Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles

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Siu, Parco M., David A. Donley, Randall W. Bryner, and Stephen E. Alway. Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. J Appl Physiol 94: 555–560, 2003; 10.1152/japplphysiol.00821.2002.—The present study was designed to examine the acute and chronic effects of endurance treadmill training on citrate synthase (CS) gene expression and enzymatic activity in rat skeletal and cardiac muscles. Adult rats were endurance trained for 8 wk on a treadmill. They were killed 1 h (T1, n = 8) or 48 h (T48, n = 8) after their last bout of exercise training. Eight rats were sedentary controls (C) during the training period. CS mRNA levels and enzymatic activities of the soleus and ventricle muscles were determined. Training resulted in higher CS mRNA levels in both the soleus muscles (21% increase in T1; 18% increase in T48, P < 0.05) and ventricle muscles (23% increase in T1; 17% increase in T48, P < 0.05) when compared with the C group. The CS enzyme activities were 42 (P < 0.01) and 25% (P < 0.01) greater in the soleus muscles of T1 and T48 groups, respectively, when compared with that of the C group. Soleus CS enzyme activity was significantly greater in the T1 vs. T48 groups (P < 0.05). However, no appreciable alterations in CS enzyme activities were observed in the ventricle muscles in both training groups. These findings suggest differential responses of skeletal and cardiac muscles in CS enzymatic activity but similar responses in CS gene expression at 1 and 48 h after the last session of endurance training. Moreover, our data support the existence of an acute effect of exercise on the training-induced elevation in CS activity in rat soleus but not ventricle muscles.

physical activity; oxidative enzyme; gene transcriptional expression; soleus muscle; heart muscle

CITRATE SYNTHASE (CS) is one of the key regulatory enzymes in the energy-generating metabolic pathway that catalyzes the condensation of oxaloacetate and acetyl coenzyme A to form citrate in the tricarboxylic acid cycle. It has been extensively used as a metabolic marker in assessing oxidative and respiratory capacity (22). More than 30 years ago, Holloszy and his colleagues (8) reported that endurance exercise training increases the oxidative enzyme activities in skeletal muscle. After that, numerous studies were conducted to examine the effect of exercise training on muscle oxidative capacity and metabolic characteristics (7, 15, 20, 22, 29). It is generally recognized that the skeletal muscle oxidative capacity and the activities of mitochondrial enzymes are elevated by endurance exercise training.

Transient changes in skeletal muscle mitochondrial enzyme activities have been investigated after a single bout of exercise in both animals (5, 9, 21) and humans (14, 27). Data from the human studies suggested that CS activities elevate in response to acute exercise, whereas conflicting findings in the CS activity changes after an acute bout of exercise were reported in the rat studies (5, 9, 12, 13, 26). Unaltered or even decreased CS activities have been reported in skeletal muscles shortly after a single bout of exercise. Several studies have been conducted to determine the influence of exercise training in the mitochondrial enzyme adaptation in cardiac muscles (2, 10, 11, 18, 19). Although the majority of previous studies suggest that the training-induced alteration of mitochondrial enzymes is not noticeable in cardiac muscle (1, 2, 17, 18, 30), a few discrepant results regarding the changes were reported (10). Moreover, among the studies that have been conducted to examine the exercise-induced response of CS in cardiac muscle, very few have provided data regarding the transcriptional, translational, and/or posttranslational processes. Thus, whereas exercise-associated changes in CS have been well studied, the molecular mechanism for CS adaptation to endurance training in cardiac muscle is not well understood.

Adaptive elevation in the mitochondrial protein content by exercise training is suggested to be due to an increase in the rate of protein synthesis rather than a decrease in the rate of protein degradation (3, 24, 25). This raises the possibility that the modulation of mitochondrial enzyme expression in response to exercise training is regulated by pretranslational, translational, and/or posttranslational control mechanisms. In fact, an increase in any particular mRNA concentration may signal an increased synthesis of that particular protein (28). Because the underlying mechanism(s) for the changes in CS activity induced by

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endurance training and acute exercise has not been completely elucidated, the purpose of this study was to examine the acute and chronic responses of CS gene expression and enzymatic activity in rat skeletal and cardiac muscles after 8-wk endurance treadmill training. We hypothesized that the responses of cardiac and skeletal muscles to acute exercise and endurance training as measured at 1 and 48 h after the last training session of the endurance training would have similar CS gene expression and activity.

METHODS

Animals. Male adult (3 mo of age) Sprague-Dawley rats (Harlan, Indianapolis, IN) were studied. Animals were housed in pathogen-free conditions, two per cage at 20–22°C with a reversed 12:12-h light-dark cycle, and fed rat chow and water ad libitum throughout the study period.

Training protocol. Sixteen animals ran on a level motorized rodent treadmill (Columbus Instruments, Columbus, OH) 5 days weekly for a period of 8 wk. During the first 4 wk, the speed of the treadmill and duration of the training sessions were gradually increased from a speed of 10 m/min for 10 min to a running speed of 28 m/min for 55 min by the end of the fourth week. During weeks 5 through 8, a 5-min warm-up session at a speed of 20 m/min was followed by the 55-min training session at a speed of 28 m/min. During the training sessions, mild electrical shock stimulation was applied, if necessary, to maintain the running motivation.

Trained animals were killed either at 1 h (T1, n = 8) or 48 h (T48, n = 8) after the last training session to determine the acute and chronic effects of training, respectively, on CS gene expression and enzymatic activity. Sedentary animals (C, n = 8) were used as control animals and were killed at the same approximate time as T48 animals. The acute effect of treadmill running on trained rats was determined by evaluating CS in the muscles of animals that were killed 1 h after the last exercise session. Comparison between T1 and T48 animals was used to determine the magnitude of the acute effect of exercise. The CS change resulting from acute exercise was not determined in C animals. To distinguish between the acute and the chronic effects of exercise, and to reduce the likelihood that the last bout of exercise influenced CS measures, we measured CS mRNA level and activity 48 h after the last exercise bout in a separate group of rats. Comparisons between T48 and C animals reflected the magnitude of chronic CS responses to 8 wk of endurance training. The C animals were weight matched to the trained animals at the beginning of the study. To avoid any possible confounding effect of external factors, all C animals were handled daily and subjected to the noise of the running treadmill by placing their cages next to the treadmill while the experimental animals trained. All animals were killed via CO2 inhalation followed by decapitation, at which time the soleus muscles and 4–5 mm of the apex cordis from the heart muscle were quickly removed and frozen immediately in liquid nitrogen and stored at −80°C until further analysis. All animal procedures were conducted in accordance with institutional guidelines, and ethical approval was obtained from the Animal Care and Use Committee at the West Virginia University.

RT-PCR analysis of CS mRNA. Total RNA was isolated from rat soleus and cardiac muscles with use of TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1 ml of ice-cold TriReagent. RNA was solubilized in RNase-free H2O and quantitated in duplicate by measuring the absorbance at 260 nm. Two micrograms of RNA were reverse transcribed with decaamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 μl according to standard methods (Invitrogen, Life Technologies, Bethesda, MD). Control RT reactions were done in which the RT enzyme was omitted. The control RT reactions were PCR amplified to ensure that DNA did not contaminate the RNA. One microinch of cDNA was then amplified by PCR using 100 ng of each forward and reverse CS primers, 250 μM deoxyribonucleotide triphosphate, 1× PCR buffer, and 2 units Taq DNA polymerase (Sigma Chemical, St. Louis, MO) in a final volume of 50 μl. PCR were performed by using a programmed thermocycler (Biometra, Göttingen, Germany). A primer pair was designed against CS (forward 5′-CGTGTCCATGGACCTGCGGTC-3′; reverse 5′-CCCCGCGCCACGATGTGCTC-3′). The primers were designed with an annealing temperature at 59.9°C. All PCR products were verified by restriction digestion and by sequencing. Preliminary experiments were conducted with CS primers to ensure that the number of cycles at standards f the PCR optimized the amplification efficiency and within the exponential phase of the PCR amplification for the muscle samples. The cDNA from all muscle samples was amplified simultaneously by using aliquots from the same PCR mixture. After the PCR amplification, 15 μl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Then images were captured and the signals were given in arbitrary units as optical density × band area by use of the Kodak one-dimensional image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed CS mRNA. In the present study, ribosomal 18S were used as internal controls, and all RT-PCR signals were normalized to the 18S signal of the corresponding RT product (Ambion, Austin, TX). This eliminated the measurement error from uneven sample loading and provided a semiquantitative measure of the relative changes in CS gene expression.

CS activity measurement. Ventricle and skeletal muscles (–20 mg) were homogenized on ice in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. CS activity was determined spectrophotometrically according to the method of Sørensen (23). The homogenates were frozen under liquid nitrogen and thawed four times to disrupt the mitochondria to expose the CS. The assay system contained in a total volume of 200 μl: 100 mM Tris buffer (pH 8.35), 5 mM 5,5′-dithiobis(2-nitrobenzoate) (DTNB), 22.5 mM acetyl-CoA, 25 mM oxaloacetate (OAA), and 4 μl of homogenate of muscle. The principle of the assay was to initiate the reaction of acetyl-CoA with OAA and link the release of free CoA-SH to a colorimetric reagent, DTNB (acetyl-CoA + OAA + H2O ⇌ citrate + CoA-SH, then CoA-SH + DTNB ⇌ mercuri-picolinic acid). The rate change in color was monitored at wavelength of 405 nm at 15-s intervals for a period of 3 min by using a Dynex MRX plate reader controlled through personal computer software (Revelation, Dynatech Laboratories). All measurements were performed in duplicate, in the same setting at 20–22°C. The solubilized protein extracts of the homogenates were quantified in duplicate by using bicinchoninic acid reagents (Pierce, Rockford, IL) and bovine serum albumin standards. The CS activity was then normalized to the total protein content and was reported in as nanomoles per milligram protein per minute.

Statistical analyses. Results are presented as means ± SE. One-way ANOVA was used to examine differences across groups. Post hoc differences between the means from experi-
imental groups were determined via Tukey’s tests. Significance was set at $P < 0.05$.

**RESULTS**

*Characterization of animals.* At the start of the study, animals in all groups had similar body weights (210–240 g). All animals assigned to the T1 and T48 groups successfully completed the treadmill training protocol. After the training, the animals were ~5 mo old. Trained animals (both T1 and T48) weighed less than the sedentary control animals at the time of death (T1 and T48 vs. C: 406 ± 9.9 and 386 ± 10.2 vs. 449 ± 12.9 g, $P < 0.05$).

*CS gene expression.* CS mRNA content was analyzed by RT-PCR in soleus and cardiac ventricle muscles of C, T1, and T48 animals. Representative ethidium bromide-stained gels for the CS mRNA are shown in Figs. 1 and 2. The soleus CS mRNA levels were 21 and 18% greater in T1 and T48 animals, respectively, compared with sedentary C animals ($P < 0.05$) (Fig. 1). Similarly, cardiac CS mRNA levels were 23 and 17% higher in muscle isolated from T1 and T48 animals, respectively, compared with those isolated from C animals ($P < 0.05$) (Fig. 2).

*CS activity.* CS activities were measured biochemically in soleus and cardiac ventricle muscles of C, T1, and T48 animals. CS activities increased by 42 and 25% in soleus muscles from T1 and T48 animals, respectively, relative to activities in C animals (T1 vs. C: 469 ± 18.9 vs. 330 ± 6.6; T48 vs. C: 412 ± 10.9 vs. 330 ± 6.6 nmol·mg protein$^{-1}$·min$^{-1}$, $P < 0.01$). The soleus muscle from T1 had a 14% higher level of CS activity compared with the muscle from T48 (T1 vs. T48: 469 ± 18.9 vs. 412 ± 10.9 nmol·mg protein$^{-1}$·min$^{-1}$, $P < 0.05$) (Fig. 3). However, cardiac CS activities were not significantly changed with exercise training in either T1 or T48 animals compared with C animals (Fig. 4).

**DISCUSSION**

This study demonstrated that 8 wk of endurance treadmill training in rats increases the CS activity in skeletal soleus muscle but not in cardiac ventricle muscle. Here we provide evidence that suggests that exercise has an acute effect on the training-induced increase in CS activity. These data are in agreement with human studies showing that the acute CS activity in skeletal muscle is increased after a single bout of exercise (14, 27). Although different acute and chronic adaptive responses of CS activities to endurance training were found between soleus and cardiac ventricle muscles, both of the muscle types demonstrated a sim-
ilar modulation of CS mRNA level in the animals 1 and 48 h after the final session of exercise training. However, the data in the present study do not show whether there are different molecular adaptations (translational and/or posttranslational mechanism) of CS adaptations to exercise training in rat soleus and cardiac muscles or whether the heart already has maximal levels of CS, so that training cannot further increase these levels. Further studies are needed to determine whether either mechanism can explain the different CS adaptive responses to training reported in skeletal and cardiac muscles.

Chronic response of CS activity to training. Chronic effects of exercise training (8–12 wk) on skeletal muscles CS activity have been extensively examined in rats (1, 6, 20) and humans (4, 14). In the present study, we have found that soleus muscle from the trained rats 48 h after the last training session had a 25% higher level of CS activity compared with the sedentary rats (T48 vs. C: 412 ± 10.9 vs. 330 ± 6.6 nmol-mg protein−1·min−1, P < 0.01). This is consistent with the previous findings, and the data fall into published ranges of CS activity elevation from similar duration and intensity of exercise training (1, 14, 20, 30). Moreover, we reported that the CS activities in cardiac ventricle muscle were unchanged after 8-wk endurance treadmill training in both groups of 1 and 48 h after the final session of exercise training, a finding that agrees with the majority of the previous findings. There is little evidence in the literature reporting that exercise training promotes an increase in CS activity in rat cardiac muscle (10). Our findings are consistent with previously published data indicating that CS activity is not altered after endurance training in rat cardiac muscle (1, 2, 17, 18, 30). It was suggested that myocardium has sufficient preexisting oxidative capacity to supply the energy requirement during exercise.

Acute response of CS activity to exercise. Although it appears clear that endurance exercise training promotes a chronic effect of CS activity elevation in skeletal muscle, results on the acute effects of endurance exercise on CS activity are less clear. Tonkonogi and co-workers (27) investigated the acute effect of a single bout of prolonged cycling on the oxidative function in human skeletal muscle. They observed that skeletal muscle CS activity was increased by 20% measured in millimoles per kilogram dry weight per minute (increased by 12% measured in related to protein content) immediately after an acute bout of exhaustive cycling in ~75% peak O2 uptake. A recent study conducted by Leek and colleagues (14) reported that an acute bout of exercise lasting for 30 min significantly increases the CS activity 1 h after the cessation of exercise in the skeletal muscle from both sedentary and trained humans. They have shown that there is a 50% increase in the CS activity in 1 h after the acute exercise and an 18% increase in the CS activity in the skeletal muscle after 8 wk of training. In summary, the findings from previous human studies consistently demonstrated that an acute bout of exercise upregulates the CS activity in skeletal muscle. There is evidence supporting the conclusion that a single bout of exercise (at least 30 min) exerts an increase in the skeletal muscle CS activity that would overshadow the CS activity elevation resulting from chronic endurance training in humans (14). In contrast to the human studies, several previous rat studies have reported unchanged or even decreased CS activities immediately after a single bout of exercise (9, 12, 26). In the present study, we have shown that acute exercise significantly increases the soleus CS activity by 42% (measured in relation to protein content) 1 h after cessation of prolonged exercise in trained rats compared with the sedentary control rats. In addition, we found a 25% increase in the soleus CS activity of the trained rats 48 h after the last training session compared with the sedentary control rats. The greater level of soleus CS activity in T1 animals compared with T48 animals suggests that an acute effect of exercise on the training-induced elevation in CS activity exists in rats; therefore, it is important to choose appropriate points after the last bout of exercise if the chronic effects of exercise are to be fully evaluated. Choosing a sampling time too close to the last exercise session will probably overestimate the effect of chronic exercise on CS levels. Collectively, the present findings indicate that the elevation in CS activity after an acute bout of exercise occurs at 1 h after the cessation of exercise.

CS gene expression after exercise training. Our findings demonstrated that the CS mRNA levels in soleus and cardiac ventricle muscles from both the T1 and T48 animals were elevated by ~20% compared with sedentary control animals. Previous findings suggested that upregulation of CS gene transcription occurs within 3 h after a single bout of exercise and the elevated CS mRNA level is still present 24 h after exercise (16). Our present data have added to the previous findings of others by showing that the CS mRNA level in soleus muscle is elevated by 21% when comparing rats 1 h after the last training session with sedentary control rats. We have also reported that there is an 18% increase in soleus CS mRNA level in rats 48 h after the last training session compared with sedentary control rats. We have provided novel evidence indicating that there is an upregulation of CS gene transcription in rat soleus muscle occurring at both 1 and 48 h after the

Fig. 4. CS activities for cardiac muscles in C, T1, and T48 animals. CS activity is normalized to the total protein content of the sample used in the assay. Normalized data are presented as means ± SE.
last session of endurance training. We have shown that the soleus CS activity is significantly increased by 42% when comparing rats 1 h after the last training session with sedentary control rats. This could not be completely explained by the 21% upregulation of CS gene transcription in soleus. However, we cannot rule out the possibility that CS mRNA content may have also changed at the time points other than 1 h after the last training session. Further studies are needed to determine whether modification on the active site of the existing CS proteins may be important in the regulation of CS enzymatic activity. We speculate that this may be a reasonable explanation for our findings because it was demonstrated that covalent enzyme modification may occur by phosphorylation and dephosphorylation or oxidation and reduction, which is related to the increased muscle CS activity (9).

Both the T1 and T48 animals had a higher level of CS mRNA in cardiac ventricle muscle compared with sedentary control animals. We have shown that there is an ~20% upregulation of CS gene transcription in the ventricle muscle from both the T1 and T48 animals. However, interestingly, unaltered cardiac CS activity was reported in these trained animals compared with the sedentary control animals. Our data do not permit us to know whether reduced mRNA stimulatory or different translational and/or posttranslational mechanisms might explain the difference in response of ventricle and skeletal muscles to exercise. The mechanism(s) responsible for the upregulation of ventricle CS gene transcription but without elevated CS activity is unknown. It is also possible that pretranslational mechanisms and CS turnover modulation could explain our observations.

In the present study, the training effect and the acute effect of exercise on CS gene expression and enzymatic activity in soleus and cardiac muscles have been discussed according to the findings at 1 and 48 h after the last training session of endurance training. Because our data are limited to selected time points, it is noted that they do not allow us to rule out any changes that may have occurred at the time points that were not examined in this study. Moreover, we failed to observe a large difference in CS activity between cardiac and soleus muscles. This unexpected observation may be due to a different normalizing procedure compared with previous studies.

In conclusion, the results of this study have shown significant elevations of CS activities in soleus muscle, but not in cardiac ventricle muscle, 1 and 48 h after the last session of 8-wk endurance training compared with sedentary animals. Our data are consistent with studies in humans that reported the acute increase in CS activity in skeletal muscle after a single bout of exercise (14, 27). Our results suggest that crucial sampling times are required to separate acute and chronic effects of exercise on CS in muscles of rats. We also demonstrated that there is a similar upregulation of CS mRNA content in both the soleus and cardiac ventricle muscles from those 1 and 48 h after the final session of exercise training. This study underscores the basis for future studies that distinguish between different molecular adaptations (translational and/or posttranslational modification) of CS adaptations to exercise training in rat soleus and cardiac muscles.

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