HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats

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Smolka, Marcus B., Claudio C. Zoppi, Armindo A. Alves, Leonardo R. Silveira, Sergio Marangoni, Lucia Pereira-Da-Silva, Josec C. Novello, and Denise V. Macedo. HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats. Am J Physiol Regulatory Integrative Comp Physiol 279: R1539–R1545, 2000.—Given the potential of reactive oxygen species to damage intracellular proteins during subsequent bouts of muscle contractions, it was suggested that, when this production exceeds the antioxidant capacity, the preexisting antioxidant pathways may be complemented by the synthesis of the defense mechanism represented by heat shock proteins (HSPs), stress proteins with the function of repair and maintaining protein folding. To test this hypothesis, we analyzed reactive carbonyl derivatives in plasma and the expression of HSP72 and activities of enzymes from the oxidative and antioxidant defense systems in the soleus muscle of sedentary rats and rats trained by two protocols: continuous and intermittent. We analyzed all three groups at rest and 2 h after acute exercise. After 8 wk of training, the animals from both groups clearly demonstrated higher resistance to exercise. Both trained groups showed significantly higher citrate synthase, catalase, and glutathione reductase activities than the control group (P < 0.01). After acute exercise, catalase and glutathione reductase activities significantly decreased (P < 0.01) and plasma reactive carbonyl derivatives significantly increased (P < 0.05) in the sedentary group, suggesting an oxidative-stress condition as responsible for exhaustion in this group. Finally, after acute exercise, the induction of HSP72 expression occurred only in the sedentary group, suggesting that HSP72 acts as a complementary protective mechanism in exercise-induced oxidative stress.

heat shock protein 72; reactive carbonyl derivatives; catalase; glutathione reductase; intermittent training; continuous training

MANY GENERATED STRESS CONDITIONS can result, at the molecular level, in a fast production of heat shock proteins (HSPs), stress proteins with the chaperone function of maintaining and repairing protein conformation. Despite the name, these proteins are implicated not only in the protection of cells from heat stress, but also against different types of proteotoxic insults such as oxidative stress, exposure to amino acid analogs, heavy metals, and others (12, 37). Some of these insults, such as oxidative stress, heat stress, and low pH resulting from lactic acid accumulation, are generated during exercise (7, 19, 25, 27, 32). Thus HSP expression could represent an important protection mechanism against exercise-induced damage to muscle. The induction of HSPs is well established in many organisms and some tissues, but the importance of such proteins in the protection against exercise stress is a topic that needs further study. It was shown that sedentary rats submitted to a bout of intense exercise have increased muscular expression of HSP72 after a period of 2 h (12, 19, 25, 28), indicating that HSP72 is rapidly synthesized in response to exercise stress. Moreover, ischemia-reperfusion studies in the cardiac muscle provide evidence for a direct role of HSP72 in the prevention of oxidative-stress damage in this tissue (16, 20, 33).

Increased energy demand during physical exercise induces a multifold increase in oxygen supply to active tissues (26). This results in an increased production of reactive oxygen species (ROS), mainly due to elevated rates of mitochondrial respiration and increased xanthine oxidase activity (27). When ROS production overcomes the capacity of the antioxidant defense system, an oxidative stress occurs and many cellular constituents, such as lipids, proteins, and DNA, may suffer oxidation due to ROS attack, deeply compromising cellular functions. There is a large variety of biochemical parameters affected by an increased level of ROS. It includes the oxidation of one or more proteins of the excitation-contraction-coupled process (5) and the production of protein reactive carbonyl derivatives, leading to loss of catalytic activity and increased susceptibility to proteolytic degradation, as already described under different pathophysiological conditions (24). Moreover, free radical formation and lipid peroxidation...
observed after a bout of exhaustive exercise performed by sedentary or trained rats led to the proposition of a cause-and-effect relationship between oxidative stress and muscle fatigue (7, 27, 35). The occurrence of fatigue, however, seems to be dependent on the exercise conditions (e.g., intensity, type, duration) used in different studies (26).

A fundamental change in skeletal muscle in response to endurance training is the up adaptation in the antioxidant and oxidative enzyme activities (6, 23, 26, 34, 35). These and other adaptations seem to minimize disruption of homeostasis during an exercise bout, permitting the performance of physical work for a longer duration before fatigue (4). Thus increased activity of antioxidant defense system during physical work could contribute to a delay in the time required for the oxidative process to impair cell functions. The potential of ROS to damage intracellular proteins during subsequent bouts of muscle contractions led to the hypothesis that the preexisting antioxidant pathways may be complemented by the synthesis of another defense mechanism represented by HSPs (9).

To test this hypothesis, we analyzed the expression of HSP72, the activity of enzymes from the oxidative and antioxidant defense systems in the soleus muscle, and the plasma reactive carbonyl derivatives of sedentary rats and rats trained for 8 wk by two different protocols: continuous (CT; moderate intensity) and intermittent (IT; high intensity). The same parameters were also investigated after submitting all groups to a bout of exhaustive exercise performed in the case of the rats submitted to the EX protocol (5 animals), enabling comparison of a resting situation (48 h after exercise) with an intense exercise situation under which HSP72 accumulation occurs 2 h after exercise (12, 19, 25, 28). Animals were anesthetized with an intraperitoneal injection of chloral hydrate 10% (wt/vol), 0.3 ml for each 100 g of body weight, and right and left soleus muscles were carefully dissected and immediately frozen in liquid nitrogen and maintained at −70°C until further analysis. The left muscles were used for the biochemical assays, and the right muscles were used for HSP72 analysis.

**MATERIAL AND METHODS**

Animals. The experiments were performed using 2-mo-old male Wistar rats. They were maintained on an inverted 12-h light-dark cycle at 22°C with food and water ad libitum.

Exercise training protocol. Thirty rats that showed willingness to run on a motorized treadmill were randomly assigned to a sedentary control group (Sed) or to one of two exercise training groups (10 animals in each group). IT or CT exercise training protocols were used as outlined in Table 1. Forty-eight hours after the end of the 8-wk training period, five rats from each of the three groups were also submitted to 82 min of exercise (EX), as described in Table 2, 82 min being the maximum time that the Sed rats could run before exhaustion, when they refused to continue running.

**Materials and Methods**

Muscle samples. The soleus muscles were removed either 48 h after the end of the training protocol (5 animals) or 2 h after running in the case of the rats submitted to the EX protocol (5 animals), enabling comparison of a resting situation (48 h after exercise) with an intense exercise situation under which HSP72 accumulation occurs 2 h after exercise (12, 19, 25, 28). Animals were anesthetized with an intraperitoneal injection of chloral hydrate 10% (wt/vol), 0.3 ml for each 100 g of body weight, and right and left soleus muscles were carefully dissected and immediately frozen in liquid nitrogen and maintained at −70°C until further analysis. The left muscles were used for the biochemical assays, and the right muscles were used for HSP72 analysis.

Enzyme assays. All tissue samples were homogenized in a solution of (in mM) 440 cold sucrose, 50 MOPS, 0.01 phenylmethylsulfonyl fluoride (PMSF), and 100 EDTA (pH 7.2). Homogenization was performed using a polytron homogenizer at its highest speed for 30 s. Homogenates were then centrifuged (4°C) for 20 min at 40,000 g (38). The supernatant was decanted and assayed for citrate synthase (CS; EC 4.1.37), glutathione reductase (GR; EC 1.6.4.2), and catalase (CAT; EC 1.11.1.6) activities as biomarkers of oxidative metabolism and antioxidant system, respectively. We choose these antioxidant enzymes because the methods to measure CAT and GR activities are less sensitive to small variations in reagent concentrations; in these assays, they are not rate limiting (3). All enzyme assays were performed in triplicate according to the following methods.

CS activity was measured in a medium containing 0.1 mM DTNB and 0.3 mM acetyl CoA by monitoring the decrease in absorbance at 412 nm for 3 min after the addition of 0.5 mM oxaloacetic acid (30). The soleus CS activity was expressed as international units per milligram wet tissue. GR activity was measured using a medium containing 2 mM EDTA, 0.1 mM NADPH, and 0.75 mM DTNB. Glutathione disulfide (1 mM) was added into the cuvette to start the reaction, and the absorbance was followed at 412 nm for 3 min (29). The soleus GR activity was expressed as international units per milligram wet tissue. CAT activity was

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Animals trained 5 days/wk. Grade was always −3°.
measured in 50 mM phosphate buffer (pH 7.0) by monitoring the decrease in absorbance at 240 nm for 30 s after the addition of 10 mM hydrogen peroxide. One unit of catalase activity is the amount of enzyme present that decomposes 1 μM H₂O₂/min at 25°C (1).

**Plasma samples.** The blood samples (5 ml) were collected through hepatic vein using heparinized syringes. Plasma was separated from blood cells by centrifugation at 1,000 g for 10 min, transferred to a fresh tube, and stored at −80°C until analysis.

**Plasma reactive carbonyl derivatives.** The plasma reactive carbonyl derivatives were determined by carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH), using the spectrophotometric method (10). Plasma (200 μl) was mixed with 1 ml H₂O and 2 ml 20% TCA and centrifuged at 1,000 g for 10 min. The pellet was resuspended in 1 ml of 10 mM DNPH and incubated for 60 min at 37°C. For control, 1 ml of 1 M HCl was used instead of DNPH. Subsequently, 1 ml of 20% TCA was added, and the sample was centrifuged at 1,000 g for 10 min. The pellet was washed with 1:1 ethanol-ethyl acetate solution and centrifuged at 1,000 g for 10 min. The pellet was mixed with 1 ml of 6 M guanidine (diluted in 20 mM H₃PO₄, pH 2.3). Finally, the sample was incubated for 40 min at 37°C. The absorbance was measured at 380 nm. The analyses were done in triplicate, and the mean was used for statistical analysis.

**HSP72 analysis.** The whole right muscle was carefully minced and homogenized at 4°C in three volumes (wt/vol) of a buffer (pH 7.2) containing (in mM) 300 NaCl, 100 KH₂PO₄, 50 K₂HPO₄, 10 EDTA, and 2 PMSF. The homogenate was sonicated in an ice bath for 5 min and then centrifuged at 500 g for 5 min. The supernatant was diluted 1:1 in sample buffer (pH 6.8) containing 180 mM Tris·HCl, 30% glycerol, 6.9% SDS, and 200 mM dithiothreitol. These samples were stored at −20°C until SDS-PAGE separation using 11% acrylamide gels, in which 80 μg of total muscle protein was loaded. The gels were used either for total protein staining using Coomassie blue or for electrotransfer of proteins to polyvinylidene difluoride (PVDF) membranes (Waters) using a TE mini gel transfer system (Amersham Pharmacia, Uppsala, Sweden). After protein transfer, PVDF membranes were blocked with 5% nonfat dried milk powder in Tris-buffered saline (TBS; 150 mM NaCl and 20 mM Tris·HCl, pH 7.5) for 1 h. Blots were washed for 1 min in TBS + 0.05% Tween 20 (TBST) and incubated overnight at 4°C with a monoclonal antibody specific for HSP72 (SPA-810, StressGen, Canada) diluted 1:1,000 with TBST. The membranes were then washed three times (10 min each) with TBST and incubated for 1 h with the secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase; Promega) diluted 1:2,000 in TBST. Blots were washed three times (10 min each) in TBST and developed by immersion in a buffer (100 mM Tris·HCl, 100 mM NaCl, and 5 mM MgCl₂, pH 9.8) containing commercially specified amounts of p-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Promega). Quantification of bands from blots was performed by scanning with a Sharp scanner and using the computer program ImageMaster 1D Elite (Amersham Pharmacia). Linearity was analyzed by standard curves.

**Statistical analysis.** The data are expressed as means ± SE (indicated by vertical bars in the figures). Each group was composed of five animals. The program “Statistics for Windows” version 4.3 (Statsoft, 1993) was used for statistical analysis using P < 0.01 and P < 0.05 as the confidence limit.

**RESULTS**

The effect of different training protocols and a bout of exhaustive exercise on oxidative metabolism. Figure 1 shows the soleus CS activity of the three groups (Sed, CT, and IT) measured at the end of the 8-wk training period. The activities were determined either at rest (48 h after the last exercise of the training protocol in the case of the trained rats) or 2 h after EX. We can observe that the different training protocols resulted in different degrees of increased oxidative capacity. The IT group showed significantly higher CS activity compared with the CT group. Compared with the Sed group, muscle CS activity was 3.75-fold higher for the CT and 5.5-fold higher for the IT group. The data also show that, compared with the respective resting value, no significant variation on CS activity occurred after EX for all groups examined.

The effect of different training protocols and a bout of exhaustive exercise on biomarkers of oxidative stress. Figure 2 shows plasma reactive carbonyl derivatives (RCD) as a biomarker of protein oxidative attack (Fig. 2A) and the activities of soleus enzymes GR and CAT as biomarkers of an antioxidant system (Fig. 2, B and C) measured under the same experimental conditions described for the CS activity. We can observe that, after 8 wk of training, a significant increase in plasma RCD occurred only in the IT group compared with control group (Fig. 2A). On the other hand, the activities of the antioxidant system were significantly greater in both trained groups (Fig. 2, B and C), although a significantly higher increase was found in CT compared with either IT (1.6-fold for GR and 1.5-fold for CAT) or control groups (3.3-fold for GR and 5.2-fold for CAT). We can also observe that 2 h after EX, only in the Sed group, the activities of the antioxidant enzymes GR and CAT significantly decreased compared with the resting values. For both trained groups, there was no significant decrease in the activities of GR or CAT.

Figure 3 shows the 48-h kinetics of plasma RCD in Sed group after EX. We can observe a peak after 6 h that could reflect the ROS-induced damage in the tis-
sue proteins. When the peak concentrations of plasma RCD in the Sed group at rest and after exhaustion are compared, a significant increase is observed. Taken together, these findings are consistent with an increase in oxidative stress level under exhaustive conditions for the control group.

The effect of different training protocols and a bout of exhaustive exercise on soleus HSP72 expression. Figure 4 shows representative Western blot detections of HSP72 in soleus muscle of rats from the three groups at rest and after 82 min acute exercise. Data are means ± SD (n = 5). *P < 0.01 compared with Sed group. #P < 0.01 compared with IT group.

**DISCUSSION**

**HSP72 as complementary protection against exercise-induced oxidative stress.** According to the model proposed by Essig and Nosek (9), the higher the activity of the preexisting antioxidant enzymes, the more efficient the inhibition of ROS attack on proteins, thus requiring less synthesis of HSP72. In agreement with this proposal, we have shown in sedentary rats exercised to exhaustion a significant increase in HSP72 levels parallel to a decrease in the antioxidant enzymes’ activities. A study from Ji (14) analyzing the activities of muscle antioxidant enzymes of sedentary rats after exhaustive exercise did not detect any decrease in such activities, differing from our results. This could be explained by the different time used to collect the samples. In the present work, the muscles were taken for analysis 2 h after the end of exercise, whereas in the work by Ji, the muscles were taken immediately after the exercise. This difference in the time of muscle collection could reflect the kinetics of protein ROS.
attack. This idea is supported by the experiment in which it was shown that plasma RCD shows a peak of maximum concentration 6 h after exhaustive exercise, possibly reflecting tissue damage. In this situation, we have shown an unbalance condition between antioxidant defense and oxidative attack, indicative of a higher degree of oxidative stress that is parallel to an important increase in HSP72 expression.

An interesting point explored here is that both untrained and trained rats were submitted to the same bout of EX. The time of exhaustion was determined by the untrained rats. So, the effect of training in response to EX could be directly analyzed. After 8 wk of training, the animals from both trained groups clearly demonstrated higher resistance to exercise as they were able to run for longer than the 82 min of the EX protocol if they were allowed to. Moreover, EX did not result in induction of HSP72 or on decreased activity of the antioxidant enzymes in either group of trained animals. Our interpretation is that EX was less stressful to the trained rats due to a higher activity of the preexisting antioxidant enzymes GR and CAT in the soleus muscle of these animals. Although plasma RCD was significantly higher in the IT group at the end of training, the HSP72 levels were not. Possibly, the significant increase in the antioxidant enzymes’ activities compared with the control group could be sufficient to promote the resistance to EX observed in this group.

Experiments from other groups using rat heart reinforce the idea of the HSP72 as a complementary protection against oxidative stress when the preexisting antioxidant defense system cannot efficiently combat ROS attack. Turrens et al. (33) showed that protection from reperfusion injury by preconditioning hearts does not involve increased antioxidant defenses. Kukreja et al. (16) demonstrated that oxidative stress increases HSP72 mRNA in perfused rat heart. Demirel et al. (8), analyzing hearts from trained and untrained rats, showed that training provides protection against myocardial lipid peroxidation induced by ischemia-reperfusion and that this protection was not associated with increased antioxidant activity, but with increased levels of HSP72. In heart, training does not seem to upregulate antioxidant enzymatic activity; so, the high HSP72 expression observed could represent a way of complementing the protection against ROS damage.

In agreement with others, we have shown that the antioxidant enzymatic activities from soleus muscle, differently from heart, may suffer modulation by exercise. It is possible that ROS attack on proteins in the muscle of rats from the Sed group could be the signal for HSP72 induction. The model proposed by Morimoto (21) and Voellmy (36) suggests that, under normal conditions, HSP72 is bound to heatshock factor and, in a proteotoxic stress condition, HSP72 binds to unfolded proteins leaving the HSF free to trimerize, entering the nucleus and activating HSP expression. In this sense, our data suggest that HSP72 is part of a secondary antioxidant defense system acting to provide fast additional protection when the main system is also attacked. Probably, due to higher levels of HSP72 in soleus muscle, oxidized or partially denatured muscle enzymes could be more rapidly stabilized, possibly preventing loss of function. On the other hand, if the preexisting antioxidant enzymatic system is sufficiently efficient to avoid or minimize the ROS attack to antioxidant enzymes, the HSP72 induction will not be triggered or will be low.

Muscle adaptations to different training protocols.

The question of whether exercise intensity and duration increase the skeletal muscle antioxidant status still remains controversial. Whereas some authors have shown increased antioxidant enzymatic activity, mainly induced by high intensity endurance training, others do not report any exercise effect on these enzymes (2, 17, 22). The upregulation seems to be fiber-type specific, with highly oxidative muscles being most responsive (17, 18, 22). Additionally, information on the differential effects of the aerobic continuous versus intermittent high-intensity exercise training on skeletal muscle antioxidant status is scanty. Thus these ambiguous results could be related to interstudy differences in the exercise used to train the animals and/or differences in the muscle fiber types studied. This led us to compare the effects with two-experiment protocol training in the soleus muscle of rats.

An original finding here reported is that intermittent training results in a significant higher increase of the oxidative capacity but induces a lower increase in the activity of the antioxidant enzymes CAT and GR compared with continuous training. The data shown in Fig. 1 reflect, at the molecular level, the requirement of relatively high amounts of intensive exercise to push the performance capacity to its upper limit in athletic training (15). With the use of another antioxidant enzyme, Criswell et al. (6) have shown that intermittent exercise was superior to moderate continuous exercise in upregulating muscle glutathione peroxidase and that both resulted in a similar increase in superoxide dismutase activity. However, in contrast to our data, they observed almost the same increase in CS activity in trained rats compared with control group, independently of the training protocol used (6).
The cellular signals that regulate gene expression for individual antioxidant enzymes are still unclear, but there is the proposition that an increased rate of free radical formation may act as potential stimulus for the upregulation of antioxidant enzyme activity in muscle (14, 6). Our results indicate, however, that there is a limit of ROS production able to increase the activities of both enzymes analyzed. It seems that if the radical production was too high, the antioxidant enzyme itself could be oxidatively attacked. This could explain the lower increase in the activity of antioxidant enzymes and the higher plasma carbonyl derivative concentration observed at the end of training in the IT compared with CT group, as shown in Fig. 2. These results suggest that the animals trained by the intermittent protocol (high intensity and short duration) may be more vulnerable to an oxidative stress situation than the animals trained by the continuous protocol (low intensity and longer duration). Reinforcing our data, it was recently demonstrated that there were no alterations in thiobarbituric acid reactive substances or levels of reactive carbonyl derivatives after continuous swim training, reflecting a beneficial effect of exercise (24). Conversely, CS activity upregulation seems to be dependent on a higher ROS production level. In the CT group, in which the higher antioxidant enzymatic activities were sufficient to decrease ROS attack, the CS activity was lower compared with the IT group.

Perspectives

This work demonstrates that HSP72 may represent an important mechanism of protection against oxidative damage of proteins caused by ROS. The involvement of HSP72 (and other HSPs) in the antioxidant defense system is a topic still unclear and that certainly needs to be better analyzed. It is possible that the regulation of HSP72 expression represents an important point in muscle resistance to exercise stress. HSP72 may be an important biomarker of fatigue. In a physiological view, to achieve the best condition to compete, an athlete must be submitted to increasing intensities of training. One of the main problems is to detect an overtraining condition in the beginning, before the onset of tissue damage. So, it is extremely important to find appropriate biomarkers to finalize the installation of oxidative stress at its very beginning. The biomarkers used here, as CAT and GR activities, plasma reactive carbonyl derivatives, and HSP72, show a close correlation between exercise intensity and oxidative damage. In this sense, the HSP72 detection could be a useful tool to balance the intensity of training exercises preventing the athlete from losing his or her performance.

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