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Regular Training Modulates the Accumulation of Reactive Carbonyl Derivatives in Mitochondrial and Cytosolic Fractions of Rat Skeletal Muscle

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The oxygen flux into the mitochondria of skeletal muscle increases with exercise. However, the extent of oxidative damage to mitochondrial proteins of skeletal muscle has only been estimated. We studied the alteration of reactive carbonyl derivatives (RCD) in mitochondrial and cytosolic fractions of skeletal muscle following 9 weeks of swimming training in rats. The RCD content of mitochondria was significantly elevated compared with the cytosolic fraction of both control and exercised animals. Accumulation of RCD in muscle mitochondria of the exercised group was also significantly elevated ($P < 0.05$). On the other hand, alteration of the accumulation of RCD was not apparent in the cytosolic fraction of skeletal muscle. The activity of proteasome complex, however, was increased in the cytosolic fraction of exercised muscle ($P < 0.05$). The data suggest that mitochondria of skeletal muscle accumulate significantly larger amounts of RCD than the cytosolic fraction and the tendency of the accumulation varies in cell fractions. Exercise training increases the accumulation of protein damage in mitochondria of skeletal muscle but cytosolic proteins are protected by increased activity of proteasome complex and possibly by other antioxidant enzymes.

Key Words: **exercise; free radicals; oxidative stress; reactive carbonyl derivatives (RCD); mitochondria; proteasome; adaptation.**

The mitochondrial electron transport chain is the major intracellular source of reactive oxygen species (ROS), which are generated as byproducts during transfer of electrons from NADH or FADH2 to O_2 under normal physiological conditions (1–3). During physical exercise there is a significant need of enhanced ATP production, which is associated with a large oxygen flux into the mitochondria of working skeletal muscle. Direct evidence suggests that exhaustive physical exercise increases the rate of ROS generation in skeletal muscle (4). However, there is a paradox regarding the effects of exercise. While exercise can induce ROS formation, which may be detrimental to cellular functions, it might reduce a variety of age-related diseases, extend life-span, and improve quality of life in general (5, 6). The biochemical mechanisms by which regular exercise significantly benefits health and well being, including depression of the incidence of diseases, are not well understood. Mitochondria, which house the site of the electron transport chain, could be the main targets of ROS resulting in modification of certain amino acids (1, 3, 7–9). Oxidative damage of mitochondrial proteins might have serious consequences because it could effect and defect the vital respiration process in the mitochondria (1). ROS induced oxidation of arginine, lysine, threonine, or proline amino acid residues generates reactive carbonyl derivatives (RCD), which can be readily measured by reaction with 2,4-dinitrophenyl hydrazine (10–12). In addition to ROS, lipid peroxidation by-products such as aldehydes, especially the very reactive 4-hydroxynonenal, and ketons can also generate the formation of RCD. The determination of RCD is not without prob-

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lems, however, simultaneous application of spectrophotometric and immunoblot measurements could decrease the possible errors of currently used method (13–16). Indeed, the appearance of RCD leads to inactivation of proteins and accumulation of RCD is linked to a variety of diseases and the aging process (13, 17–22).

The effect of regular exercise on the accumulation of RCD in skeletal muscle mitochondria is not known. Therefore, the present investigation was designed to study whether 9 weeks swimming modulated the accumulation of RCD in mitochondrial and cytosolic fractions of skeletal muscle. The accumulation of oxidatively modified proteins is dependent upon the rate of protein degradation. Most native and modified proteins in eukaryotic cells are believed to be degraded by nonlysosomal pathways involving 20S and 26S proteasomes (23). It is assumed that 20S and 26S forms of proteasomes coexist in cells and 20S proteasome is activated by a variety of treatments such as sodium dodecyl sulfate (SDS). In the present study we measured the basal peptidase activity of proteasome complex (24).

METHODS

Twelve Wistar rats (4 weeks old) were randomly assigned to control and swimming-exercised trained groups. All animals were cared for according to the "Guiding Principles for the Care and Use of Animals." The exercised rats were exposed to swimming exercise for 9 weeks. The water temperature was maintained at 32°C and the swimming duration was 60 min per day for 5 days a week for 6 weeks. The training regimen was increased to 90 min per day for 5 days a week, for the remaining 3 weeks.

One day after the last training session the rats were sacrificed by decapitation. The mitochondria of gastrocnemius muscle were separated as described by Erstner and Nordenbrand (25). In brief, 3 g of skeletal muscle were minced and homogenized in 10 vol of ice cold Chappell–Perry medium (0.1 M KCl, 0.05 M Tris–HCl buffer (pH 7.4), 0.001 M Na-ATP, 0.005 M $MgSO_4$, and 0.001 M EDTA) with a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 650*g* for 10 min. The supernatant fraction was decanted into a new tube and recentrifuged, as before, to remove residual myofibrils. The resulting supernatant was centrifuged at 14,000*g* for 10 min. The pellets were resuspended in Chappell–Perry medium and recentrifuged at 14,000*g* for 10 min. This washing was repeated one more time and then the washing medium was discarded and the mitochondrial pellet was resuspended in 0.25 M sucrose and stored at −80°C for analysis. The purity of the mitochondrial section was apprised, when the Cu,Zn-SOD (cytosolic enzyme) protein content was measured by ELISA method (26). Cu,Zn-SOD was undetectable indicating that the contamination of mitochondrial fraction was not significant.

The RCD was detected by spectrophotometry and immunoblot methods using antibodies against 2,4-dinitrophenyl hydrazones (DNPH) of oxidized bovine serum albumin as described earlier (10, 11). In brief, proteins precipitated with trichoroacetic acid were suspended and incubated in a solution containing 10 mM DNPH and 2 N HCl for 1 h at 15°C. The resulting protein hydrazones were pelleted in a centrifuge at 11,000*g* for 5 min. The pellets were washed three times with ethanol-ethyl acetate (1:1) and then once with acetone. The final precipitates (1 mg protein) were dissolved in 1 ml buffer containing 8 M urea and 5% 2-mercaptoethanol using a sonicator for 10 min. The protein content was remeasured and following the RCD spectrophotometric measurement the same samples were further used for Western blot. Duplicate polyacrylamide gel electrophoresis of derivatized proteins was carried out in 10% polyacrylamide gels containing 0.1% SDS. After the electrophoresis the proteins were transferred to nitrocellulose membranes. Then the membranes were soaked in PBS containing 3% skim milk, 0.05% Tween and 0.05% sodium azide and then treated with anti-DNPH antibody (10). After washing the buffer without antibodies, the membranes were treated with ^{125}I-Protein A (0.02 μCi/ml). Finally, the radioactive signals were quantified by BAS 2000 Bioimaging Analyzer (Fuji Film Co., Japan).

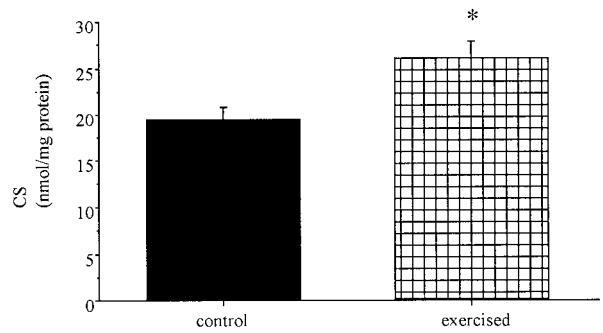

FIG. 1. CS activity increased significantly in skeletal muscle of exercised rats. Values are mean ± SD of six animals per group (n = 6). *$P < 0.05$.

Proteasomes are a cytosolic enzyme complex, which have at least five distinct peptidase activities (23) and among these, two types of peptidase activities were measured as described previously by Hayashi and Goto (24). These peptidase activities were determined fluorometrically by measuring the release of 7-amino-4-methyl-coumarin from the peptides succinyl-Leu-Leu-Val-Tyr-MCA (SUC-LLVY-MCA) and butyloxycarbonyl-Leu-Arg-Arg-MCA (BOC-LRR-MCA) for chymotrypsin-like and trypsin-like activities, respectively. Incubation was carried out at 37°C for 30 min in buffer containing 100 mM Tris–HCl (pH 9.0) and 20 ml of gradient fraction, using samples with 5 μg of protein content, in a total volume of 50 ml. Fluorescence intensity was measured by the excitation at 380 nm and emission at 440 nm.

Citrate synthase (CS) catalyzes the reaction between acetyl-CoA and oxaloacetate to form citrate. The activity of CS was measured by standard techniques as previously described by Shephard and Garland (27) to appraise the training effect on the tricarboxyl cycle. Two-way ANOVA was applied to determine the differences in variables between C and E groups. When applicable, an unpaired Student's t test was used. Significance was set at $P < 0.05$.

RESULTS

Nine weeks of swimming increased the activity of CS in the exercised group, indicating that the given training regime induced adaptation to oxidative metabolism (Fig. 1). The data revealed that the accumulation of RCD was more enhanced in mitochondria compared with the cytosolic fraction ($P < 0.05$). The mitochondrial fraction of skeletal muscle of exercised animals contained significantly more RCD than that of control rats ($P < 0.05$), as measured by spectrophotometry and Western blot (Figs. 2 and 3). The Western blot signals indicated that the accumulation of RCD is de-

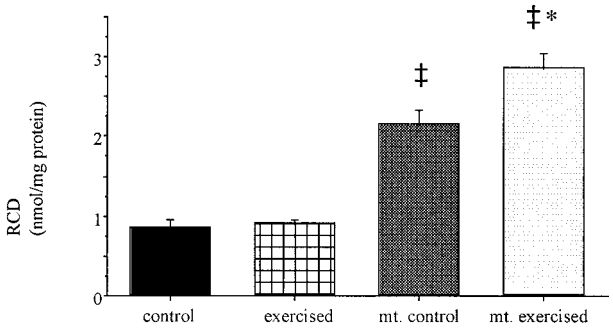

FIG. 2. Accumulation of reactive carbonyl derivatives (RCD), measured by spectrophotometer, was significantly higher in mitochondrial fraction of skeletal muscle than cytosol. Exercise training increased the accumulation of RCD in mitochondria (mt) of skeletal muscle. The amount is normalized by protein content. Values are mean ± SD of six animals per group. $^{‡}P < 0.05$ cytosol vs mitochondria; $^{*}P < 0.05$ control vs exercised.

pendent upon the amount of protein; a stronger protein band is associated with a stronger RCD band. However, some proteins with similar concentrations accumulated more RCD in mitochondria obtained from exercised animals (Fig. 3). There is a possibility that the exercise caused a physiological challenge, which made these proteins prone to accumulate RCD. On the other hand, no apparent alteration occurred in the accumulation of RCD in the cytosolic fraction of the skeletal muscle. Exercise training increased both kinds of peptidase-like activity of proteasome complex in the cytosol (Fig. 4).

DISCUSSION

We tested the hypothesis that regular swimming causes oxidative stress leading to oxidative protein damage of cytosol and/or mitochondria. The data revealed that the accumulation of RCD was more enhanced in the mitochondria of exercised rats than control animals. This could be due to the fact that the mitochondrial ROS production is markedly increased during exercise (4), although, in general, regular exercise at sea level tends to maintain or decrease the oxidative damage of proteins (14, 15, 28). Even though Witt *et al.* (29) reported an increase in RCD accumulation. It is possible that the type of exercise (running vs swimming) differently effect the antioxidant and repair systems or the extent of oxidative damage. Therefore, it cannot be excluded that swimming causes hyperthyroidism that may lead to mitochondrial swelling and proliferation. The increased cytochrome *c* associated with mitogenesis may partly account for the increased level of mitochondrial RCD. However, our earlier findings indicate that swimming training results in adaptation against oxidative stress measured by decreased nuclear DNA damage, maintained RCD level and increased resistance against chronic hydrogen peroxide treatment (15, 30). The interpretation of elevated RCD levels in mitochondria of exercised animals is complex, since direct evidence is not available on the effect of regular exercise on mitochondrial ROS production. An increase in the activity of mitochondrial superoxide dismutase can be regarded as indirect evidence, because ROS production might serve as a stimulator of antioxidant enzymes (1). Oxidative damage markers might also be regarded as an indirect tool, since the accumulation of oxidative damage is dependent on the efficiency of antioxidant and repair systems (15).

The increase in exercise mediated accumulation of oxidative protein damage in mitochondria could be due to the increased ROS production and/or the poor proteolytic breakdown of oxidatively modified proteins. Mitochondria are equipped, however, in a limited extent with a proteolytic system. Incubation of mitochondria in air with ROS generators such as xanthine oxidase, enhance the degradation of labeled leucine, while

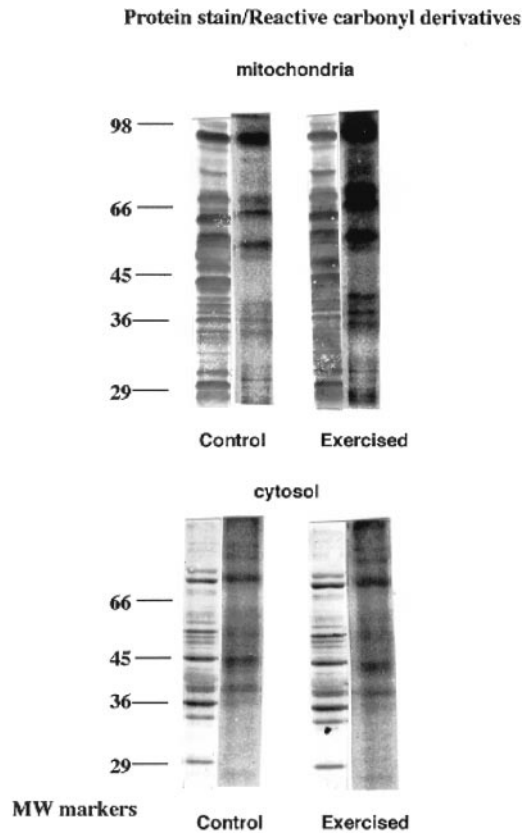

FIG. 3. The same mitochondrial and cytosolic fractions were used in Western blot as in spectrophotometric measurements. Western blot data revealed that proteins with a molecular weight around 84 kDa (probably aconitase) are significantly carbonylated in mitochondrial fraction. The RCD accumulation is more enhanced in exercised group. The RCD signals are to the right of Coomasine blue panels, and the molecular weight markers are at left.

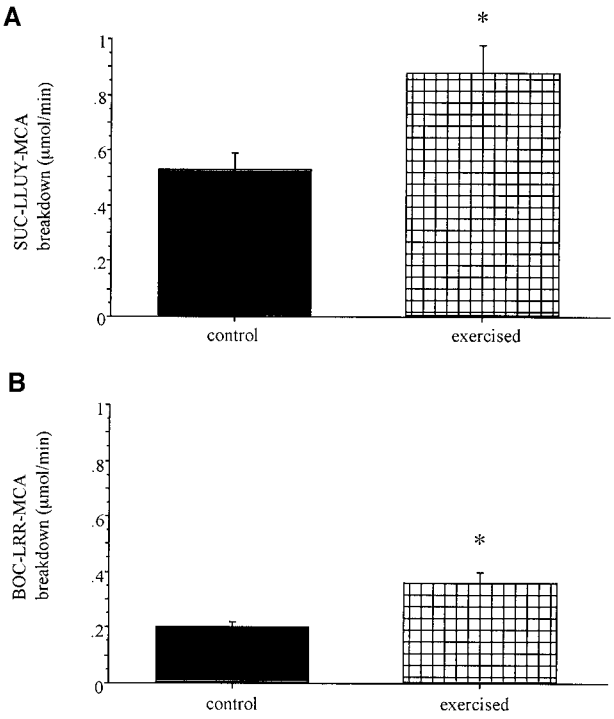

FIG. 4. The chymotrypsin-like activity (breakdown of SUC-LLVY-MCA) of proteasome complex is shown in A, while the trypsin-like activity (breakdown of BOC-LRR-MCA) is shown in B. Exercise increased both kind of activity. Values are mean ± SD of six animals per group. $*P < 0.05$ control vs exercised.

no stimulation is observed under incubation in a nitrogen atmosphere (31). Indeed, in general, oxidative modifications of proteins activate the proteolitic system (20, 21). However, when the rate of oxidative damage exceeds the removal capability of the cell or cell fractions an accumulation occurs. The peptidase activities of proteasome complex were induced by hydrogen peroxide treatment and with an increase in RCD concentration (30, 31). Hence, the ability of cytosol to remove oxidatively modified proteins might be greater than mitochondria, since cytosol hosts the proteasome complex, which is the major enzyme to break down oxidatively modified proteins (32, 33). It cannot be ruled out that similar to the poor repair capability of DNA damage (19) the ability of mitochondria to repair/breakdown oxidatively modified proteins is also limited. Therefore, it appears that the RCD removing ability of mitochondria is not adequate to prevent the exercise-induced accumulation of RCD.

Yan *et al.* (9) have demonstrated that mitochondrial aconitase is especially sensitive to accumulated RCD in the aged housefly. In our mitochondrial preparation, we have also observed accumulation of RCD in proteins having molecular weights around 80 kDa (the molecular weight of aconitase is 84 kDa). However, the extent of RCD accumulation in this rat mitochondrial fraction is not as massive as reported by Yan *et al.* (9). This might be due to a species difference and/or the tissue measured. As a result of attack by ROS, aconitase could release free iron, thereby promoting further protein damage (34, 35). It is well known that during mild oxidative stress the oxidative modification of proteins takes place in a selective manner and iron-containing proteins, like aconitase, are especially prone to oxidative damage (20, 21). The possible damage of aconitase might have serious consequences since it is involved in the processes of the citric acid cycle. However, oxidative modifications of proteins might activate proteolysis and faster protein turnover (20–22). It can be suggested that newly made proteins are able to cope with greater physiological challenges than old proteins. Therefore, oxidative modifications of proteins, to a certain extent, might be a signal for adaptive response.

Long-term regular exercise decreases the rate of lipid peroxidation in rat organs (35). Recently, we demonstrated that accumulation of RCD after regular exercise decreases in some proteins (15). In the present study we found that the RCD content in the cytosol did not change as a result of exercise. There is little doubt that exercise increase ROS production (4, 36). However, it seems that this increase is compensated for by the activity of antioxidant and repair enzymes which results in maintenance of RCD content in cytosol. To our knowledge there is only one study that measured the activity of proteasome as a result of exercise (15). In concurrence with that finding we observed an increase in proteasome activity in cytosol, indicating that the removal of oxidatively modified proteins is faster in exercise trained muscle. The maintenance of the accumulation of RCD with an increased level of proteasome activity might indicate a constant oxidative damage and efficient removal of oxidatively modified proteins. The study of Hayashi and Goto (24) revealed that without SDS induction of 20S proteasome the peptidase activity was almost exclusively found in the 26S complex. The 26S proteasome is known to be responsible for the degradation of ubiquitinated proteins, which accumulate as a function of age (24, 25). The 20S proteasome, on the other hand, is believed to be responsible for the degradation of oxidatively modified proteins (32). The method used in this study does not allow exclusively naming the 20S or 26S proteasome as an exercise-inducable form; however, we hypothesize that 20S are mostly activated by exercise to eliminate the oxidatively modified proteins. Nevertheless, the data indicate that regular exercise-induced adaptation involves the up-regulation of proteasome complex which serves as a repair and antioxidant enzyme (37).

In summary, we have shown that mitochondria of skeletal muscle contain greater amounts of RCD than cytosol and that regular swimming training increases the accumulation of RCD in mitochondria of skeletal

muscle. Our data indicate that mitochondria are one main source and target of exercise-induced ROS production and attack. The regular exercise induced beneficial adaptive process might include increases in the removal of oxidatively modified proteins in different cell fractions.

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