Eur J Appl Physiol (2000) 81: 114–119 © Springer-Verlag 2000

ORIGINAL ARTICLE

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Alterations in the expression and activity of creatine kinase-M and mitochondrial creatine kinase subunits in skeletal muscle following prolonged intense exercise in rats

Accepted: 18 July 1999

Abstract Creatine kinase (CK) isoenzymes are important structural and energy metabolism components in skeletal muscle. In this study, CK isoenzyme alterations were examined in male rats, with an 8% body mass weight attached to their tail. The rats were either forced to swim for 5 h (5S, $n = 51$), or were pre-trained for 8 days and then forced to swim for 5 h (T5S, $n = 48$). Rats were sacrificed either immediately (0 h PS), 3 h (3 h PS), or 48 h post-swimming (48 h PS). Serum CK was increased significantly ($P < 0.01$) 6.2- and 2.0-fold at 0 h PS following the 5S and T5S protocols, respectively. However, training (T5S protocol) significantly ($P < 0.01$) decreased CK release. Soleus and white gastrocnemius (WG) CK activity was significantly decreased following the 5S protocol ($P < 0.05$), but not following the T5S protocol. The CK-M activity of the soleus muscle was significantly ($P < 0.05$) decreased at 0 h PS following both the 5S and T5S protocols, and returned to control values at 3 h PS. The CK-M activity of the WG was significantly ($P < 0.05$) decreased at 0 h PS following the 5S protocol. Sarcomeric mitochondrial CK (sCK-Mit) was decreased significantly ($P < 0.01$) at 0 h PS (20%), 3 h PS (14%), 24 h PS (22%), and 48 h PS (15%) following the 5S protocol. However, sCK-Mit was decreased significantly ($P < 0.01$) only at 0 h PS (7%) following the T5S. The results of this study demonstrate that prolonged intense exercise causes a loss of skeletal muscle CK-M and sCK-Mit activity and that training prior to the prolonged intense exercise attenuates the exercise-induced CK-M and sCK-Mit loss in both red and white skeletal muscles.

Key words Mitochondrial creatine kinase · Muscle fatigue · Muscle injury · Swimming · Creatine kinase isoenzymes

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Introduction

Creatine kinase (CK, EC 2.7.3.2) isoenzymes catalyze the reaction that generates ATP from ADP and phosphocreatine, and plays a central role in the energy metabolism of myocardial and skeletal muscle (Haas and Strauss 1990; Wallimann et al. 1992). Four highly conserved mammalian CK subunits have been identified. Each is located on distinct genes and is expressed in a tissue-specific manner: two cytosolic forms, CK-M and CK-B, and two mitochondrial forms, uCK-Mit (ubiquitous) and sCK-Mit (sarcomeric). The CK-M and CK-B subunits form three dimeric cytosolic isoenzymes, CK-MM, CK-MB, and CK-BB, all with an approximate molecular weight of 80 kDa (Wallimann et al. 1992). CK-M predominates in mature skeletal muscle and the myocardium. CK-B predominates in the adult mammalian heart, and is also expressed in striated muscles during development (Wyss et al. 1992). The uCK-Mit and sCK-Mit subunits also exist in dimeric form (molecular weight 80 kDa; Wyss et al. 1992). However, sCK-Mit is present only in striated muscle (Payne and Strauss 1994), localized along the outer mitochondrial inner membrane and at sites where the outer and inner mitochondrial membranes are close to each other (Erickson-Viitanen et al. 1982; Wyss et al. 1992). In skeletal muscle, over 95% of CK expression occurs as CK-MM, with 1–3% as CK-MB and sCK-Mit (Wallimann et al. 1992; Yamashita and Yoshioka 1991). Compared to slow-twitch fibers, fast-twitch skeletal muscle fibers contain more CK-M and less sCK-Mit and CK-B (Apple and Tesch 1989; Jansson and Sylven 1986; Yamashita and Yoshioka 1991).

Prolonged intense exercise is known to cause muscle injury and muscle dysfunction, as demonstrated by swelling, disruption, and a reduction in the number of mitochondria (Gollinick and King 1969; Marciniak et al. 1983; McCutcheon et al. 1992; Nimmo and Snow 1982), disarrangement and disruption of the myofilaments (Fridén et al. 1988), reduction in the force and shortening velocity (Allen et al. 1995; Fitts 1997), and increased serum total CK and CK-MB (Rogers et al. 1985; Schwane and Armstrong 1983). The effects of acute and chronic exercise on skeletal muscle CK-M and CK-B have been studied. Few studies, however, have examined the mitochondrial CK composition of skeletal muscle following acute prolonged intense exercise (Apple and Rogers 1986). However, the changes in CK isoenzymes, especially sCK-Mit, that occur following prolonged intense exercise have yet to be determined fully (Apple and Rogers 1986). An insufficient supply of ATP and phosphocreatine in skeletal muscle has been proposed as one important mechanism for exercise-induced muscle dysfunction (Korge and Campbell 1995). A study of CK subunit alterations in skeletal muscle following prolonged intense exercise may therefore improve our understanding of the mechanism underlying exercise-induced muscle dysfunction, which is found commonly in both human and animal subjects. The purpose of this study was, therefore, to determine whether prolonged intense exercise with and without pretraining, would alter the skeletal muscle CK isoenzyme compositions in rats. We designed the study to quantitate total CK activity, and CK-M and mitochondrial-CK subunits expression in the soleus and white gastrocnemius (WG) muscles in non-exercising controls and up to 48 h following either 5 h of forced swimming, or after an 8-day pre-training regimen followed by 5 h of swimming.

Methods

Animals and animal care

Male Sprague-Dawley rats, weighing 285–335 g (body mass weight on the day before swimming), were used in these experiments. The rats were housed in cages in rooms regulated for temperature (23 ± 1°C), humidity (45–55%), and light/dark cycle (lights on: 0600–1800 h), and were provided with laboratory rat chow and water ad libitum. The research involving rodents in this study conforms with the current ''Guide for the care and use of laboratory animals'' as set by the NIH, and was approved by the Institutional Animal Care and Use Committee.

Experiment groups and exercise protocol

The rats were divided into two protocols. Protocol 1 used 63 rats, of which 12 were used as sedentary controls. Fifty one rats swam for 5 h (5S) in a swimming tank with 8% body mass weights attached to the tail (25 or 26 rats swam together at each time). Three rats were removed from this protocol since they were unable to complete the required forced swimming bout. After the forced swimming, the rats were weighed, and then sacrificed either immediately (0 h PS, $n = 12$), 3 h (3 h PS, $n = 12$), or 48 h post swimming (48 h PS, $n = 12$). In protocol 2, 48 rats were trained for 8 days prior to a 5-h swim (T5S). Pre-training included 25 min of swimming on day 1, increasing to 60 min on day 6, with no weight attached to their tails. The rats swam with a 5% tail weight for 20 min on day 7 and 30 min on day 8. After the training, a 2-day rest period was allowed. Twelve rats were then used as pre-trained control group. The other 36 rats then swam for 5 h with 8% body mass weights attached to the tail (each time, 18 rats swam together). After the forced swimming, the rats were weighed, and then sacrificed at either 0 h PS ($n = 12$), 3 h PS ($n = 12$) or 48 h PS ($n = 12$). The body-mass of the 48 h PS groups in both protocols was monitored for up to 48 h post-exercise. In both protocols, the 8% body-mass weight load attached to the tail determined exercise intensity. The water temperature was 35°C, and the water depth was 26 cm.

Most of the rats could constantly keep their noses and mouths above the water surface by paddling their legs during swimming. Some of the rats were unable to keep their body floating in the water, and would occasionally sink to the bottom of the swimming tank, and then press the tank bottom forcefully with their hind legs to keep their nose and mouth out of the water. Immediately after the 5-h swimming bout, the rats were very tired but were not exhausted, since they were still able to catch the trainer's hands to get out of the water, were able to move around in the cage, and were soon actively cleaning the water on their fur with their front legs and mouths.

It should be noted that the swimming model used in this study has been shown to result in more metabolic stress to skeletal muscles (based on alterations in blood glucose, blood lactate, and muscle glycogen) than long-duration, low-intensity treadmill running (unpublished data from our laboratory).

Animal sacrifice and tissues sampling

Rats were anesthetized with Nembutal (50 mg/kg intraperitoneal injection). The abdominal cavity was quickly opened and 10 ml of blood was drawn out from the abdominal aorta, and the serum was harvested. The soleus and WG muscles were excised, frozen on dry ice and stored at −80°C until biochemical analysis.

Sample preparation

Frozen muscle samples were cut into small pieces on dry-ice-cooled sample plates and then added to 2 ml of ice-cold buffer (200 mM potassium phosphate pH 7.4, 5.0 mM ethylene glycol-bis(oxonitrilo) tetraacetic acid, 5.0 mM β-mercaptoethanol, and 10% v/v glycerol, Apple and Billadello 1994). The samples were homogenized in an ice bath for 3 × 10 s at high speed with a Polytron tissue homogenizer (Brinkman Instruments, Westbury, N.Y., USA). This was followed by centrifugation at 3,000 ×*g* for 30 min at 4°C (Apple and Billadello 1994). The supernatants were used for Western blotting and total CK activity determination. Protein concentrations were determined using a modified Lowry method (Lowry et al. 1951) with bovine serum albumin as a standard. Total CK activities of the serum and tissue homogenates were measured at 37°C on a kinetic enzyme analyzer with N-acetylcysteine-activated reagents (Calbiochem-Behring; Rosalki 1967).

Antibodies

A mouse monoclonal antibody specific for the CK-M subunit was purchased from OEM Concepts (Toma River, N.J., USA; Hoang et al. 1997). A rabbit polyclonal antibody, which was generated with a synthetic peptide immunogen (DAREQHKLFPC) derived from the rat sCK-Mit subunits (Payne and Strauss 1994), was a gift from Washington University School of Medicine (laboratory of R.M. Payne, St. Louis, MO., USA; Hoang et al. 1997).

Western blot analysis

Tissue homogenates were size fractionated on 12% sodium dodecyl sulfate polyacrylamide gels (Towbin et al. 1979) and subsequently

transferred to Hybond nitrocellulose membranes (Amersham, Arlington Heights, Ill., USA). Non-specific binding sites were blocked by incubating the membranes in a blocking buffer (5.0% non-fat dry milk in Tris-buffered saline: 20 mM Tris-HCl pH 7.6, 137 mM NaCl: TBS) for 1 h. Primary antibodies were diluted in antibody buffer (1.0% non-fat dry milk in TBS) and incubated with the membranes for 2 h on a rotating cylinder. The membranes were washed three times with Tween-Tris-buffered saline (TTBS) for 30 min. Horseradish-peroxidase-labeled secondary antibodies were then incubated with the membranes for 1 h at a dilution of 1:3000. The membranes were again washed three times in TTBS buffer prior to a 1-min incubation with chemiluminescent substrate (Amersham). Light emission was detected by exposure to a Fuji RX autoradiography film in the presence of Cronex intensifying screens. Signal intensities within the linear range were quantitated using laser densitometry (Molecular Dynamics, Sunnyvale, Calif., USA). To make sure proteins on different sides of the gel were equally transferred, a control sample was loaded onto all of the gels in at least two lanes, as internal control lanes (on each side of the gels), allowing for blotting quality control.

Statistical analysis

Data from all groups were analyzed using a two-way analysis of variance to determine main effects across time after the swimming. Tukey's post-hoc test for multiple comparisons was used to determine significant differences from pre-exercise values when significant main effects were found. The level of statistical significance was set at $P < 0.05$. All results are reported as mean (SD) unless stated otherwise. The values obtained from Western blots are presented as a percentage of the control group value, which was designated 100%.

Results

Rat body weights decreased by 9.4% ($P < 0.01$ vs before exercise) and 9.9% ($P < 0.01$ vs before exercise) following the 5S and T5S protocols, respectively. Body weights remained decreased ($P < 0.01$) for up to 48 h in the 5S protocol, and for up to 24 h in the T5S protocol.

Figure 1 shows the serum total CK alterations that were observed following the 5S and T5S protocols. Serum total CK activity of pre-swimming controls of the T5S protocol was significantly greater compared to that of the 5S protocol ($P < 0.01$). At 0 h PS, the 6.2-fold serum CK increase that was observed following the 5S protocol ($P < 0.01$ vs controls) was greater ($P < 0.05$) than the 2.0-fold serum CK increase observed following the T5S protocol ($P < 0.01$ vs controls).

Table 1 shows the alterations in CK activity of the soleus and WG muscles following the 5S and T5S protocols. The total CK activity of the WG was threefold greater ($P < 0.01$) than that of the soleus muscle. The CK activities of the controls were not significantly different between the 5S and T5S protocols. In the 5S protocol, the CK activity of the soleus muscles showed significant decreases of 9% ($P < 0.05$) at 0 h PS and 11% ($P < 0.01$) at 48 h PS. The CK activity of WG in the 5S protocol showed significant decreases of 9% ($P < 0.05$) at 0 h PS, 7% ($P < 0.05$) at 3 h PS, and 11% ($P < 0.05$) at 48 h PS. In contrast, the CK activity of the soleus and WG showed no significant changes in any post-exercise group following the T5S protocol. However, the CK activity of the WG at 0 h PS and 3 h PS in the T5S protocol was significantly ($P < 0.05$) greater than at similar times in the 5S protocol.

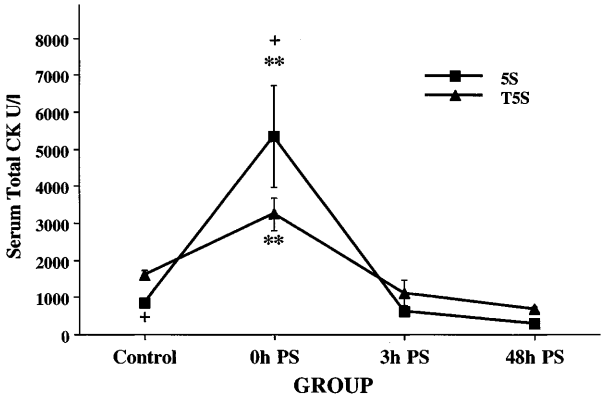

Fig. 1 Serum total creatine kinase (CK) concentrations of rats following the 5S (*filled squares*) and T5S (*filled triangles*) protocols. Data are given as the mean ± SE. $**P < 0.01$ compared with controls; $+P < 0.01$ compared between groups

Figure 2 shows a representative Western blot analysis of the CK-M and sCK-Mit subunits of the soleus muscles following the 5S protocol at 0 h PS, 3 h PS, and in the controls. A single protein band migrated to a molecular weight position corresponding to approximately 36 kDa and 38 kDa for CK-M and sCK-Mit, respectively.

The alterations of CK-M, determined by Western blot analysis, in the soleus and WG muscles following the 5S and T5S protocols are shown in Figs. 3 and 4, respectively. For the soleus muscle, CK-M subunit expression decreased significantly ($P < 0.05$) only at 0 h PS in both the 5S (7%) and T5S (6%) protocols. No significant differences were noted between the CK-M alterations in the 5S and T5S protocols at any post-

Table 1 Alterations in total creatine kinase in the soleus and white gastrocnemius (WG) muscles of untrained (5S) and trained (T5S) rats following 5 h of swimming. Values are given as the mean (SD). (*PS* Post-swimming)

Protocol	Muscle	Control	PS (h)		
			0	3	48
5S	Soleus	32.2 (3.3)	29.5 (3.2)*	30.3 (3.3)	28.5 (2.4)**
	WG	110.4 (9.9)	100.4 (10.2)*	102.4 (7.4)*	98.6 (12.8)*
T5S	Soleus	31.2 (1.6)	32.1 (3.5)	32.8 (3.9)	33.4 (2.2)*
	WG	107.1 (17.8)	117.0 (24.5)	116.9 (17.6)	111.4 (19.5)

$*P < 0.05$ compared to controls
$**P < 0.01$ compared to controls

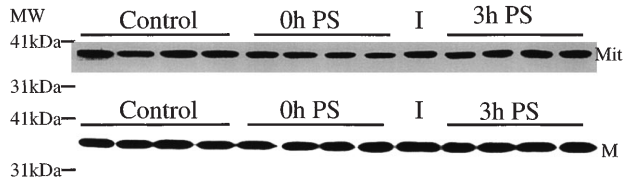

Fig. 2 Representative Western blot analysis of CK-M (M), and sarcomeric mitochondrial CK (Mit) from the soleus muscles of control, 0 h PS, and 3 h PS groups following the 5-h swimming protocol. [*I* Internal control (pooled tissue samples) used for determining the equivalence of protein transfer across each gel, *MW* molecular weight]

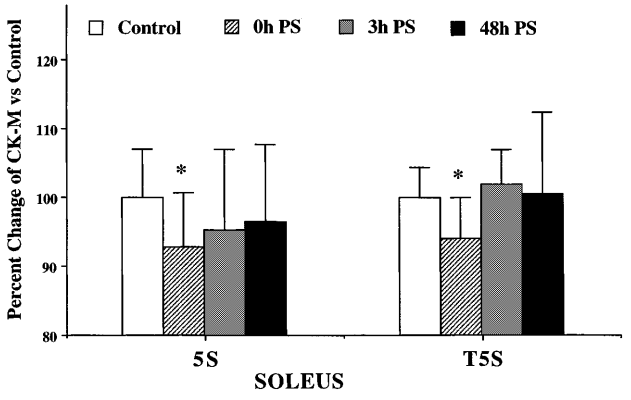

Fig. 3 Alterations in CK-M subunits activity as determined by Western blot analysis of soleus muscles following the 5S and T5S protocols compared to non-swimming controls. $*P < 0.05$ compared with controls within the same group

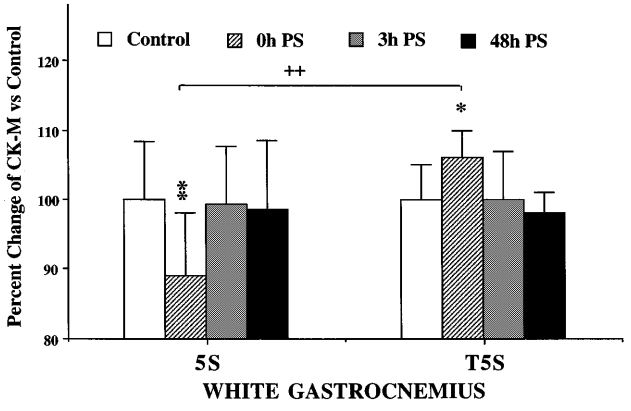

Fig. 4 Alterations in CK-M subunits activity as determined by Western blot analysis of white gastrocnemius muscles following the 5S and T5S protocols compared to non-swimming controls. $*P < 0.05$, $**P < 0.01$ compared with controls; $++P < 0.05$ compared between groups

exercise point. For the WG muscle, CK-M expression was significantly decreased ($P < 0.01$), by 11%, only at 0 h PS following the 5S protocol. In contrast, CK-M expression was increased by 6% ($P < 0.05$) at 0 h PS following the T5S protocol.

sCK-Mit was undetectable in the WG muscles. Figure 5 shows the alterations of sCK-Mit expression,

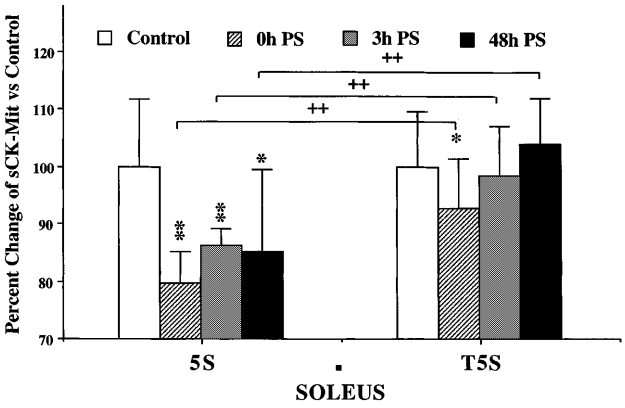

Fig. 5 Alterations in sCK-Mit as determined by Western blot analysis of soleus muscles following the 5S and T5S protocols, compared to non-swimming controls. $*P < 0.05$, $**P < 0.01$ compared with controls; $++P < 0.01$ compared between groups

as determined by Western blot analysis, in the soleus muscle following the 5S and T5S protocols. Following the 5S protocol, sCK-Mit was significantly decreased ($P < 0.05$ or $P < 0.01$) at all times as follows: 0 h PS by 20%, 3 h PS by 14%, and 48 h PS by 15%. Following the T5S protocol sCK-Mit was significantly ($P < 0.05$) decreased (7%) only at 0 h PS. The decrease in sCK-Mit observed following the 5S protocol was significantly ($P < 0.01$) greater than that observed following the T5S protocol at 0 h PS and 3 h PS.

Discussion

The current study is unique in that it demonstrates that an 8-day period of training prior to exercise had a protective effect against an exercise-induced decrease in mitochondrial CK, which is likely to be due to a training effect against injury. In concert with the observed loss of muscle CK-M in both red-fiber muscles (soleus) and white-fiber muscles (WG) following the 5S exercise protocol, these alterations could therefore impair the CK-phosphocreatine shuttle (Wallimann et al. 1992), disturb the ATP supply, delivery, and utilization processes, and affect muscle performance.

Compared to the untrained rats of the 5S protocol, the trained rats of the T5S protocol demonstrated less serum CK release at 0 h PS. This is consistent with previous studies in which it was reported that training prior to exercise resulted in a reduction in the release of CK into the circulation (Hyatt and Clarkson 1998; Schwane and Armstrong 1983). Our present study also showed that alterations in total CK activity did not occur in either the soleus or WG in the trained rats following 5 h of swimming, a result that is also consistent with previous studies in humans (Apple and Rogers 1985; Siegel et al. 1983). In contrast, we found that untrained rats showed a significant decrease in total CK activity in both the soleus and WG muscles following the 5-h swimming protocol (Table 1). Since there were few

significant changes in the T5S protocol compared to those observed in the 5S protocol, it follows that there would be significant CK activity differences demonstrated between muscles at the same time periods between the 5S and T5S protocols (Table 1). Decreased muscle CK activity in the current study may be attributable to the acute nature of the high-intensity, high-volume exercise coupled with the lack of pre-conditioning of the rats.

In the present study, the total CK activity of the fast-twitch WG was approximately three times higher than the total CK activity of the predominantly slow-twitch soleus. This was in agreement with previous studies that have reported fast-twitch fibers with higher total CK activity (Apple and Tesch 1989; Jansson and Sylven 1986; Yamashita and Yoshioka 1991). Furthermore, in the current study we also found that sCK-Mit was undetectable in the WG. However, it has been reported that the fast-twitch glycolytic fibers of the extensor digitorum longus muscle contain detectable levels of both the sCK-Mit and CK-B subunits (Yamashita and Yoshioka 1991). Differences in these finding may due to the fact that the same fiber types from different muscles vary in their CK activity and isoenzyme composition (Takekura and Yoshioka 1990).

Following the T5S protocol, both the CK-M and sCK-Mit subunits of the soleus muscle decreased at 0 h PS, while the total CK activity did not decrease. Mechanisms that may explain this finding were not studied. It could be hypothesized that the decreased levels of CK-M and sCK-Mit subunits were balanced by an increase in the CK-B subunit; thus, no overall alteration in the total CK activity would be detected. However, this study was limited in that the expression of CK-B subunits was not studied. Furthermore, no explanation has been provided for the observed increase of CK-M expression in WG at 0 h in the T5S group. Previous studies have reported shifts of CK subunits in skeletal muscle after training and long-distance running (Apple and Rogers 1986; Siegel et al. 1983; Yamashita and Yoshioka 1992).

The mitochondrial injury indicated by the loss of CK-Mit in this study is consistent with previous studies in which it has been reported that prolonged swimming (Gollnick and King 1969) and running induced ultrastructural alterations including mitochondrial swelling (McCutcheon et al. 1992; Zamora et al. 1995), mitochondrial disruption (Bowers et al. 1974; Gollnick and King 1969; Nimmo and Snow 1982), reduction in the volume and number of mitochondria (Hochli et al. 1995; Marciniak et al. 1983), and a decline in muscle respiratory capacity (Gollnick et al. 1990). Using a myocardial ischemic model, one study reported that increased serum mitochondrial CK was directly associated with mitochondrial injury, with the extent of myocardial protein release being directly related to the degree of myocardial injury as monitored by electron microscopy (Ishikawa et al. 1997). However, contrary studies have reported the mitochondrial structure to be unchanged following running to exhaustion (Gale 1974; Terjung et al. 1972). Unfortunately, no electron microscopy experiments were performed in the current study. However, our previous unpublished study (using the same swimming model) show no delayed-onset muscle damage in the soleus and gastrocnemius muscles following the same forced swimming in untrained rats (data not shown).

The reduced mitochondrial damage of trained rats (Fig. 4) observed in the current study is consistent with studies in which it has been reported that prolonged exercise induced less muscle mitochondrial damage and less muscle fiber degeneration in trained animals as compared to untrained and detrained animals (Gollnick and King 1969; Nazar et al. 1993). Contrary to the current finding, one study has reported that sCK-Mit increased significantly in gastrocnemius muscle in humans during training for, and 1 day after a marathon race (Apple and Rogers 1986). However, no data are available regarding sCK-Mit alterations immediately following prolonged intense exercise in either trained or untrained subjects. The mechanism involving the effects of training in protecting the mitochondria is not clear. It has been proposed that training reduces the accumulation of calcium in mitochondria (Bonner et al. 1976) and increases the antioxidant capacity (Venditti and Di Meo 1997) of muscle fibers.

Following the T5S protocol, the decreased expression of the CK-M and sCK-Mit subunits in the soleus muscle returned to control values by 3 h post-exercise. This suggests that the decrease in CK-M and sCK-Mit was recoverable during the post-exercise period. However, the decreased expression of sCK-Mit in the soleus muscle did not return to control values during the 48-h post-exercise period studied (Fig. 4). The recovery of cellular proteins might be due to resynthesis of CK-M and sCK-Mit in the same damaged fibers, or up-regulation of mRNA for CK-M and sCK-Mit synthesis in non-damaged cells. The current study did not determine whether the CK-M and sCK-Mit subunits were controlled by increased transcription at the mRNA level. However, a previous study has demonstrated that increased CK-B subunit expression following training was controlled partially by an increase in mRNA for CK-B (Apple and Billadello 1994).

In conclusion, our study shows that prolonged intense exercise causes a loss of skeletal muscle CK-M and mitochondrial CK subunits and that training prior to the prolonged intense exercise provides a protective effect against CK-M and mitochondrial CK loss in skeletal muscles. The molecular mechanisms responsible for these alterations still need to be addressed.

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