

THE EFFECT OF EXERCISE TRAINING ON OXIDATIVE DAMAGE OF LIPIDS, PROTEINS, AND DNA IN RAT SKELETAL MUSCLE: EVIDENCE FOR BENEFICIAL OUTCOMES

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Abstract—Moderate daily exercise is known to be beneficial to health, reducing risks of a number of age-related disorders. Molecular mechanisms that bring about these effects are not clear. In contrast, it has been claimed that some types of prolonged physical exertion are detrimental to health because active oxygen species are generated excessively by enhanced oxygen consumption. Using two age groups of rats, young (4 week) and middle aged (14 months), we investigated the effects of long-term swimming training on the oxidative status of phospholipids, proteins, and DNA. The concentration of thiobarbituric acid reactive substances and 4-hydroxynonenal protein adducts did not differ in the gastrocnemius muscle between exercised and nonexercised animals in the two age groups. The extent of carbonylation in a protein of molecular weight around 29 KDa and the amount of 8-hydroxydeoxyguanosine in nuclear DNA were smaller ($p < .05$) in the exercised rats than in the sedentary animals. Activities of DT-diaphorase (C1: 29.3 ± 1.9 ; C2: 36.1 ± 2.6 ; E1: 27.2 ± 1.3 ; C2: 33.4 ± 2.9 nmol/mg protein) and proteasome, a major proteolytic enzyme for oxidatively modified proteins were significantly higher in the exercised animals of both age groups ($p < .05$). The adaptive response against oxidative stress induced by moderate endurance exercise constitutes a beneficial effect of exercise. © 1999 Elsevier Science Inc.

Keywords—Oxidative damage, Exercise, DT-diaphorase, Proteasome complex, Skeletal muscle, Free radicals

INTRODUCTION

Reactive oxygen species (ROS) are formed during normal physiologic processes by nonenzymatic and enzymatic sources and continuously cause damage to lipids, proteins and nucleic acids.

Peroxidation of unsaturated fatty acid residues of phospholipids in cell membranes might result in significant loss in membrane integrity, that is one of the most striking effects of oxidative damage [1,2] leading to generation of potentially harmful aldehydes and alkanes. Unfortunately, if 4-hydroxynonenal (4-HNE) is excluded, the detection of membrane adducts is not directly measurable. Therefore, the nonspecific test of thiobarbi-

turic acid reactive substances (TBARS) is very often applied to assess the pressure of oxidative stress. In addition, oxidative modification of amino acids has significant effects on cell functions as oxidatively modified proteins lose their physiological activity and tend to be very sensitive to proteolytic degradation [3]. Oxidative modifications of amino acid residues include derivatization of amino acid residues such as proline, arginine, and lysine to reactive carbonyl derivatives (RCD) and links have been established between the degree of RCD accumulation and a variety of pathophysiological conditions [4,5]. RCD can be determined by carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH) using spectrophotometric and immunoblot methods. The cytotoxic effects of ROS are also targeting DNA bases and the unprepared mutations could lead to concomitant change in DNA genotype. The 8-hydroxydeoxyguanosine (8-OHdG), a

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guanine base modification, for instance, induces G-C to T-A transversion during DNA replication [6].

Regular physical exercise retards the aging process and extends average life-span of experimental mammals [7]. Numerous studies on humans have suggested that life long regular exercise reduces the incidence of cardiovascular diseases and certain types of cancer, and thereby prolongs life-span [8,9]. The biochemical mechanisms by which regular exercise has beneficial effects are not well understood. Single bouts of exercise of moderate intensity and long duration or of relatively short duration and high intensity might lead to increases in the content of TBARS, RCD or 8-OHdG [10–13]. It is likely, however, that regular exercise induces adaptive responses in antioxidant and repair systems and the combined effects of these changes results in enhanced protection against ROS and a decrease in the accumulation of oxidative damage [14].

The aim of our investigation was to assess whether regular exercise induced adaptations to reduce the extent of oxidative damage of lipids, proteins, and DNA. Results of our recent study were interpreted to suggest that the underlying mechanisms of lipid peroxidation and oxidative modification of proteins in muscle vary according to specific conditions [15] and DNA damage could differ as well. Accumulation of oxidative damage is dependent upon the rate of ROS generation as counteracted by the activity of repair systems, or ROS removal by degradation of the whole molecule, or a combination of these processes. To obtain a more substantial view of the mechanisms of damage accumulation we measured peptidase activities of proteasomes that are known to be involved in the degradation of oxidatively modified proteins [16].

It is well established that quinones and semiquinones play an important role in electron leakage; hence, we measured the activity of DT-diaphorase, that detoxifies quinones and semiquinones and curbs the formation of ROS and oxidative damage [17]. The anti-carcinogenic effects of DT-diaphorase are well documented [18,19]. Therefore, we were interested in whether regular exercise induces this enzyme and hence could contribute to the anticarcinogenic effects. Our hypothesis was that appropriate exercise training would not increase the level of oxidative damage of lipids, proteins, and DNA *in vivo*. Rather, we evaluated the possibility that increases in proteasome and DT-diaphorase activities would reduce the generation and accumulation of exercise-induced oxidative damage.

MATERIALS AND METHODS

Animals

Twelve young male Wistar rats aged 4 weeks, and 12 middle-aged (age, 14 month), were used in the study and cared for according to the *Guiding Principles for the*

Care and Use of Animals [20]. Six rats were randomly assigned to each of four groups: young control (C1), young exercised (E1), middle-aged control (C2), and middle-aged exercised (E2).

Training protocol

All exercised rats were exposed to swimming five times/week for 9 weeks. Water temperature was set at 32°C. Swimming duration was 60 min for 6 weeks and then it was increased to 90 min for the remaining 3 weeks. Swimming was selected because muscle trauma caused by prolonged running, exercise-stimulated electric shock, and plyometric contractions could be avoided. These factors alone could induce oxidative stress. One day after the last training session rats were killed and gastrocnemius muscles were excised and frozen in liquid nitrogen and stored at –80°C for later analysis. Subsequently, samples were homogenized for several biochemical assays.

Assays

For the estimation of the degree of lipid peroxidation the TBARS method was used [21]. To obtain more reliable data on lipid peroxidation and related oxidative damage the 4-HNE modified proteins in Western blot were measured by a commercially available monoclonal antibody as described by the supplier (Japan Institute for the Gerontology, Saitama, Japan). The measurement of RCD was carried out as described earlier [15] by a spectrophotometric method and by anti-DNPH antibodies using Western blot. In brief, proteins precipitated with trichloroacetic 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 $11,000 \times g$ 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. Duplicate polyacrylamide gel electrophoresis of derivatized proteins was carried out in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After 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 [11]. After washing the sheet with the buffer, the membranes were treated with ^{125}I -Protein A (0.02 $\mu\text{Ci/ml}$). Finally, the radioactive signals were quantified by BAS 2000 Bioimaging Analyzer (Fuji Film Co., Japan).

The isolation of nuclear DNA and the measurement of 8-OHdG was done as described by Kaneko et al. [22]. In brief, after the isolation of DNA the aqueous solution containing 50 μg DNA was adjusted to 45 μl , and 5 μl of 200 mM sodium acetate buffer (pH 4.8) and 5 μg of nuclease P1 were added. After a purge with a nitrogen stream, the mixtures were incubated at 37°C for 1 h to digest the DNA to nucleotides. Then, 5 μl of 1 M Tris-HCl (pH 7.4) and 0.65 units of alkaline phosphatase were added and the mixture was incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides. Nucleosides in samples were analyzed by the HPLC/ECD system, that consisted of a Pegasil ODS column connected to a Shimadzu LC-10 pump (Tokyo, Japan) coupled to an ECD (ESA Coulechem II 5200; Bedford, MA). The solvent system used was a mixture (pH 5.1) of 6% methanol, 12.5 mM citric acid, 30 mM sodium hydroxide, 25 mM sodium acetate, and 10 mM acetic acid. The flow rate was 1.4 ml/min. dG was calculated from absorbance at 260 nm using an UV detector (Shimadzu UVD-10). 8-OHdG was measured simultaneously by ECD. The amount of 8-OHdG in the sample was expressed relative to the amount of dG.

Proteasomes have at least five distinct protease activities [23] and among these, two types of peptidase activities were measured for each fraction of the gradient as described previously [24–26]. These peptidase activities were determined fluorometrically by measuring the release of 7-amino-4-methyl-coumarin (AMC) from the peptides succinyl-Leu-Leu-Val-Tyr-MCA (SUC-LLVY-MCA) and butyloxycarbonyl-Leu-Arg-Arg-MCA (BOC-LRR-MCA) for chymotrypsinlike and trypsinlike activities, respectively.

For the determination of DT-diaphorase activity the Erstner [27] method was used. The assay mixture contained 25 mM Tris-HCl (pH 7.4), 0.3 mM NADH, 0.04 mM 2,6-dichloroindophenol and 0.2% Tween-20. The reaction was started by the addition of the muscle extracts and absorbance was followed at 600 nm. To appraise the effects of physical exercise, the activity of citrate synthetase (CS) was measured as described by Shepherd and Garland [28].

Statistical analysis

Statistical significance was assessed using ANOVA, followed by Scheffe's posthoc test. When applicable, an unpaired Student's *t*-test was used. The significance level was set at $p < .05$.

RESULTS

Nine weeks of swimming did not result in significant differences in body mass between the exercised and

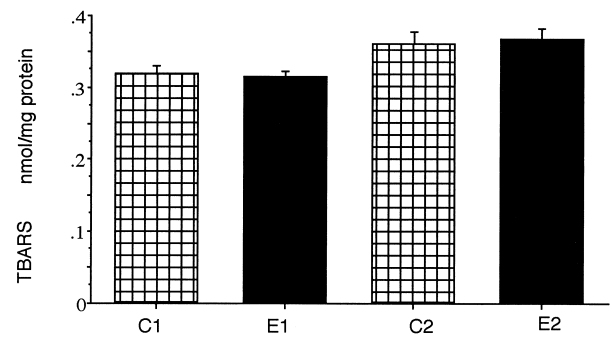


Fig. 1. Nine weeks of swimming did not result in significant alteration in the level of TBARS in gastrocnemius muscle of exercised animals compared with controls. Values are means \pm SE ($n = 6$).

control groups. The activity of CS was used as a marker of oxidative adaptation of skeletal muscle to endurance training. The training load significantly increased the activity of CS from 0.28 ± 0.02 (C1) to 0.35 ± 0.02 mol/mg protein (E1) ($p < .05$) and from 0.27 ± 0.02 (C2) to 0.41 ± 0.05 mol/mg protein (E2) ($p < .01$).

Thiobarbituric acid reactive substances level of exercised and control rats were not significantly different (Fig. 1). The content of 4-HNE was not detectable in Western blots by the commercially available antibodies indicating a very low level of 4-HNE modified proteins in all groups. Similar to lipid peroxidation data the RCD content was not significantly different between groups measured by a spectrophotometric method (Fig. 2). The densitometric data, based on Western blot analysis, however, showed that the extent of RCD in proteins having a molecular weight of approximately 29 KDa (most probably carbonic anhydrase) are higher ($p < .05$) in control groups (C1: 40 arbitrary units and C2: 44 arbitrary units) than in the exercised groups (E1: 30.4 arbitrary units, E2: 32 arbitrary units, Fig. 3). The 8-OHdG content of exercised rats was significantly less than in the age matched control groups (Fig. 4). The activity of DT-diaphorase

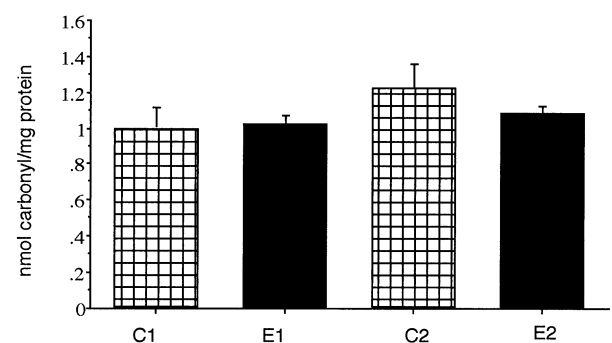


Fig. 2. The reactive carbonyl derivatives content measured by the spectrophotometric method did not show significant changes in all groups. Values are means \pm SE ($n = 6$).

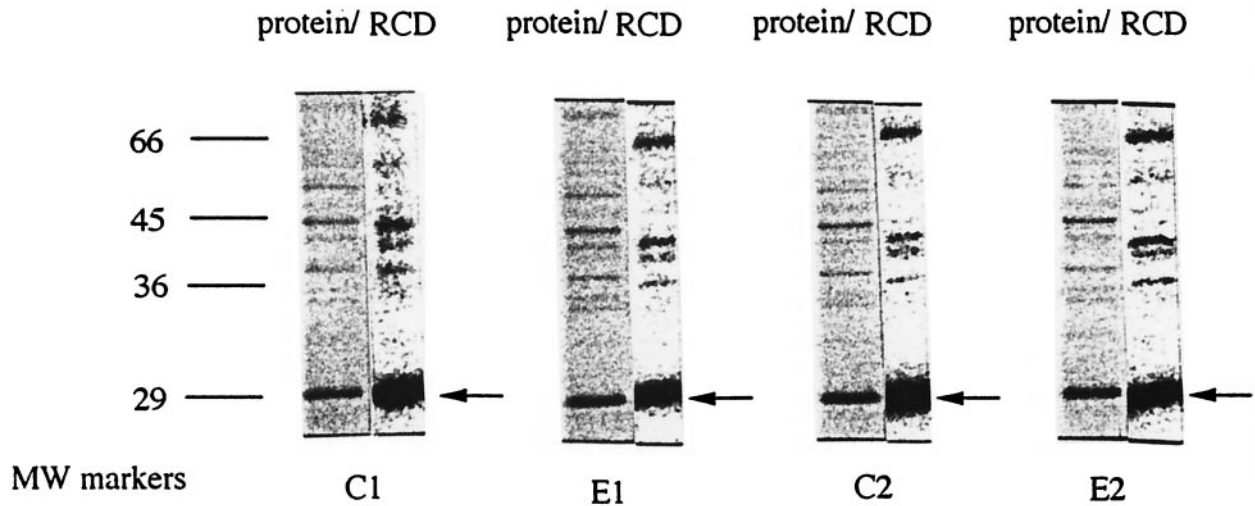


Fig. 3. Western blot data in triplicate revealed a significant increase ($p < .05$) in reactive carbonyl derivative content in proteins having molecular weight around 29,000 (most probably carbonic anhydrase) in muscle of control animals compared with exercised animals. Molecular mass markers are shown on the left of the Coomassine Blue panel.

increased in both exercised groups when compared with the parallel control groups ($p < .05$, Fig. 5).

The peptidase-like and chymotrypsin-like, trypsin-like activities of proteasome complex increased in skeletal muscle of exercised rats compared with sedentary groups ($p < .05$) indicating faster protein breakdown of exercised muscles (Fig. 6).

DISCUSSION

Despite the paradox that exercise might induce ROS production, regular exercise is known to bring about significant benefits to health and to improve quality of life. In the present study regular exercise decreased the accumulation of nuclear 8-OHdG content in skeletal muscle of exercised rats, a finding that might have important beneficial consequences, because the mutagenic

and carcinogenic effects of this modification are well established [29,30]. The reduction of 8-OHdG steady-state content indicates decreased level miscoding, mutation [6,31] and more efficient reproduction of DNA. The mechanism by which exercise reduced the accumulation of 8-OHdG is unclear, although it could involve the upregulation of both scavenger and repair systems. DT-diaphorase might be an active contributor as it is involved in the reductive activation of several cytotoxic quinones and nitrobenzenes protecting DNA and other macromolecules [18,19,32]. The exercise-induced activation of DT-diaphorase, might be an important tool to reduce the incidence of cancer as has been reported in epidemiological studies [8,9]. The reduced 8-OHdG content after regular exercise might also be the result of an induced repair system as previously shown by the results of Inoue *et al.* [33] and Pilger *et al.* [34]. Other studies indicate that a single bout of exhaustive exercise or

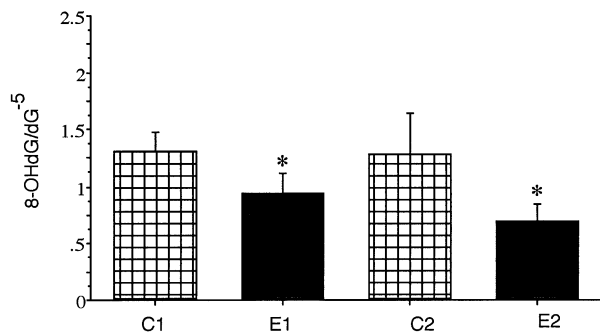


Fig. 4. The comparison of 8-OHdG content in gastrocnemius muscle of exercised and nonexercised animals shows that nine weeks of swimming significantly reduced DNA damage in muscle of exercised rats. Values are means \pm SE ($n = 6$). $*p < .05$.

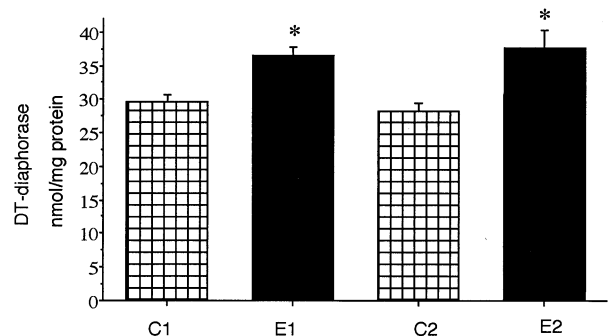


Fig. 5. The activity of DT-diaphorase was induced by exercise and showed significantly higher activity in muscle of exercised rats compared with control animals. Values are means \pm SE ($n = 6$). $*p < .05$.

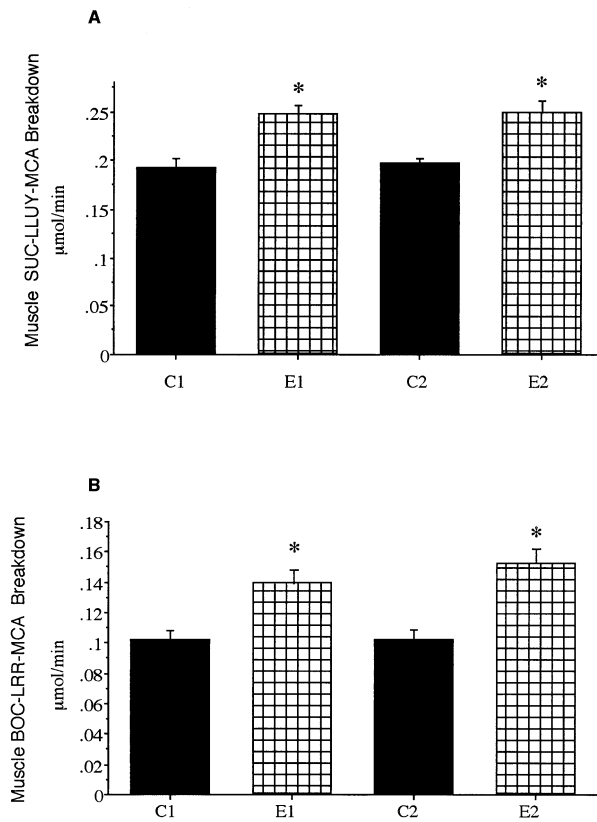


Fig. 6. The peptidase-like activity (breakdown of SUC-LLVY-MCA) of proteasome complex is shown on the upper panel (A), the chymotrypsinlike, trypsinlike activity (breakdown of BOC-LRR-MCA) on the lower panel (B) of the figure. Exercise training significantly induced both kind of activities of proteasome. Values are means \pm SE ($n = 6$). * $p < .05$.

exercise with moderate intensity maintains or increases the level of oxidative damage in macromolecules [10–13,33,34]. Hence, it seems very unlikely that the decrease in 8-OHdG content is due to the effects of only the last training session. In particular, the decrease in RCD content in a protein with a molecular weight of 29 KDa indicates that the measured values are a result of the adaptive process of skeletal muscle to regular training. The 9 weeks of swimming used in this study brought about similar changes as has been previously shown using anticancer drugs [18], such as reduced DNA damage and increased DT-diaphorase activity.

The RCD content changed significantly only in protein with molecular weight of approximately 29 KDa that stresses the reliability of immunoblot methods when compared with spectrophotometric techniques. The reduced RCD content in this particular protein could be due to the faster turnover rate of the protein, that might be associated with the increases in the activity of proteasome complex. The accumulation of oxidatively modified proteins is dependent upon the extent of oxidative stress and the rate of

protein degradation [35–37]. In the current study the RCD content in the skeletal muscle of exercised and control rats was not significantly different in proteins except for protein with molecular weight near 29 KDa. The peptidase-like and chymotrypsin, trypsinlike activities of proteasome complex were elevated. To our knowledge this is the first report on proteasome complex activity in an exercise study; although it has been reported that exercise increases the rate of both protein synthesis and degradation [for review see 5]. The increase in some proteasome complex activity with maintained level of oxidatively modified protein concentration suggests that the ROS formation was increased during exercise. This is in agreement with numerous of studies [10,18,35,38], but the ROS formation was balanced by antioxidant and repair systems. It has been previously shown that oxidative modifications of proteins induce protein degradation [36,37]. The proteasome complex could be induced by some other factors, such as cell division, antigen processing and NF- κ B [37]. Interestingly, the NF- κ B activity can be induced by exercise [39] and therefore our finding of an increase in proteasome complex activities is supported by other mechanisms.

The rate of lipid peroxidation did not change significantly that suggests a balance between ROS-induced damage to lipids and antioxidant-repair systems. The fact that the changes in the measured oxidative damage indices of DNA, proteins and lipids are not consistent, indicates that light repeated oxidative stress, such as regular exercise, affects the macromolecules or their repair systems differently. Whether the oxidative damage is repaired in a selective manner according to its physiological importance, i.e., the DNA damage, then protein and finally lipid, cannot be answered by the results of this study.

In conclusion, we suggest that regular exercise-induced beneficial effects on health are partly due to an adaptive process, that involves reduced accumulation of 8-OHdG, and increased activity of DT-diaphorase and the proteasome complex. A link is possible between the decreased level of 8-OHdG and the increased DT-diaphorase activity and the reduced incidence of cancer noted in physically active people. The degree of accumulation of oxidative damage in DNA, proteins and lipids is different after regular exercise. This is possibly due to different mechanisms or different activities of specific antioxidant and repair systems.

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ABBREVIATIONS

- C1—control young rats
 C2—control middle aged rats
 DNPH—2,4-dinitrophenylhydrazine
 E1—exercised young rats
 E2—exercised middle aged rats
 4-HNE—4-hydroxynonenal
 8-OHdG—8-hydroxydeoxyguanosine
 RCD—reactive carbonyl derivatives
 ROS—reactive oxygen species
 TBARS—thiobarbituric acid reactive substances