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Effects of Endurance Training on Three Superoxide Dismutase Isoenzymes in Human Plasma

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The effects of endurance training and acute exhaustive exercise on plasma levels of three superoxide dismutase (SOD) isoenzymes and the ability of superoxide generation in neutrophils were studied. Eighteen healthy male students, aged 17–22 years, who volunteered for this study, underwent three months of endurance training in swimming or running. Before and after the training course, they performed acute exercise and blood samples were collected before and after this exercise. The endurance training significantly increased maximal oxygen uptake ($\dot{V}O_2max$) in all subjects. Neither the endurance training nor the acute exercise affected the plasma CuZn-SOD level. Acute exercise after the training, but not before the training, increased both the plasma Mn-SOD and extracellular SOD (EC-SOD) levels by 33.6 and 33.5%, respectively. The training decreased the EC-SOD level at rest by 22.2%. Acute exercise after the training, but not before the training, increased the plasma lipid peroxide level, suggesting higher oxidative stress in trained subjects during exhaustive exercise. The ability of neutrophils to generate superoxide was increased by the acute exercise, but induction of the superoxide was suppressed after training. These results indicate that EC-SOD levels were changed in a different manner from the CuZn-SOD and Mn-SOD: it was decreased by training but was increased by acute exercise, suggesting that endurance training increases the reserve of EC-SOD in tissues. The results also suggest the possibility of plasma EC-SOD assay as a new index of endurance training.

Keywords: Endurance training; Acute exercise; EC-SOD; Mn-SOD; Oxidative stress

INTRODUCTION

It is well known that physical exercise imposes oxidative stress on the body due to the generation of reactive oxygen species (ROS), particularly the superoxide anion ($O_2^{\bullet -}$).[1,2,3] Oxygen consumption may lead to an increase in ROS production, which may limit exercise performance. Therefore, the function of antioxidant enzymes appears to be a factor in aerobic physical performance.

Mammalian tissues contain enzymatic and non-enzymatic antioxidant defense systems that protect against or minimize oxidative tissue damage caused by ROS. Because most of the ROS are thought to be generated as $O_2^{\bullet -}$ *in vivo*, superoxide dismutase (SOD), which is a scavenger of $O_2^{\bullet -}$, is one of the most important enzymes in the antioxidant defense system.[4,5] Therefore, SOD is involved in the first step in the metabolism of ROS. Mammals have three SOD isoenzymes: CuZn-SOD which is localized predominantly in the cytosol;[6] Mn-SOD which is present mostly in the mitochondrial matrix;[7] and extracellular SOD (EC-SOD) which is localized in extracellular fluid, such as plasma, and in the extracellular matrix of tissues.[9,10] EC-SOD contains copper and zinc and exists in a secretory form. One of the unique properties of EC-SOD is its affinity for heparin analogues.[8,11] It is thought that,

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after being released into the extracellular space, it is distributed to specific regions of extracellular matrix or to the cell surface because of its affinity for heparin analogues.[12,13] This can allow EC-SOD to efficiently scavenge superoxide in specific regions of the extracellular matrix.[9,12,13]

Several investigators, including our group, have been focusing on the relationship between antioxidant enzymes, such as Mn-SOD and CuZn-SOD, and physical exercise.[2,3,14–17] In general, exercise is associated with an increase in the production of ROS in tissues,[1,18] thus resulting in an increase in expression of antioxidant enzymes, especially Mn-SOD, in tissues such as skeletal muscle,[16,17,19] heart and liver.[20]

There are very few reports on EC-SOD in physical exercise and most of the researches have been done using laboratory animals. In this report, we carried out a human study. Because assaying EC-SOD is difficult owing to interference by intracellular CuZn-SOD and Mn-SOD, we have employed an enzyme-linked immunosorbent assay (ELISA) using a specific antibody against human EC-SOD produced by our laboratory.[21] In contrast with other cytosolic and mitochondrial enzymes, EC-SOD has a secretory nature which enables it to be distributed around the entire body on demand. Therefore, EC-SOD may possibly play an important role in the metabolism of ROS under stressful conditions such as physical exercise.

MATERIALS AND METHODS

Subjects

Eighteen healthy male students, aged 17–22 years, volunteered for this study. None of the subjects had previously undergone endurance training and nobody took regular physical exercise. Every subject was informed of the purpose and the nature of the experiments before his voluntary consent was obtained, and the procedures were approved by our Institutional Review Board. The characteristics of the subjects are shown in Table I.

Training Protocol

The 18 subjects were divided randomly into two groups of nine. One group underwent endurance training in swimming and the other underwent endurance training in running. They trained 2 h per day, five days per week for three months at an intensity of 80–90% of maximum heart rate estimated during an acute exercise test performed before the three-month training course. The acute exercise test, specifically ramp exercise, was repeated at the end of the training course. During the acute exercise, peak maximal oxygen uptake ($\dot{V}O_2max$), maximum heart rate and maximum ventilation volume were determined as described below.

Assessment of Maximal Oxygen Uptake

Exercise was performed on a cycle ergometer. After a 3-min rest, subjects pedaled the ergometer, maintaining 60 rpm as the load was increased such that the energy output increased at a rate of 15 W/min. The subjects stopped the exercise by their own volition. Throughout the test session, heart rate and ventilation volume were monitored.

SOD Isoenzymes Assays

Just before and after the acute exercises, venous blood was collected and centrifuged to separate the plasma and blood cells. Plasma concentrations of three SOD isoenzymes were determined using an ELISA. For CuZn-SOD and Mn-SOD, commercial kits (Amersham, Piscataway, NJ) were used according to the manufacturer's instructions. The EC-SOD concentration was measured using an ELISA kit designed by our group.[21] Purified human EC-SOD was used as an assay standard.

Plasma Lipid Peroxide

The plasma lipid peroxide level was determined by the thiobarbituric acid (TBA) method using malondialdehyde (MDA) as a standard.[22]

TABLE I Subject characteristics

		Total		Swimmers		Runners	
Training		Before	After	Before	After	Before	After
n		18		9		9	
Age	years	18.7 ± 0.3		19.0 ± 0.5		18.4 ± 0.2	
Height	cm	175 ± 1		176 ± 1		174 ± 1	
Body weight	kg	68.4 ± 1.6	68.7 ± 1.5	66.4 ± 1.7	66.9 ± 1.5	70.5 ± 2.6	70.4 ± 2.7
Body mass index	kg/m^2	22.4 ± 0.5	22.4 ± 0.5	21.5 ± 0.5	21.6 ± 0.5	23.4 ± 0.6	23.3 ± 0.7

Values are mean ± SEM.

Assay for the Ability of Neutrophils to Generate Superoxide

The ability of neutrophils in four of the running-trained subjects to generate $O_2^{\bullet -}$ was determined by measuring the intensity of 2-methyl-6(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-a]pyrazine-3-one (MCLA, Tokyo Kasei, Tokyo)-dependent chemiluminescence according to the method of Nakano and co-workers[23] with a slight modification. The neutrophils were isolated by buoyant density centrifugation as previously described.[24] Briefly, blood (3 ml) anticoagulated with EDTA was layered over Polymorph-Prep (3 ml; Nycomed, Oslo) in a 14-ml polypropylene centrifuge tube and then subjected to centrifugation at $500g_{max}$ at 20°C for 30 min. The polymorphonuclear leukocyte fraction was isolated by adding an equal volume of medium A [Hank's balanced salt solution (magnesium-, calcium-, phenol- and bicarbonate-free; Life Technologies, New York) supplemented with 100 μM DTPA (diethylenetriaminepentaacetic acid), pH 7.20)] that had been diluted with water (1:1, v/v)]. The cells were then washed twice by centrifugation in medium A. Residual red blood cells (RBC) were removed by hypotonic lysis at 4°C. The resultant neutrophils were resuspended at 1×10^7 cells/ml in medium A and were stored at 0°C for no longer than 2 h prior to use. Zymosan (Sigma, St Louis) was opsonized in human serum at 37°C for 30 min and then stored at −80°C until needed. MCLA was dissolved in distilled water and also stored at −80°C until needed. A reaction mixture was prepared containing 1×10^5 neutrophils, 2.0 mg of opsonized zymosan (OZ), 1 μM of MCLA and 0.5 mM sodium azide (NaN_3) in 2.0 ml of continuously stirred medium B [magnesium- and calcium-containing Hank's balanced salt solution (Life Technologies) supplemented with 100 μM DTPA, pH 7.20]. Since MCLA reacts with singlet oxygen ($^1O_2$) as well as $O_2^{\bullet -}$, NaN_3 was also added to the reaction mixture as a scavenger of $^1O_2$. The intensity of luminescence was monitored with the luminescence reader BLR-301 (Aloka Co., Tokyo) set at 37°C. The reactions were started by the addition of MCLA and OZ. At the end of the reaction, CuZn-SOD (Wako Chemicals, Tokyo) was added and the maximum intensity of OZ-stimulated neutrophils was measured as a peak value. The reaction without OZ was monitored as a control value, and the difference between the peak and control values was regarded as the ability of neutrophils to generate $O_2^{\bullet -}$.

Statistical Analysis

The results are expressed as the mean ± SEM. Scheffé test was applied to the data when significant F ratios were obtained using analysis of variance (ANOVA). All differences stated as "significant" were at a level of $P < 0.05$.

RESULTS

Training Data

All 18 subjects completed the three months of training and the acute (ramp) exercise tests. Table I shows the characteristics of the subjects; there were no significant changes in body weight or in the body mass index (B.M.I.) during the training period. Table II shows the results of a blood test; the post-training acute exercise significantly increased the amount of hematocrit (Ht), red blood cells (RBC), white blood cells (WBC) and hemoglobin (Hb), suggesting hemoconcentration in response to exhausting exercise. Based on the amount of increase in Ht and RBC, the degree of plasma concentration could be estimated as about 5%. As shown in Table III, $\dot{V}O_2$max, maximum heart rate and maximum ventilation volume were significantly increased after the three-month training by 13.9, 7.6 and 31.5%, respectively. Therefore, the aerobic exercise performance of each subject improved significantly, and thus the training program was sufficiently effective. There were no significant differences in physical data, blood chemistry or training effects between the swimmers and the runners (data not shown).

Plasma SOD Levels

Figure 1 shows the effects of the endurance training and the acute exercises on concentrations of the three SOD isoenzymes in plasma. Either the training or the acute exercises did not significantly affect

TABLE II Hematological data of 18 volunteers before and after acute exercise performed before and after 3 months of training

		Before training		After training	
		Pre-test	Post-test	Pre-test	Post-test
Haematocrit	%	48.0 ± 0.4	50.2 ± 0.7	48.0 ± 0.5	52.3 ± 0.78*
RBC	cells/mm^3	518 ± 6	529 ± 4	524 ± 8	552 ± 6**
WBC	cells/mm^3	6090 ± 420	9480 ± 410*	6300 ± 280	10,930 ± 750*
Hb	g/dl	16.0 ± 0.2	16.3 ± 0.2	15.8 ± 0.2	17.1 ± 0.2*

Significantly different vs. pre-test value. ($p < 0.001$*, 0.02**).

TABLE III Maximum oxygen consumption, maximum heart rate and maximum ventiration volume of 18 subjects

Training		Before training	After training
$n = 18$			
$\dot{V}O_2max$	ml/min/kg	44.5 ± 1.0	50.7 ± 1.1*
Maximum heart rate	beats/min	184 ± 1	198 ± 2*
Maximum ventiration volume	l/min	111 ± 5	146 ± 5*

Values are mean ± SEM. *$p < 0.002$ vs. before training value.

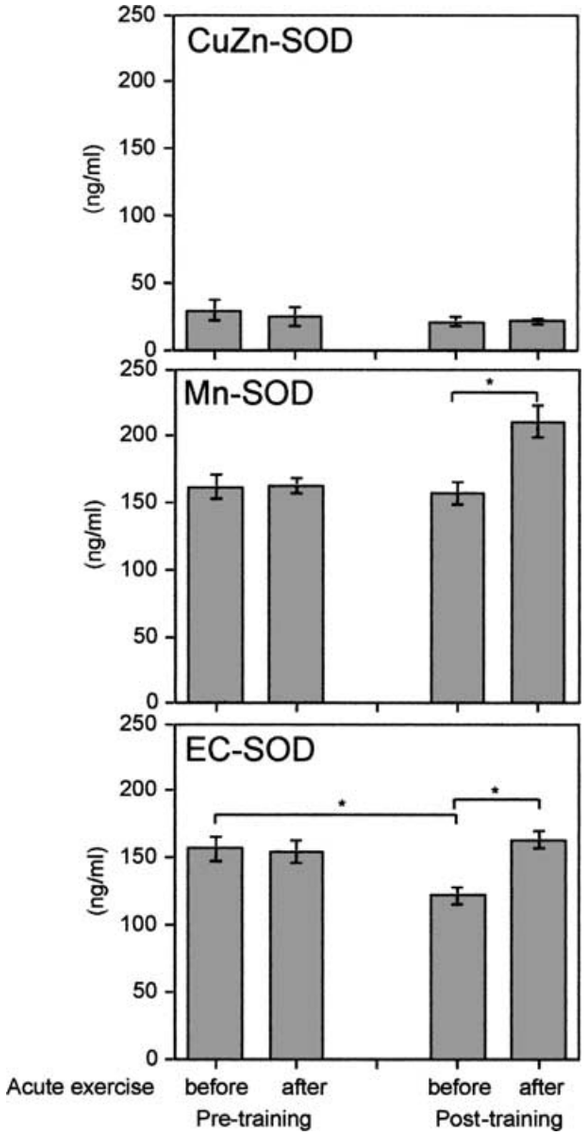

FIGURE 1 Changes in plasma CuZu-SOD (upper panel), Mn-SOD (middle panel) and EC-SOD (lower panel) levels before and after acute exhaustive exercise performed before and after three months of endurance training. Values are means ± SEM. *Significantly different $P < 0.05$.

the CuZn-SOD levels. The endurance training did not affect the Mn-SOD level at rest. While the acute exercise did not affect the Mn-SOD levels in the subjects before training, it significantly increased the isoenzymes after training (33.6%). Training caused a significant decrease in the EC-SOD level at rest (22.2%). While acute exercise tended not to affect the EC-SOD levels before training, the exercise increased it after training (33.5%). Although 2–6% of the general population show polymorphism of the EC-SOD gene, involving a single amino acid substitution (Arg213Gly) at the heparin-binding domain[25,26] and causing higher plasma EC-SOD levels than in people with the normal genotype, none of our subjects had more than 250 ng/ml of plasma EC-SOD, indicating all of them had the normal genotype. Moreover, there was no significant difference in the levels of the SOD isoenzymes between the swimmers and the runners.

Oxidative Stress *In Vivo*

To confirm that the oxidative stress arose from the acute exhaustive exercise, the plasma lipid peroxide level was assayed by the TBA method using MDA as an assay standard. As shown in Fig. 2, the acute exercise induced lipid peroxidation only after training, although the training itself did not affect the lipid peroxide level in the subjects at rest.

The Ability of Neutrophils to Generate Superoxide

Figure 3 shows the effects of endurance training and acute exercise on the ability of neutrophils to generate $O_2^{\bullet -}$. There was no difference in the ability of neutrophils to generate $O_2^{\bullet -}$ before and after training in subjects at rest. On the other hand, the acute exercise induced the ability of neutrophils to generate $O_2^{\bullet -}$, and this induction was more

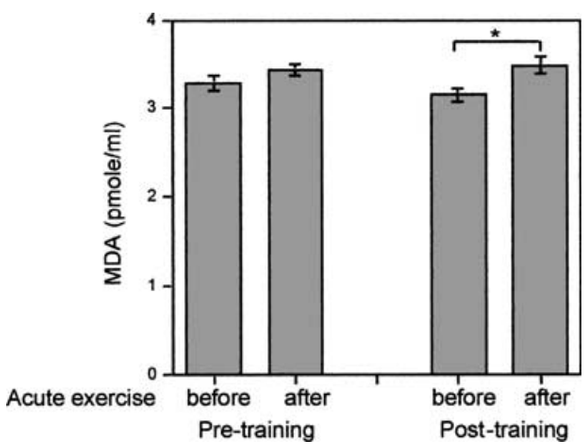

FIGURE 2 Changes in plasma lipid peroxide level before and after acute exhaustive exercise performed before and after three months of endurance training. Values are means ± SEM. *Significantly different, $P < 0.05$.

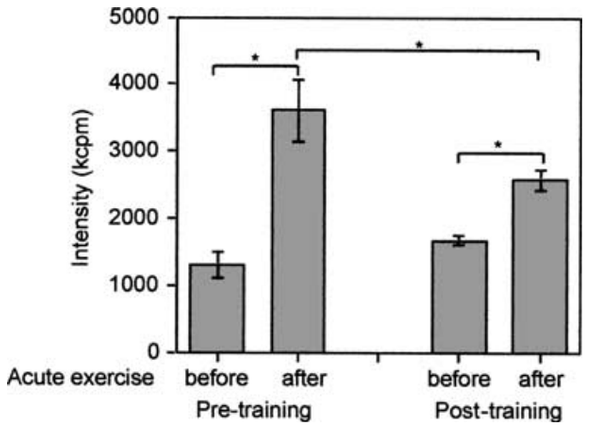

FIGURE 3 Changes in ability of neutrophils of generate superoxide before and after acute exhaustive exercise performed before and after three months of endurance training. Values are means ± SEM. *Significantly different, $P < 0.05$.

pronounced with the pre-training subjects than with the post-training subjects.

DISCUSSION

We employed two kinds of exercise, swimming and running for the current study. As mentioned in the "Results" section, both training events were equally effective for the improvement of the aerobic performance and we could not observe any significant differences in physical data, training effects, plasma SOD levels or estimated oxidative stress between two groups. Therefore, it seems reasonable not to distinguish swimmers and runners in the following discussions in addition to the "Results" section.

One of major benefits on non-exhaustive exercise is that it stimulates the expression of certain antioxidant enzymes in response to the induced mild oxidative stress.[2] This is mediated by the activation of redox-sensitive signaling pathways. For example, acute bout of exercise induces gene expression of muscle mitochondrial Mn-SOD with activation of NF-kappaB and AP-1 binding.[19] An increase in the *de novo* protein synthesis of antioxidant enzymes usually requires repeated bouts of exercise. As for the induction mechanism of EC-SOD, Marklund[27] has reported that some cytokines induce the expression of EC-SOD in cultured fibroblast cell line. Furthermore, Fukai *et al.*,[28] have reported that three weeks of treadmill running upregulates aortic EC-SOD expression and induces endothelial nitric oxide synthase (eNOS) expression simultaneously in mice. Moreover, in the same study, NO donor also induced EC-SOD expression in time- and dose-dependent manners in human aortic smooth muscle cells.

The physiological significance of plasma SOD levels remains controversial. It is well known that the plasma level of CuZn-SOD is much affected by hemolysis induced by mechanical stress on the capillary or by artifacts such as venous puncture. In the present study, the acute exercise definitely increased the plasma CuZn-SOD levels in two subjects (data not shown). These increases might also be due to minor hemolysis. Moreover, because its molecular weight is comparatively small,[29] CuZn-SOD is readily filtrated by the glomerulus and lost in urine rapidly. The source of plasma Mn-SOD is thought to be the leakage of the enzyme from the tissue; therefore, an increased level of Mn-SOD may reflect either tissue damage or increased expression. Our previous study showed that very stressful strenuous training increased the plasma Mn-SOD level, along with muscle damage.[30] On the other hand, well-trained athletes have a significantly higher plasma level of Mn-SOD compared with sedentary controls.[31] Mn-SOD levels in skeletal muscles increased in rats subjected to regular treadmill exercise for 10 weeks.[17] These results suggest that adequate endurance training increases the amount of Mn-SOD in skeletal muscle and improves tolerance against oxidative stress induced by physical exercise. Thus, it appears that Mn-SOD may provide a good index of physical training. In the present study, we have no data about muscle enzyme levels, but the plasma level of Mn-SOD was increased by acute exercise only after the training. If the origin of the plasma Mn-SOD was leakage from skeletal muscle, the increased level might be due to either a higher level of muscle Mn-SOD in trained subjects or to damage of skeletal muscle through more vigorous exercise made by the improved maximal oxygen uptake.

To our knowledge, there are no previous reports on the relation between human EC-SOD and exercise. It is well known that the low heparin binding genotype of EC-SOD (Arg213Gly) gives much higher levels of plasma isoenzyme than the normal genotype:[32] Marklund *et al.*,[25] found that 3.8% of 4900 randomly selected Swedes had the Arg213Gly genotype with approximately eight-fold higher levels of plasma EC-SOD than the normal genotype. They also reported that the genotype was correlated with increased risk of cardiovascular disease through higher levels of serum cholesterol, triglycerides and fibrinogen as well as of BMI. This may suggest that high plasma levels of EC-SOD are not always beneficial. The plasma level of EC-SOD may be affected by its expression and its heparin-binding nature of both the enzyme and target molecules on tissues. As summarized by our group, the amount of EC-SOD in tissues does not reflect its level of expression in some mouse tissues,[12] suggesting that EC-SOD migrates from

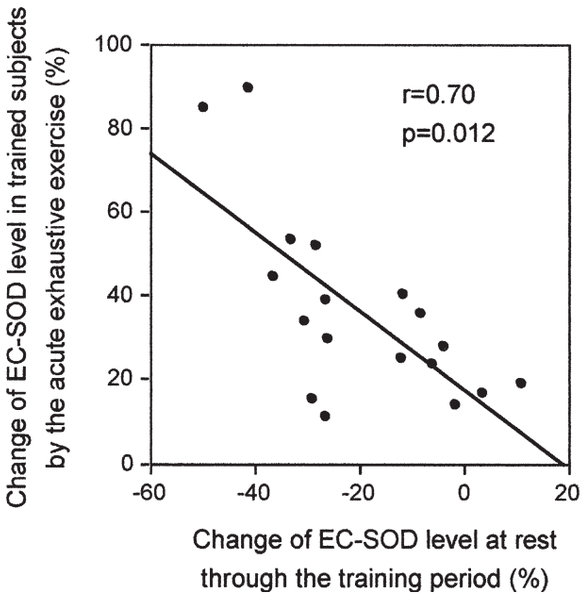

FIGURE 4 Relationship between changes in EC-SOD levels at rest after the endurance training and in the enzyme level after the post training ramp test.

highly expressive tissues, such as kidney, lung and adipose tissues, to other tissues. Moreover, in our recent study, recombinant EC-SOD migrated into cultured pre-adipocytes from the cultured medium.[33] In the present study, the plasma EC-SOD level was downregulated through the training (Fig. 1, lower panel), suggesting that EC-SOD might more readily accumulate in tissues upon incorporation into the cells made easier through training. The fact that intravenous administration of heparin induces a three-fold increase in plasma EC-SOD levels[34] may be evidence for EC-SOD accumulating in tissues. On the other hand, the acute exhaustive ramp exercise increased not only the plasma Mn-SOD level but also the EC-SOD level after training. Because it is obvious that the improved oxygen uptake after training brought about much stronger exercise strength in the post-training ramp test, they might be exposed to more severe oxidative stress in the post-training ramp test than in the pre-training ramp test as shown in Fig. 2. In this case, the increased EC-SOD after exhaustive exercise could be considered as release of a tissue reserve. The extent of the downregulation of EC-SOD level in trained subjects at rest had a significant inverse relationship with the increment of its level by the acute exhaustive exercise (Fig. 4). From these viewpoints, the training effect could be designated as the expansion of a reserve for antioxidant enzymes in tissues. In addition, there is a possibility that a plasma EC-SOD assay may serve as a new index of endurance training.

As shown in Fig. 3, in spite of the higher exercise intensity in the post-training ramp test, the induction of $O_2^{\bullet -}$ generation in neutrophils upon acute exercise was much greater before training than after training, although the endurance training did not affect it at rest. From these results, the effect of endurance training on the ability of $O_2^{\bullet -}$ generation in neutrophils seems to be rather suppressive. Several cytokines [e.g. interleukin (IL)-6, IL-8, tumour necrosis factor-α][35] and an ischemia[36] may exert a role in the priming process of neutrophils for the reactive oxygen formation. Although the effects of exercise on TNF-α expression are unclear, Greiwe *et al.*,[37] reported that resistance exercise for three months decreased the expression of TNF-α in skeletal muscles of elderly humans. On the other hand, the endurance training may improve the resistance against the tissue ischemia during the acute exhaustive ramp exercise. The downregulation of the cytokine expression such as TNF-α and the improvement of resistance against tissue ischemia may be involved in the suppressive effect of endurance training on priming of neutrophils. $O_2^{\bullet -}$ production in neutrophils may be utilized in the normal inflammation process to kill phagocytized microorganisms, however, overproduction of ROS in inflamed tissues may cause tissue damage. Our results suggest that endurance training suppresses overproduction of $O_2^{\bullet -}$ in neutrophils and thus may protect tissues, especially inflamed tissues, from damage by the $O_2^{\bullet -}$.

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