such as increased \( O_2 \) delivery) that precede the later mitochondrial adaptation (>5–12 days). The present finding of a significant and transient rise in skeletal muscle CS with acute exercise may help to explain the different results across studies in which experimental design dictates muscle sampling after acute exercise.

The present study cannot determine the exact time course of the effect of acute exercise on CS. However, observations and inferences can be made. First, there was a large increase in CS at 50–70 min in acutely exercised muscle compared with rested muscle in both trained and untrained muscle. Second, the trained, rested muscle, sampled 72 h after the last exercise bout, demonstrated an increase in CS similar to other chronic training studies (16). Thus it is probable that the acute exercise effect had somewhat subsided at this time point, suggesting a transient phenomenon. Third, in contrast to the human study of Tonkonogi et al. (30), several previous studies of rat skeletal muscle have reported unaltered or even decreased CS immediately (minutes) after acute exercise. This suggests that in rat muscle, an increase in CS due to acute exercise does not occur during exercise itself or immediately on cessation (3, 13–15, 29). Fourth, Savard et al. (23) reported a 30% increase in CS when rat skeletal muscle was harvested 24 h after acute exercise. In agreement with the time course of the majority of these observations, Neufer and Dohm (18) found a significantly elevated gene transcription for CS 3 h after acute exercise and that it was still present 24 h after exercise. Thus it appears that the major increase in CS may not occur immediately on cessation of exercise. In summary, the present data taken in combination with the available data in the literature (cautionary note: much of it from different animal species) suggest that CS significantly increases 1 h after acute exercise, remains elevated for at least 24 h, and then falls to baseline levels within 72 h.

Mechanisms of Increased CS

From the present data, it is impossible to isolate the exact cause of the increased CS, because exercise induces a wide range of neural, physiological, and hormonal stimuli that may act at a molecular or enzymatic level in either isolation or in combination with each other. Although speculative, potential explanations for the current findings are as follows.

Mitochondrial membrane breakdown. CS is directly related to the extent of mitochondrial membrane disruption achieved in the assay. Hence, the method of homogenization has been documented to influence CS (5). Acute exercise has been associated with damage to mitochondrial membranes, although the validity of this observation and the extent to which this occurs are not universally accepted (Fig. 3) (10, 29). Additionally, Lidocaine, the anesthetic used in this study, independently and synergistically with epinephrine, has been documented to damage skeletal muscle structure (34). Although the Lidocaine used in the present study did not contain epinephrine, it is possible that elevated endogenous epinephrine levels, associated only with the acutely exercised muscle, may have acted synergistically with the Lidocaine and resulted in greater membrane damage in these samples. Such a possibility is supported by previous EM analyses that have demonstrated mitochondrial swelling and ultrastructural damage attributed to lidocaine use (24) as well as the qualitative assessment of the present EM analysis (Fig. 3) that suggests that this effect may be more significant after acute exercise. However, for these effects to be a nontrivial contribution to the increase in CS, this membrane disruption would need to be significant relative to that which occurs in every tissue sample during the analysis (homogenization, sonication, and freezing). Since the completion of the present study, we have performed a follow-up investigation using rat muscle that appears to exonerate lidocaine as the cause of the elevated CS levels (20).

CS concentration and molecular upregulation. Because the CS assay is performed with excess substrates, the method is designed to assess CS concentration [i.e., CS increases with the number of active sites (27)]. As the half-life of CS has been reported to be 7–8 days (1), the 1-h increase in CS appears not to be concentration dependent. As mentioned earlier, direct assessments of the upregulation of CS mRNA in rats (18) also indicate that elevated gene transcription for CS does not occur immediately, but within 3 h after acute exercise, and that it is still present 24 h after exercise. Therefore, it seems unlikely that the increase in CS we found 1 h after an acute exercise bout can be attributed to molecular upregulation. In addition, Dohm et al. (3) found an increase in CS 1 wk after acute exercise without a significant increase in mRNA, which further casts doubt that the currently observed CS elevation was caused by a molecular signal. How-
ever, the possibility exists that there is an alternative CS isofrom that is transcriptionally upregulated in response to acute exercise, as previously documented for the enzyme hexokinase (19).

Vascular-to-intracellular fluid shifts caused by exercise also have the potential to influence CS concentration and the CS assay. However, it is well documented that fluid enters the skeletal muscle with acute exercise, resulting in an intracellular dilution (25) and thus would tend to lower CS. However, the cellular fluid state 1 h after exercise, when these samples were taken, is less clearly understood. Because measurements were made on “wet” muscle in this study, we cannot rule out the effects of fluid movements.

Allosteric modification. Because CS is measured in excess reagents, it is assumed that CS is assessed at maximal reaction velocity, and there is also no dependence of the reaction on the kinetics of substrate availability (27). However, negative allosteric regulators of CS include the reaction product citrate and the two end products of cellular respiration, ATP and NADH (33). Thus a fall in the concentration of these CS inhibitors at the end of exercise could contribute to the observed increase in CS. Although perturbations in the ATP and NADH concentrations 1 h after exercise are unlikely in such a postexercise anabolic environment, excess acetyl-CoA can be transferred out of the mitochondrial matrix into the cytosol in the form of citrate, where it is converted to fatty acids. This reduces the allosteric inhibition of CS by citrate and thus increases CS (33). Evidence of an anabolic environment in skeletal muscle 1 h after exercise has been previously documented by the increase in non-insulin-dependent glucose uptake over the same period (12).

In summary, there was a significant effect of acute exercise on CS that was apparent 1 h after the cessation of exercise both in untrained and trained human skeletal muscle. The 50% increase in CS after acute exercise in untrained and exercise-trained muscle was superimposed on the 18% increase attributed to training. Although the present study cannot identify the mechanism of this increase, the magnitude and reproducibility of this phenomenon independent of muscle training status may help to explain the large variation in CS values reported in the literature. Additionally, these data highlight the importance of timing muscle samples in research that involves both exercise and CS.

We are indebted to the subjects who took part in this research. Dr. Richardson was funded by a fellowship from the Parker B. Francis Fellowship Foundation during this research. This study was concurrently supported by the National Heart, Lung, and Blood Institute Grant HL-17731.

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