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Journal of Nutritional Biochemistry 15 (2004) 479–484

THE JOURNAL OF Nutritional Biochemistry

Different effects of exercise tests on the antioxidant enzyme activities in lymphocytes and neutrophils

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Abstract

We have determined the effects of maximal and submaximal cycloergometer tests on the antioxidant enzyme defences of neutrophils and lymphocytes. We also compared the neutrophil and lymphocyte basal enzyme antioxidant activities. A total of 17 well-trained amateur athletes, runners, and cyclists participated in this study. Two tests were performed on an electromagnetic reduction cycloergometer: the maximal exercise test, and the submaximal prolonged exercise test. Blood samples were taken before and after the tests. Basal enzyme activity of superoxide dismutase was higher in lymphocytes but neutrophils presented higher activities of catalase and glutathione peroxidase. The maximal test increased the circulating number of lymphocytes and the activities of catalase and glutathione peroxidase. No changes were observed in lymphocyte number or in lymphocyte antioxidant enzyme activities after the submaximal test. The circulating number of neutrophils increased significantly after the submaximal test. Maximal and submaximal tests decreased the activities of neutrophil glutathione dependent antioxidant enzymes (glutathione peroxidase and glutathione reductase), but no changes were observed in catalase or superoxide dismutase activities after either test. Neither the maximal nor submaximal test produced increases in serum activities of lactate dehydrogenase and creatine kinase (CK).

Keywords: Oxidative stress; Antioxidant enzymes; Neutrophils; Lymphocytes; Exercise

1. Introduction

Exercise increases production of reactive oxygen species (ROS) exceeding the capacity of antioxidant defences [1,2]. Mitochondria, as well as catecholamine metabolism, xanthine oxidase reaction, hemoglobin oxidation, and activated neutrophils, are oxygen radical sources that may be responsible for oxidative stress during exercise [3,4]. The ROS has been shown to damage lipids, proteins, and DNA [5].

It is well recognized that exercise causes perturbations to the immune system. The effects of exercise on the circulating number of immunocompetent cells have been well described [6–10]. Neutrophilia is well documented after exhaustive exercise such as a marathon race [11–14]. However, lymphocyte concentration increases during exercise and falls below basal values after intense exercise of long duration [15].

It has become clear that the immune response to exercise could be related to exercise intensity. Although moderate exercise stimulates the immune system, strenuous exercise induces immunosuppression in the recovery period and may explain the increased risk of infection in athletes [16]. The mechanisms underlying exercise-associated immune changes are multifactorial and include neuroendocrinological factors such as adrenaline, noradrenaline, growth hormone, and cortisol [17,18], as well as oxidative stress.

Despite substantial evidence indicating that strenuous exercise induces oxidative stress and acute immune response, information concerning interactions between antioxidant defence and the immune system within exercise is scanty. It has been reported that antioxidant vitamins such as vitamin E and ascorbate are present in large concentrations in neutrophils and lymphocytes, and that they play an important role in order to maintain the immune cell integrity and functionality [19]. However, it is also of interest to understand the adaptations to exercise of the endogenous antioxidant defences of these immune cells in order to calibrate the intake requirements of antioxidant nutrients.

Human blood mononuclear cells, as well as neutrophils, express superoxide dismutase, catalase, and glutathione peroxidase; and all these scavenging enzymes provide cellular

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0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2004.03.002

defence against endogenous and exogenous ROS. We have recently evidenced the effects of strenuous exercise on neutrophil antioxidant enzyme activities [20]. Intense exercise induced a decrease in neutrophil activities of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase. Taking into account that exercise intensity could affect oxidative stress and the immune response, it is important to know the effects of exercise intensity on the antioxidant defences of the immune cells.

The aim of this study was to determine the effects of two different cycling exercise tests on the circulating number of lymphocytes and neutrophils, on neutrophil and lymphocyte antioxidant enzyme activities and on lactate dehydrogenase (LDH) and CK serum activities. The exercise tests were a maximal intensity exercise test, representative of high intensity and short duration, and a submaximal exercise test, representative of prolonged, continuous, intense exercise.

2. Methods and materials

2.1. Subjects and exercise tests

The subjects who volunteered to participate in this study were 17 amateur sports enthusiasts, who were all athletes, runners, and cyclists. They trained 14 ± 1 hours each week. Their mean (±SEM) age was 23.3 ± 2.0 years, height 168 ± 3 cm, weight 70.8 ± 1.2 kg, and body mass index 24.5 ± 1.3 kg/m^2. Two tests were performed on an electromagnetic reduction cyclo-ergometer (Ergometrics 900, MedGraphics, St. Paul, MN): the maximal exercise test and submaximal prolonged exercise test. In the maximal exercise test, subjects warmed up for 3 minutes at 30 watts before starting the test. The test started at 50 watts and the subjects' work rate was increased by 30 watts every 3 minutes. The test ended when increased work did not increase or decrease oxygen consumption; this value was the VO_2max. During this maximal exercise test the participants did not drink anything. The submaximal prolonged exercise test was carried out one week after the maximal test. The cycloergometer resistance was adjusted so that the athletes worked at 80% of their maximal capacity of oxygen consumption. This test was prolonged for 1.5 hours and all subjects drank 500 mL of spring water. All tests were performed after an overnight fast at the same time, in the same room, and with the same temperature and humidity conditions.

The study protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Ethical Committee of Universitary Hospital Son Dureta).

2.2. Experimental procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. The basal venous blood was obtained from all the subjects participating in this study on the morning of the exercise test day, after 12 hours of overnight fasting. The postexercise sample was taken immediately after the exercise. One blood vacutainer was used to purify neutrophil and lymphocytes following an adaptation of the method described by Boyum [21]. Blood was centrifuged at 900 × *g*, 4°C for 30 minutes after carefully introducing on Ficoll in a proportion of 1.5:1. The lymphocyte layer was carefully removed. The plasma and the organic phase were discarded. The phase at the bottom contained the erythrocytes and neutrophils. Erythrocytes were hemolyzed with 50 mL of ammonium chloride 0.15 mol/L at 4°C. The suspension was centrifuged at 750 × *g*, 4°C for 15 minutes and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with 50 mL of ammonium chloride 0.15 mol/L and then with 20 mL of PBS. The slurry was centrifuged again as above. Finally, neutrophils were lysed with distilled water (1:10).

The lymphocyte slurry was then washed twice with PBS and centrifuged for 10 minutes at 1000 × *g* and 4°C. Finally, the cellular precipitate of lymphocytes was lysed 1:10 with distilled water.

The lysed neutrophils and lymphocytes were used to determine antioxidant enzyme activities. Neutrophils and lymphocytes were quantified in fresh blood using an autoanalyzer system (Technicon H*2; Bayer, Pittsburgh, PA, USA) [22].

2.3. Enzymatic determinations

We, and glutathione peroxidase activities in lymphocytes and neutrophils and, also, the glutathione reductase activity in neutrophils and the cytochrome oxidase activity in lymphocytes. All activities were determined with a Shimadzu UV-2100 spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) at 37°C.

Catalase activity was measured by the spectrophotometric method of Aebi [23] based on the decomposition of H_2O_2.

Glutathione reductase activity was measured by a modification of the Goldberg and Spooner [24] spectrophotometric method. This assay required oxidized glutathione as the substrate.

Glutathione peroxidase activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [25]. This assay required H_2O_2 as a substrate and glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an enzyme indicator.

Superoxide dysmutase (SOD) activity was measured in the neutrophil preparation by an adaptation of the method of McCord and Fridovich [26]. The xanthine/xanthine oxidase system was used to generate superoxide anion. This anion produced the reduction of cytochrome c, which was monitored at 550 nm. The superoxide dismutase of the sample removed the anion superoxide and produced an inhibition of the reduction. The value of this reduction was used as a measure of enzyme activity.

Cytochrome oxidase activity in lymphocytes was measured by an adaptation of the spectrophotometric method described by Wharton and Tzagoloff [27].

2.4. CK and LDH determinations

These determinations were made in an autoanalyzer Technicon DAX system. The LDH activity determination is based on the measurement of the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH [28]. The rate of oxidation is proportional to LDH activity. The activity is monitored by measuring the decrease in absorbance at 340 nm.

In the determination of the CK activity the enzyme reacts with creatine phosphate and adenosine diphosphate to form adenosine triphosphate, which is coupled to the hexokinase/guanosine diphosphate reaction generating NADPH [29]. The CK activity is proportional to the rate of increase in the concentration of NADPH. The reaction is monitored at 340 nm.

2.5. Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS 9 for Windows; SPSS, Chicago, IL). Results are expressed as mean ± SEM, and a value of $P < 0.05$ was considered to be statistically significant. The statistical significance of the data was assessed by the two-way analysis of variance (ANOVA). The statistical factors analyzed were the different test performed (T) and the exercise (E). In the sets of data in which there was a significant ExT interaction or significant effects of T factor or E factor, one-way ANOVA was used to determine the differences between the groups involved. Basal enzyme activities in neutrophils and lymphocytes were compared by using the Student *t* test for unpaired data.

3. Results

Figure 1 shows the basal neutrophil and lymphocyte antioxidant and COX enzyme activities expressed on a cellular basis. COX activity was undetected in neutrophils, as we expected, because neutrophils do not have mitochondria. The catalase activity was approximately 1.8 times significantly higher and glutathione peroxidase was about 1.5 times higher in neutrophils than in lymphocytes. SOD activity was about 3.8 times higher in lymphocytes than in neutrophils.

The changes in the lymphocyte number and in the lymphocyte antioxidant enzyme and COX activities expressed per cell are shown in Table 1. A significant interaction between the exercise and the ANOVA factors was found in the lymphocyte number. The submaximal test did not affect this parameter, but the maximal test produced a significant increase (30.2%). A significant interaction between the two

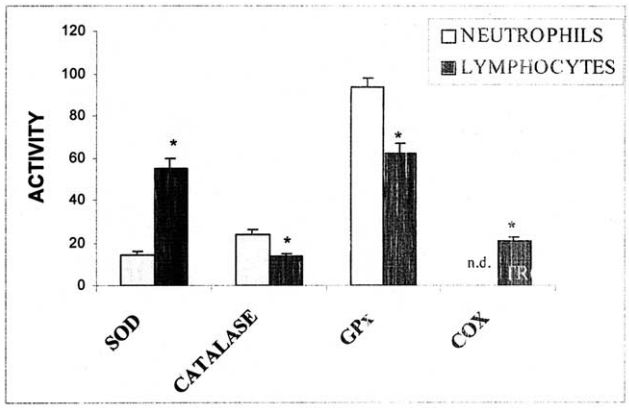

Fig. 1. Basal enzyme activities in neutrophils and lymphocytes. * Indicates significant differences between neutrophil and lymphocyte activity. Cellular activities: COX (pkat / 10^9 cells); SOD (pkat / 10^9 cells); catalase (K / 10^9 cells); GPx (nkat / 10^9 cells). n.d.: not detected.

ANOVA factors analyzed was found in the catalase and glutathione peroxidase activities. The maximal test significantly decreased (40.7%) the catalase lymphocyte activity expressed per cell, but the submaximal test maintained the basal values. Glutathione peroxidase activity expressed per cell decreased significantly after the maximal test (31.6%), whereas the submaximal test produced a slight but nonsignificant increase. Both glutathione peroxidase and catalase activities after submaximal test were higher than those after the maximal test. No changes were observed in the lymphocyte SOD activity after the maximal and submaximal tests.

Table 2 shows the neutrophil number and the neutrophil antioxidant enzyme activities during the duathlon competition and the recovery. The kind of test and the exercise significantly influenced the neutrophil number. The maximal test maintained the basal values but the submaximal test increased neutrophil number about 58.6%. The neutrophil number after the submaximal test was significantly higher than the value after the maximal test. No changes were observed in the neutrophil catalase or SOD activities during the maximal and submaximal tests. The glutathione peroxidase activity was significantly affected by the exercise. The maximal and submaximal tests slightly decreased this activity. A significant effect of the exercise was found in the glutathione reductase activity, with no changes after the maximal test and a significant decrease (27.2%) after the submaximal test.

No statistically significant changes were found in the serum activities of CK and LDH after the maximal or submaximal tests (Fig. 2).

4. Discussion

Lymphocytes and neutrophils present a different antioxidant enzyme activity profile, probably related to the exis-

Table 1
Effect of maximal and submaximal exercise tests on lymphocyte number and on antioxidant enzyme activities

	Maximal test		Submaximal test		ANOVA		
	Before	After	Before	After	T	E	T × E
Lymphocytes ($10^3/\mu L$)	1.79 ± 0.16[a]	2.33 ± 0.16[b]	2.12 ± 0.15[a]	1.98 ± 0.19[a]			X
Catalase ($K/10^9$ lymphocytes)	13.9 ± 1.1[a]	8.24 ± 0.41[b]	12.7 ± 1.3[a]	13.1 ± 1.4[a]*		X	X
GPx ($nkat/10^9$ lymphocytes)	61.7 ± 8.3[a]	42.2 ± 2.9[b]	62.5 ± 6.1[a]	76.7 ± 8.0[a]*	X		X
SOD ($pkat/10^9$ lymphocytes)	62.8 ± 8.0	44.2 ± 6.5	59.2 ± 7.8	60.7 ± 10.1			
COX ($pkat/10^9$ lymphocytes)	23.6 ± 2.3	22.9 ± 3.2	18.4 ± 2.2	23.1 ± 3.4			

Factor T represents the test type; factor E represents the realization of the test (before–after test); T×E represents the interaction between the two factors.
* Significant differences between the after maximal and after submaximal test values. Different superscript letters indicate significant differences between before and after the test.

tence of mitochondria in the lymphocyte but not in neutrophils, in accordance with the nondetected COX activity in neutrophils. Because of the existence of a mitochondrial isoenzyme, the Mn-SOD, the higher SOD levels in lymphocytes than in neutrophils could be related to the high superoxide anion production in the mitochondria. It has been pointed out that about 5% of the oxygen reduced in the mitochondria is transformed into superoxide anion [30]. On the other hand, during the oxidative burst, the neutrophil produces high amounts of H_2O_2 and ClOH [31]. Then, the higher catalase and glutathione peroxidase activities could protect the neutrophil against these ROS. It has been pointed out that high neutrophil catalase activity appears to explain their high resistance against exogenous H_2O_2 [32].

The maximal test produced a significant lymphocytosis. This result is in agreement with the usual increase in the lymphocyte number during exercise [15]. It has been reported that the lymphocyte number increases during moderate exercise but after the exercise falls below resting values [15]. The increase in circulating number of lymphocytes is due to recruitment of all lymphocyte subpopulations to the blood [33]. We can suspect that the short duration of the maximal test and the work at maximal VO_2 at the end of the test induced the increase in the circulating number of lymphocytes. The submaximal test, with a longer duration, produced a slight decrease in the lymphocyte concentration. The combination of duration and intensity of the exercise is a basic factor to determine the changes in the circulating lymphocyte number [11,15]. Taking into account the characteristics and the results obtained after the short maximal test, we can suspect that after the submaximal test, with a long duration, the lymphocyte number was decreasing from high values attained at the beginning of the test.

The maximal test produced a general decrease in the lymphocyte antioxidant enzyme activities. It seems that the first effect of oxidative stress induced by exercise on these enzymes was a partial inactivation, as we observed in the erythrocytes [34]. However, antioxidant enzyme activities after the submaximal test did not decrease, and even glutathione peroxidase activity increased after this submaximal test. It has been established that ROS and cytokines such as TNF-α and IL-1 [35–37] can regulate the expression of some antioxidant enzymes [35]. Lymphocyte antioxidant enzymes show adaptation to oxidative stress induced by long duration exercise. Glutathione peroxidase activity increased in lymphocytes as a consequence of the long duration exercise. We suspect that the oxidative stress during the submaximal test, with a longer duration, could exceed an inactivation stage and initiate the activation or the synthesis induction of antioxidant enzymes by the ROS. In this sense, the submaximal test could be considered a prolongation of the maximal test.

The response of cytochrome c oxidase activity to the maximal test was different to that described above for antioxidant enzymes. After the maximal test, cellular cyto-

Table 2
Effect of maximal and submaximal exercise tests on neutrophil number and on antioxidant enzyme activities

	Maximal test		Submaximal test		ANOVA		
	Before	After	Before	After	T	E	T×E
Neutrophils ($10^3/\mu L$)	3.05 ± 0.25[a]	3.72 ± 0.33[a]	3.38 ± 0.32[a]	5.36 ± 0.58[b]*	X	X	
Catalase ($K/10^9$ neutrophils)	27.4 ± 3.8	20.0 ± 1.6	23.8 ± 2.8	28.2 ± 2.8			
GPx ($nkat/10^9$ neutrophils)	105 ± 11[a]	94.3 ± 13.3[a]	85.7 ± 5.2[a]	70.8 ± 10.8[a]		X	
G. Reductase ($nkat/10^9$ neutrophils)	98.0 ± 7.9[a]	78.8 ± 7.5[a]	100 ± 9[a]	72.8 ± 6.2[b]		X	
SOD ($pkat/10^9$ neutrophils)	13.6 ± 0.2	13.7 ± 0.8	11.7 ± 1.6	10.6 ± 1.2			

Factor T represents the test type; factor E represents the realization of the test (before–after test); T×E represents the interaction between the two factors.
* Significant differences between the after maximal and after submaximal test values. Different superscript letters indicate significant differences between before and after the test.

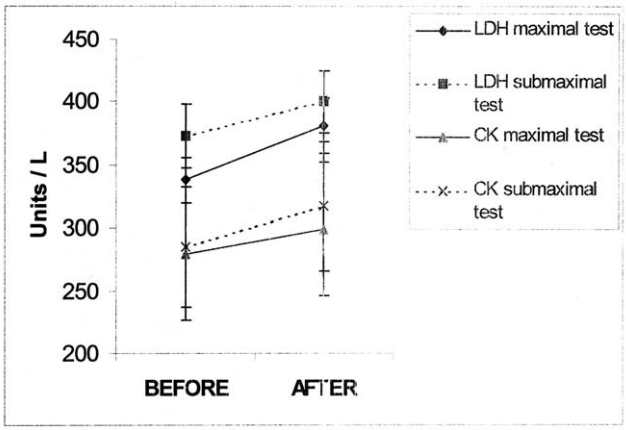

Fig. 2. LDH and CK serum activities before and after maximal and submaximal tests. Enzyme activities were expressed as enzyme unit activities per litre of serum. No significant differences were found.

chrome c oxidase activity is maintained, thus indicating possible different regulating mechanisms.

Immediately after the submaximal test, significant neutrophilia was observed. It has been pointed out that physical exercise produces pronounced neutrophilia, which is ascribed to the mobilization of cells from marginal pools [11–14]. The maximal test produced a slight increase in the number of circulating neutrophils, thus indicating that the short duration and the time during which the athletes exercised at high intensities were not enough to induce neutrophilia.

We have shown that neutrophil priming induced by intense and prolonged exercise such as cycling stage includes an important decrease in the activity of antioxidant enzymes in neutrophils [20]. In the present study we point out that both maximal and submaximal tests decreased the neutrophil glutathione-dependent enzyme activities (glutathione reductase and glutathione peroxidase). By contrast, these exercise tests did not affect the cellular activity of catalase or SOD. The exercise effects on the neutrophil antioxidant enzymes may depend mainly on the enzyme, inasmuch as the maximal and submaximal tests produced similar effects. However, the maximal test did not produce neutrophilia, indicating that the decrease in antioxidant enzyme activities is induced before neutrophilia.

It has been reported that exercise increases plasma activities of cytosolic enzymes such as LDH and CK [38]. Thus, LDH and CK are used as muscle damage indicators. Both maximal and submaximal tests induced slight increases of these activities in plasma but, because the variability, the differences are not significant. However, the response of the neutrophil antioxidant enzyme activities, mainly the glutathione-dependent enzymes, was evident before the appearance of serum muscle damage indicators.

In summary, cycloergometer test performed at different intensities produced different effects on the circulating number of neutrophils and lymphocytes and also on the antioxidant enzyme activities of these cells. It seems the effects on antioxidant enzymes in neutrophils depend mainly on the enzyme, whereas in lymphocytes they depend mainly on the type of test. These changes could be used as damage indicators because in this study they are more sensible than changes in serum LDH or CK activity.

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