UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of endurance-trained compared to untrained subjects

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UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of endurance-trained compared to untrained subjects

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Abstract Uncoupling protein 3 (UCP3) is a muscle mitochondrial protein believed to uncouple the respiratory chain, producing heat and reducing aerobic ATP production. Our aim was to quantify and compare the UCP3 protein levels in type I, IIa and IIx skeletal muscle fibers of endurance-trained (Tr) and healthy untrained (UTr) individuals. UCP3 protein content was quantified using Western blot and immunofluorescence. Skeletal muscle fiber type was determined by both an enzymatic ATPase stain and immunofluorescence. UCP3 protein expression measured in skeletal muscle biopsies was 46% lower ($P=0.01$) in the Tr compared to the UTr group. UCP3 protein expression in the different muscle fibers was expressed as follows; $IIx>IIa>I$ in the fibers for both groups ($P<0.0167$) but was lower in all fiber types of the Tr when compared to the UTr subjects ($P<0.001$). Our results show that training status did not change the skeletal muscle fiber hierarchical UCP3 protein expression in the different fiber types. However, it affected UCP3 content more in type I and type IIa than in the type IIx muscle fibers. We suggest that this decrease may be in relation to the relative improvement in the antioxidant defense systems of the skeletal muscle fibers and that it

might, as a consequence, participate in the training induced improvement in mechanical efficiency.

Keywords Exercise · Oxidative phosphorylation · Skeletal muscle

Introduction

Uncoupling protein 3 (UCP3) belongs to the family of mitochondrial carrier proteins and is expressed predominantly and abundantly in rodent and human skeletal muscle [1]. UCP3 is thought to uncouple oxidative phosphorylation, reducing aerobic ATP production and dissipating energy as heat. If this is the case UCP3 may play an important role in the regulation of human energy expenditure [2, 3, 4, 5]. Alternatively, based on UCP3 mRNA data it was proposed that UCP3 may also be involved in the exit of fatty acids out of the mitochondrial matrix [6], insulin sensitivity [7], protection against both reactive oxygen species [8] and the accumulation of nonesterified fatty acids inside the mitochondrial matrix [9]. Recent protein data have suggested that UCP3 may also play a role in regulating glucose homeostasis [10].

Endurance training has been frequently used as a model of altered energy metabolism. It has been observed to increase resting metabolic rate [11] and gross energy efficiency during exercise in humans [12]. UCP3 mRNA is decreased in rat muscle after 8 weeks of forced treadmill running [13] and is also reduced in endurance-trained (Tr) humans when compared to untrained (UTr) controls [14, 15]. These observations support a role for UCP3 as a regulator of energy efficiency.

The study of the expression of the UCP3 protein was delayed as compared to that of the mRNA due to the lack of specific UCP3 antibodies. Recent measurements of the UCP3 protein level using validated UCP3 antibodies have revealed differences between the magnitude of changes for the UCP3 mRNA and protein. Conditions such as starvation [16], fasting [17] and triiodothyronine (T3) treatment [18] caused changes of UCP3 expression which

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were found to be several-fold higher at the UCP3 mRNA than the protein level. It has also been shown that under certain conditions, such as leptin treatment, the effects on UCP3 mRNA and protein are divergent [17]. All these results suggest that UCP3 can be regulated at the posttranscriptional level [17] and therefore underscore the need for examining UCP3 protein content rather than mRNA levels.

In a recent review, results from a preliminary study demonstrated a reduced UCP3 protein expression after 3 months of endurance training in previously untrained subjects [9]. An initial aim of the present study was to determine if the UCP3 protein content is lower in a group of UTr when compared to Tr subjects

Recently, it has been demonstrated in healthy and type II diabetic humans that the UCP3 protein is expressed the least in oxidative type I, more in oxidative-glycolytic type IIa and the most in glycolytic type IIx (also known as IIb) muscle fibers [19]. Since endurance training improves oxidative capacity of all muscle fiber types [20] it was of great interest to test its possible effect on the hierarchy of UCP3 protein expression in the different muscle fiber types. The second aim of this study was to measure and compare the UCP3 protein expression in the different muscle fiber types of the UTr and Tr groups.

Materials and methods

Ten recreationally active, but untrained male subjects (UTr) and eight highly trained endurance male cyclists (Tr) participated in the present study. Subject details are provided in Table 1. All subjects signed informed consent and volunteered to participate in the present experiment that was approved by the Deakin University Ethics Committee. The subjects were asked not to participate in any physical activity 24 h prior to testing and to abstain from eating for at least 3 h prior to testing.

Exercise tests

Each subject performed an incremental exercise test on an electromagnetically braked cycle ergometer (Lode Excalibur) to determine maximal oxygen consumption ($\dot{V}O_{2\text{ max}}$) and maximal power output (MPO). Exercise was performed until exhaustion or until the subject could no longer maintain a pedaling rate of 90 rpm. Oxygen consumption and carbon dioxide production were measured using open-circuit spirometry (Medical Graphics, Cardio2 and CPX/D System). $\dot{V}O_{2\text{ max}}$ was defined either when a plateau in $\dot{V}O_2$ with increasing work rates was observed or as the highest 30-s $\dot{V}O_2$ value measured during the test. A plateau was deemed to be reached when $\dot{V}O_2$ did not increase by more than 100 ml/13-W increase. MPO was deemed as the highest power output at a cadence of 90 rpm that could be maintained for a period of 60 s during the $\dot{V}O_{2\text{ max}}$ test. On a separate day, using the same equipment as for the measurement of $\dot{V}O_{2\text{ max}}$, the mechanical efficiency (%) of seven Tr and seven UTr subjects was calculated as steady-state energy expenditure ($\dot{V}O_2$)/power output (W) when cycling at 50% of $\dot{V}O_{2\text{ max}}$ power.

Muscle sampling, treatment and analysis

Skeletal muscle samples were obtained under local anesthesia (xylocaine, 1% plain) from the belly of the vastus lateralis muscle using a percutaneous needle biopsy technique [21] modified to include suction. A single incision was made in the skin and two muscle samples were taken from a single insertion of the biopsy needle. After the first biopsy cut the needle was rotated and a second cut was performed. Each muscle sample weighed between 50 mg and 80 mg. One muscle sample was mounted in embedding medium and frozen in isopentane, that was previously cooled to its freezing point. These samples were used for Western blotting, muscle fiber typing and immunohistochemistry. From the other muscle sample approximately 10 mg of muscle was homogenized in a solution consisting of 175 mM KCl and 2 mM EDTA (pH 7.4) and then freeze-thawed twice. The homogenate was then centrifuged for 1 min at 10,000 rpm and the supernatant analyzed for citrate synthase (CS) activity using spectrophotometric detection [22]. CS activity correlates highly with in vitro measurements of skeletal muscle mitochondrial oxidative capacity [23] and has been commonly used as a marker of muscle oxidative capacity [24, 25].

Histochemical staining

Serial cross sections 10 μm thick were cut from the mounted muscle sample in a cryostat maintained at -20°C . The sections for fiber typing were preincubated at pH 4.30 and 4.54, and were classified as either type I, IIa or IIx according to the acid lability of different isoforms of myosin ATPase [26]. For each subject, a minimum of 200 fibers were classified and each fiber type was expressed as a percentage of the total number counted.

Western blotting

For each muscle sample, sections approximately 40 $\mu\text{m} \times 20 \mu\text{m}$ were cut at -20°C . They were placed in 400 μl of ice-cold PBS containing 0.4 mM phenyl methyl sulfonyl fluoride (PMSF) in ethanol and 1 mM EDTA pH 7.4 and vortexed for 5 s [27]. The sample was then homogenized using a Polytron for 3 $\times$ 10 s on ice and thereafter the homogenates were sonicated for 3 $\times$ 5 s. Subsequently, two volumes of muscle homogenate and one volume of SDS-sample buffer (2.3% SDS, 62.5 mM TRIS HCl pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 0.05% bromophenol blue) were boiled for 4 min followed by centrifugation for 5 min at 13,000 rpm. Two 12% polyacrylamide gels containing 0.1% SDS were run. The first gel was used to calculate protein concentrations by densitometry scanning using Imagemaster (Pharmacia Biotech), after Coomassie staining. On the second gel equal amounts of protein were loaded in each lane based on the protein calculations from the first gel and electrophoresis performed using a Mini-Protein 3 electrophoresis cell. Following electrophoresis, blotting was performed using a Mini Trans-Blot electrophoretic transfer cell and a nitrocellulose membrane (0.45 μm , Bio-Rad Laboratories) for 1 h at 100 V in a cold (4°C) buffer containing 25 mM TRIS pH 8.8, 192 mM glycine, and 20% methanol. After protein transfer, nitrocellulose membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20 and thereafter antibody incubation was performed with gentle shaking overnight at room temperature at a dilution of 1:5,000 in the 5% nonfat dry milk in 0.5% Tween-20/PBS. A rabbit anti-human UCP3 antibody (code:1331, kindly provided by L.J. Sliker, Eli Lilly) prepared against a 20-amino acid (aa) peptide (human sequence aa 147–166), which recognizes both the long and short UCP3 isoforms and was previously shown not to recognize UCP2, was used [19, 27].

After the primary antibody incubation, the membranes were incubated for 60 min with a horseradish peroxidase-conjugated swine anti-rabbit Ig (DAKO, Glostrup, Denmark) at a dilution of 1:10,000. The membranes were then washed for 1.5 h in 0.05% Tween20/PBS and again for 20 min in PBS. Subsequently, they were treated for 1 min with chemiluminescence substrate (Super

Signal West Dura Extended Duration Substrate; Pierce, Rockford, Ill., USA). Finally, X-ray film (CL-Xposure Film; Pierce, Rockford, Ill., USA) was exposed to the nitrocellulose membranes for 1 min. The reaction product of each blot was analyzed by densitometry using Imagemaster (Pharmacia Biotech).

Immunofluorescence

Serial sections (5 μ m) were cut for immunofluorescence determination of UCP3, type I and type IIa muscle fibers. Each section was placed on glass slides and air-dried for 2 h at room temperature. The sections were then fixed in methanol for 5 min at room temperature and acetone (-20°C) for 1 min. Following this the sections were incubated in a solution of 50% normal goat serum (NGS) and 3% BSA in PBS for 30 min at room temperature. The primary UCP3 antibody was diluted 1:400. For the detection of type I and IIa muscle fibers respectively, a monoclonal antibody (A4.951; IgM) raised against adult human slow myosin heavy chain at a dilution of 1:20 and a monoclonal antibody (N2.261; IgG₁) reactive with adult human fast IIa myosin heavy chain at a dilution of 1:20 were used. Both antibodies were developed by Dr. Blau [28]. The three antibodies were diluted in 10% NGS and 1% BSA in PBS. After overnight incubation at 4°C the sections were washed 3×5 min in PBS. The primary antibodies were visualized by the following secondary antibodies: goat anti-rabbit Ig conjugated with fluorescein isothiocyanate [GARIGFITC; Southern Biotechnology Associates (SBA), Birmingham, Ala., USA; goat anti-mouse IgM conjugated with Texas Red (GAMIGMTxRd; SBA) and goat anti-mouse IgG₁ conjugated with Alexa Fluor 350 (GAMIG₁ Alexa Fluor 350; Molecular Probes Europe, Leiden, The Netherlands)] for 30 min at room temperature. Due to difficulty in obtaining suitable morphological muscle cross sections (small cross-sectional area and/or tissue damage) from all 18 subjects muscle samples were analyzed from 5 UTr and 5 Tr subjects with a mean $\pm$ SD of 75 ± 10 fibers counted. This subgroup of 10 was representative of the original 18 subjects. There was no significant difference in the [Vidiot] O_2 max, MPO or the percentages for the type I, IIa and IIx muscle fibers determined using both the myosin ATPase histochemical and the immunofluorescent staining techniques between the original group and the subgroup ($P>0.05$ for all analysis) (data not shown). All muscle sections were analyzed at the same time. A total of 390, 271 and 90 type I, IIa and IIx muscle fibers respectively, were counted. Sections were viewed and photographed using a Zeiss Axiophot I microscope mounted with an Axiocam colour CCD camera. The specific fluorescence within each fiber was quantified using the Zeiss KS400 V3.0 program. The rabbit anti-human UCP3 antibody was the same as used for the Western blotting. The specificity of the UCP3 antiserum for immunofluorescence assays was tested on rat H9C2 myoblasts (lacking endogenous UCP3 expression), which were stably transfected with human UCP3. Additionally, after preincubation of the primary antibody with the peptide (that comprised amino acids 147–166 of human UCP3) no detectable labeling was observed [19].

Statistical analysis

Two-tailed unpaired *t*-tests were used to compare subject characteristics and total muscle UCP3 expression between the Tr and UTr groups. A two (training status; between factor) $\times$ three (fiber types; within factor) factor ANOVA was used to compare the influence of training status and fiber type on UCP3 protein expression. When a significant interaction between training status and fiber type was observed stratified analysis was used to locate the significant differences. To be specific, one-way repeated measures ANOVA followed by contrasts were used to compare the percentage change in UCP3 between type I, IIa and IIx muscle fibers, for each of the UTr and Tr groups. Moreover, the percentage difference in UCP3 between the Tr and UTr group was compared, using the *t*-test, for each of the three fiber types. While the overall alpha level for the

ANOVA was set at 0.05, the Sharpened Bonferroni method was used to adjust the individual alpha level to significance of $P<0.0167$ when multiple testings were performed in the stratified analysis. The statistical power was >0.75 for all analyses, indicating that any nonsignificant results were signs of no differences rather than a consequence of small sample size. All values are reported as mean $\pm$ SD.

Results

The subject characteristics are provided in Table 1. The Tr group had a significantly higher level of physical fitness as demonstrated by a greater $\dot{V}\text{O}_2$ max ($P<0.05$), MPO ($P<0.01$), power output per kg (W kg^{-1} ; $P<0.05$), mechanical efficiency when cycling at 50% of $\dot{V}\text{O}_2$ max ($P<0.05$) and CS activity ($P<0.05$) when compared to the UTr group. Additionally the Tr group had a greater percentage of type I ($P<0.01$) and a lower percentage of type IIa ($P<0.01$) and IIx ($P<0.05$) muscle fibers as determined by the myosin ATPase histochemical technique when compared to the UTr group.

Figure 1A shows a representative Western blot of the UCP3 protein from an UTr and a Tr subject. The UCP3 protein level measured in whole muscle homogenate was 46% lower ($P<0.05$) in the Tr than in the UTr group (Fig. 1B). A negative correlation between $\dot{V}\text{O}_2$ max and UCP3 protein content was observed ($r=-0.63$; $P<0.05$; Fig. 1C).

The triple-immunofluorescent image in Fig. 2 is multiple exposures of the same cross-sectional area demonstrating that the UCP3 protein content is expressed in a hierarchical manner with more in type IIx, less in type IIa and the least in type I muscle fibers. ANOVA demonstrated a significant interaction between training status and UCP3 content in the different muscle fibers ($P<0.001$).

As shown in Fig. 3 the UCP3 protein expression in both the UTr and Tr groups was significantly more abundant in type IIx fibers than in the type IIa and in the type I fibers ($P<0.0167$). Therefore, the hierarchy of UCP3 expression was not modified by training status. As also shown in Fig. 3 UCP3 protein

Table 1 Subject characteristics (CS citrate synthase; MPO maximal power output). Values are mean $\pm$ SD

Parameter	Untrained	Trained
<i>n</i>	10	8
Age (years)	22 $\pm$ 3	23 $\pm$ 5
Mass (kg)	76 $\pm$ 11	71 $\pm$ 6
Height (cm)	183 $\pm$ 10	176 $\pm$ 5
$\dot{V}\text{O}_2$ max (ml kg^{-1} min $^{-1}$)	51.8 $\pm$ 4.4	67.0 $\pm$ 5.2*
MPO (W)	317 $\pm$ 55	377 $\pm$ 33**
Mechanical efficiency (%)	17 $\pm$ 2	21 $\pm$ 3*
W kg^{-1} body weight	4.2 $\pm$ 0.3	5.3 $\pm$ 0.4*
CS activity ($\mu\text{mol min}^{-1}$ g $^{-1}$)	20 $\pm$ 3	38 $\pm$ 8*
% of type I muscle fibers	52 $\pm$ 11	77 $\pm$ 11**
% of type IIa muscle fibers	39 $\pm$ 13	19 $\pm$ 12**
% of type IIx muscle fibers	9 $\pm$ 6	4 $\pm$ 4*

* $P<0.05$; ** $P<0.01$ compared to untrained subjects

Fig. 1 A Representative Western blot signal of uncoupling protein 3 (UCP3) from an untrained (UTr) and an endurance-trained (Tr) subject. B UCP3 protein content in the UTr ($n=10$) and Tr ($n=8$) groups $^{**}P<0.01$. The Western blot was performed as described under materials and methods. C Negative correlation between UCP3 and $\text{VO}_2 \text{ max}$ ($\text{ml kg}^{-1} \text{ min}^{-1}$; $r=-0.63$; $P<0.05$); filled circles UTr; open circles Tr. Values are mean $\pm$ SEM

content was lower in all fiber types of the Tr when compared to the UTr. We thought it interesting to see if training status had a selective effect on UCP3 expression between the fiber types. We observed that the UCP3 protein expression was 33%, 38%, and 23% lower respectively, in the type I, IIa and IIx muscle fibers of the Tr when compared to the UTr subjects ($P<0.001$). The 33% and 38% lower UCP3 protein content in the type I and IIa muscle fibers, respectively, of the Tr when compared to the UTr group were not significantly different from each other after the Sharpened Bonferroni

adjustment ($P>0.0167$). However, these reductions were significantly more than the 23% lower UCP3 content in the type IIx muscle fibers of the Tr when compared to the UTr group ($P<0.0167$). The discrepancy in the lower UCP3 level in the Tr group determined by Western blot (46%; $n=18$) and immunofluorescence (average 32%; $n=10$) may be explained by the different subjects used in these analyses. When the Western blot results for the ten subjects used in the immunofluorescence analysis are considered, UCP3 content is 38% lower in the Tr when compared to the UTr group, although the reduction in sample size results in a loss of statistical significance ($P=0.16$; data not shown).

Discussion

The first aim of the present study was to compare the skeletal muscle UCP3 protein content in a group of healthy untrained subjects (UTr) to that in endurance trained cyclists (Tr). Our results showed a lower UCP3 protein content in the Tr when compared to the UTr group. This suggests that subjects with a higher level of aerobic fitness not only have a lower level of UCP3 mRNA [14, 15] but also a lower UCP3 protein content. This result confirms data reported previously in subjects after 3 months of endurance cycling training [9]. Reductions in the UCP3 mRNA transcript in Tr when compared to UTr subjects is most likely the cause for the lower UCP3 protein content observed in the present study, however post-translation factors cannot be excluded.

We interpret the lower UCP3 content in the Tr group when compared to the UTr group to be an effect of training, however, the difference in UCP3 protein content may be attributed to the difference in fiber composition. UCP3 protein content is greater in type II than in type I fibers with the former fiber type expressed less in the Tr than in the UTr muscle. It is therefore possible that the lower UCP3 protein content in the Tr group is due to their lower percentage of type II fibers.

We observed for the first time that the UCP3 protein was expressed decrescendo in IIx>IIa>I muscle fiber types in a group of endurance trained cyclists. The same muscle fiber type hierarchy also existed in the UTr group which supports results previously reported by Hesselink et al. [19]. Type II muscle fibers have a lower oxidative efficiency than type I muscle fibers. The ratio of phosphate produced per oxygen consumed (P/O ratio) has been found to be 18% lower in type II than in type I muscle fibers [29, 30]. The higher UCP3 protein content in type II than in type I fibers might, in part, be responsible for this lower P/O ratio.

The observation in endurance trained subjects of an increased resting metabolic rate [11] despite a decrease in muscle UCP3 suggests the idea that UCP3, although it uncouples oxidative phosphorylation, might not be thermoregulatory [2, 3]. Hence other hypothesized functions have been suggested. Recently it has been reported that the increase in muscle mitochondria proton leak, induced

Fig. 2 Triple-immunofluorescent staining of type I muscle fibers stained red; type IIa muscle fibers stained blue; type IIx muscle fibers were unstained; UCP3 protein stained green. The three

images are of the same area of the same section and demonstrate that UCP3 is expressed the most in type IIx and the least in type I muscle fibers

by exogenous superoxide production in the presence of free fatty acids, was due to an increase in UCP3 activity [31]. As an extrapolation of these observations a hypothesis was put forward that the biological role of UCP3, *in vivo*, could be to decrease reactive oxygen species (ROS) production and protect against their deleterious effects.

Muscle contraction has been found to be associated with an increased production of ROS [32, 33] while an acute bout of exercise performed in the fasted state has been observed to increase UCP3 mRNA in skeletal muscle [34, 35]. Endurance training has been shown to reduce exercise-induced ROS production in erythrocytes immediately after a bout of exhausting exercise [36] but not in skeletal muscle when measured 24 h after exercise

[37]. These differences may be due to the time of sampling. High-intensity training has been found to increase skeletal muscle anti-oxidant defense [37, 38]. The latter was greater in subjects with a higher as compared to a lower level of aerobic fitness [39]. Therefore training would provide a greater protection against ROS production during exercise. UCP3 mRNA is reduced in subjects with a higher as compared to a lower level of aerobic fitness and is negatively correlated with $\dot{V}O_{2\max}$ [14, 15] and we report in the present study that the UCP3 protein also follows these trends. Therefore a positive relationship might exist at rest and during exercise in UTr and Tr subjects between ROS and UCP3 levels. This suggests that ROS might positively

Fig. 3 UCP3 protein content in the different muscle fiber types of the UTr ($n=5$) and Tr ($n=5$) subjects. *Black columns*: type I muscle fibers; *hatched columns*: type IIa muscle fibers; *empty columns*: type IIx fibers. *Significantly different from each other, $P<0.0167$; **lower expression than in the same fiber of the UTr group, $P<0.001$. Values are mean $\pm$ SEM

regulate not only UCP3 activity [31] but also UCP3 protein level in the mitochondria. Clearly further investigations are required to test this hypothesis.

UCPs have been postulated to uncouple oxidative phosphorylation with a resulting decrease in the efficiency of aerobic ATP production. The consequence of the increase in UCP3 levels during acute exercise would be a decreased mechanical efficiency. The decrease in UCP3 level observed after training on the other hand, might contribute to the training induced improvement in whole body mechanical efficiency during exercise [12]. Indeed this is supported in the present study by a higher mechanical efficiency observed in the Tr when compared to the UT group.

A new finding from the present study is the observation of a similar reduction of UCP3 content in the type I and IIa fibers (33% and 38%, respectively) in the Tr when compared to the UTr group. Antioxidative enzymes have been reported to be predominantly activated in highly oxidative muscle by endurance training in rodents [40, 41]. Our observation of a similar reduction in UCP3 in the type I and IIa fibers might mean that endurance training decreases ROS production similarly in these two types of fibers. One possible explanation for the significantly lower decrease in UCP3 in the IIx muscle fibers (28%) between the Tr and UTr subjects might be a reduced recruitment of these fibers during endurance training. Endurance training often occurs at an intensity which preferentially recruits type I muscle fibers [42]. In contrast, sprint training which recruits type II muscle fibers and has been found to increase their antioxidant capacity [43]. Sprint training might also reduce UCP3 content more in these fibers than in type I fibers.

Another proposed function for UCP3 may be to protect the mitochondrial matrix from the toxic effect of accumulating nonesterified fatty acids (NEFAs) in situations where fatty acid delivery exceeds oxidation [9]. This hypothesis is in line with the function of UCP3 as a protector against ROS production as high intracellular levels of fatty acids increase ROS production [44]. The lower UCP3 protein expression in all muscle fiber types of the Tr group may arise from a reduction in the need to protect mitochondria against high levels of accumulating NEFAs due to an improved fat oxidation capacity [45, 46].

Conclusion

We observed that the UCP3 protein content was lower in all fiber types of Tr when compared to UTr subjects. Training status did not effect the hierarchical expression of UCP3 in the different muscle fiber types, however, it affected UCP3 content more in the type I and IIa than in the type IIx muscle fibers. The exact reason for the lower UCP3 content in the Tr when compared to the UTr subjects as well as the differential skeletal muscle fiber type expression of UCP3 remains unknown. We speculate that this decrease may be in relation to the relative improvement in the antioxidant defense systems of the skeletal muscle fibers and that it might, as a consequence, participate in the training induced improvement in mechanical efficiency.

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