Exercise-induced oxidative stress affects erythrocytes in sedentary rats but not exercise-trained rats

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A NUMBER OF STUDIES HAVE SUGGESTED that destruction of erythrocytes occurs during physical training and that it may lead to an anemia state, commonly called “sports anemia” (20, 21, 24). Many hypotheses have been proposed to explain the exercise-induced erythrocyte destruction. Gastrointestinal and urinary tract bleeding, iron deficiency (dietary deficiency, reduced absorption from intestine, increased demand, loss with sweat), insufficient erythropoiesis, and intravascular hemolysis are the main mechanisms that have been proposed for explaining this situation (15, 25, 27). Intravascular hemolysis is the most emphasized mechanism. Mechanical trauma (footstrike or compression of erythrocytes in capillaries within the contracting muscles), elevated body temperature, dehydration, hemoconcentration, and oxidative stress may cause intravascular hemolysis during regular or single bouts of exercise and/or recovery period (20, 24). Smith et al. (21) previously reported that exercise-induced oxidative stress and intravascular hemolysis may coexist. However, their study did not indicate a causal relationship between these observations.

The decrement in hemolysis after enhancement of the antioxidant defense may indicate the contribution of oxidative stress to intravascular hemolysis in exercise. Besides the exercise, some conditions may lead to erythrocyte damage and hemolysis in vivo and in vitro by oxidative stress, such as sickle cell anemia, β-thalassemia, vitamin E depletion, H₂O₂ exposure, hyperglycemia, and halothane and sulphur dioxide exposure (6, 9, 11, 12, 26), and these situations could be prevented by antioxidant treatment in some conditions (9, 12, 18, 26).

The aim of the present study was to determine the contribution of oxidative stress to exercise-induced intravascular hemolysis and to examine the effect of antioxidant vitamin (C and E) treatment in sedentary and physically trained rats.

MATERIALS AND METHODS

Animals, Protocols, and Sampling

Sixty-one adult male Wistar rats, weighing 250–350 g, were used in this experimental study. All rats were given standard rat chow and tap water ad libitum and housed at 23 ± 2°C on a 12:12-h dark-light cycle. The animals were divided mainly into two groups: sedentary (Sed) and exercise
trained (ET). Each group was further divided into three subgroups: control (C; Sed-C and ET-C), exhausted (Exh; Sed-Exh and ET-Exh), and treated (T) Exh (Sed-T-Exh and ET-T-Exh). This experiment was approved by the Akdeniz University Animal Care and Usage Committee and followed the guidelines established by the American Physiological Society.

All ET groups were introduced to running on a motor-driven rodent treadmill (May, TME 9805, Ankara, Turkey). The treadmill was equipped with an electric shocking grid on the rear barrier to provide exercise motivation to the animals. Animals of ET groups started treadmill running at 25 m/min, 5% grade, and 10 min/day during 1 wk. Running time was gradually increased to 60 min/day in 1 wk, and this intensity was maintained in the same grade and speed for 4 wk (5 days/wk).

An exhaustive, acute exercise protocol was used for both Sed and ET groups except for the C subgroups. The protocol was started with 25 m/min speed, 5% grade, and reached 15% grade in 20 min. Running was continued until exhaustion. The point of exhaustion was determined by the animal’s loss of righting reflex when turned on its back.

During the training period, one subgroup of both Sed and ET groups (Sed-T-Exh and ET-T-Exh) was treated with vitamin C (ascorbic acid, 50 mg·kg⁻¹·day⁻¹, Sigma Chemical, St. Louis, MO) and vitamin E (α-tocopherol, 20 mg·kg⁻¹·day⁻¹, Sigma Chemical) by intra gastric catheter.

Twenty-four hours after exhaustion or after the last training, blood samples were obtained from the abdominal aorta of the rats under light ether anesthesia and anticoagulated with sodium heparin (15 U/ml). These samples were used for the measurement of hemolysis parameters, lipid peroxidation, and antioxidant defense system of erythrocytes.

**Erythrocyte Fragility and Mechanical Parameters**

**Osmotic fragility.** Erythrocyte fragility was determined osmotically by the method of Beutler (7). Briefly, heparinized whole blood (10 μl) was added to tubes with increasing concentration of buffered salt solution (pH 7.4; 0, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.7, 0.9%). The tubes were gently mixed and incubated at room temperature for 30 min. The samples were then centrifuged at 2,000 rpm for 10 min, and supernatants were collected. Optical density of the supernatant was measured by spectrophotometer at 540 nm. Hemolysis in each tube was expressed as a percentage, taking as 100% the maximum value of absorbance of the distilled water (0% concentration). The half-maximal (50%) concentration values were calculated from the percentage of hemolysis in buffered salt solutions at various concentrations by GraphPad Prism 3.0 software program.

**Erythrocyte deformability.** Erythrocyte deformability was estimated by measuring transit times (TTs) through pores with 5-μm diameter and 15-μm length by using a cell transit analyzer (4). The cell transit analyzer consists of an oligopore filter with 30 cylindrical pores, mounted between two reservoirs and an alternating-current conductimeter. The conductimeter operates at 100 kHz and measures the electrical resistance between the electrodes placed in each reservoir. By adjusting the level of fluid in the reservoirs, a pressure gradient is created that forces the erythrocyte suspension diluted (0.04%) in phosphate-buffered saline (pH 7.4) in one reservoir to flow through the oligopore filter. The passage of an erythrocyte through 1 of the 30 pores results in a resistance change between two reservoirs. A resistive pulse is generated at the output of the conductimeter circuit, which carries the information about the passage of that erythrocyte through the 5-μm pore. This signal is then digitized and passed onto a digital computer for analysis. The computer determines the width of each valid pulse that corresponds to the TT of an erythrocyte through a pore. TTs of 1,000 erythrocytes for each specimen were determined, and the mean was used to represent the deformability of the erythrocyte population.

**Plasma hemoglobin concentration.** A modified method, based on cyanmethemoglobin, was used for determining plasma hemoglobin concentrations, and the values are expressed in grams per deciliter (5).

**Oxidant Stress Parameters**

**Thiobarbituric acid-reactive substance.** Lipid peroxidation of erythrocytes was estimated by the measurement of thiobarbituric acid-reactive substance (TBARS), as described by Stocks and Dormandy (22) using 1,1,3,3-tetraethoxypropane as the standard. TBARS levels were determined by measuring absorbance at 532 nm after reaction with thiobarbituric acid in erythrocyte extracts. Results are expressed in nanomoles per gram hemoglobin.

**Methemoglobin.** Methemoglobin was measured by estimating the absorbance of diluted blood at 630 nm before and after its conversion to cyanmethemoglobin (17). Methemoglobin values are expressed as a percentage of total blood hemoglobin.

**H₂O₂-induced oxidative stress and hemolysis.** A 5% suspension of washed, packed erythrocytes in buffered saline was mixed with the same volume of 1% H₂O₂ solution so that the final mixture consisted of 2.5% erythrocyte and 0.5% H₂O₂. The mixtures were incubated at 37°C for 2 h. At the end of the incubation, TBARS and the extent of hemolysis were determined in the mixtures. Hemolysis was determined by measuring released hemoglobin into the supernatant by using a spectrophotometer at 540 nm and was expressed on the basis of the maximum absorbance (100%) in the aliquots of erythrocyte completely hemolyzed in distilled water.

**Antioxidant Status**

The activities of two antioxidant enzymes (catalase (CAT), superoxide dismutase (SOD)) and levels of GSH were evaluated. CAT (EC 1.11.1.6) and SOD (EC 1.15.1.1) activities were assessed by using the methods of Aebi (1) and Misra and Fridovich (16), respectively. GSH was determined by using the technique described by Fairbanks and Klee (10).

**Statistical Analysis**

The results are expressed as means ± SE, and statistical analyses were done by one-way ANOVA. Newman-Keuls posttest for multiple comparison among means was used to compare intergroup differences. P < 0.05 was accepted as significant.

**RESULTS**

At the end of 4 wk, there were no significant differences in body weight, heart weight, and body-to-heart weight ratio among groups. Four weeks of exercise training also did not alter the hematocrit and total blood hemoglobin values (Table 1). Exhaustion time was significantly longer in ET groups compared with Sed groups.
Erythrocyte Fragility and Mechanical Parameters

Osmotic fragility, erythrocyte deformability, and plasma hemoglobin concentration are shown in Fig. 1. Osmotic fragility was significantly increased after exhaustion in Sed-Exh compared with Sed-C, but this was prevented with antioxidant treatment (Sed-T-Exh). The same patterns were valid for erythrocyte deformability and plasma hemoglobin concentrations values. Whereas exhaustion lead to decreased erythrocyte deformability (increased TT) and increased plasma hemoglobin concentration levels, antioxidant treatment before exhaustion gave protection from these harmful effects in Sed rats. The osmotic fragility, erythrocyte deformability, and plasma hemoglobin concentration were not significantly different in ET groups compared with the Sed-C group.

Oxidant Stress Parameters

TBARS and methemoglobin results are presented in Fig. 2. TBARS and methemoglobin levels were significantly increased after exhaustion in Sed-Exh animals compared with the Sed-C group. Antioxidant treatment caused a significant decrease in TBARS and methemoglobin levels of Sed-T-Exh compared with Sed-Exh animals. TBARS level was significantly higher in ET-Exh than in ET-C and ET-T-Exh. However, TBARS levels were not elevated in the ET-Exh group compared with Sed-Exh. There was no significant difference in methemoglobin values in ET groups.

The ability of erythrocytes to resist a given oxidative stress was studied in H_2O_2 incubation (Fig. 3). In response to exhaustive exercise, there was a significant elevation in TBARS levels and an increased tendency for hemolysis after H_2O_2 treatment in the Sed-Exh compared with the Sed-C group. The decreased hemolysis and lower TBARS levels in Sed-T-Exh groups indicated that erythrocytes became more resistant after the administration of vitamins C and E for 1 mo. No differences in H_2O_2-induced hemolysis and TBARS levels were found in ET groups compared with Sed-C.

Antioxidant Status

CAT, SOD, and GSH levels in various groups of animals are shown in Fig. 4. There were no significant effects of exhaustion and antioxidant treatment on CAT, SOD activities, and GSH levels in Sed groups. Also, CAT activity and GSH levels were not significant differences in ET and corresponding Sed groups, P < 0.05.

Table 1. Body weight, heart weight, heart weight-to-body weight ratio, exhaustion time, and hematological values of Sed and ET groups

<table>
<thead>
<tr>
<th></th>
<th>Sed-C</th>
<th>Sed-Exh</th>
<th>Sed-T-Exh</th>
<th>ET-C</th>
<th>ET-Exh</th>
<th>ET-T-Exh</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>269 ± 13.6</td>
<td>269 ± 9.3</td>
<td>282 ± 14.1</td>
<td>274 ± 12.3</td>
<td>268 ± 11.1</td>
<td>288 ± 11.0</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>0.856 ± 0.036</td>
<td>0.855 ± 0.021</td>
<td>0.874 ± 0.034</td>
<td>0.917 ± 0.035</td>
<td>0.903 ± 0.41</td>
<td>0.909 ± 0.019</td>
</tr>
<tr>
<td>Body/heart weight</td>
<td>303.3 ± 11.8</td>
<td>315.2 ± 8.1</td>
<td>322.6 ± 8.7</td>
<td>304.4 ± 7.7</td>
<td>302.9 ± 6.7</td>
<td>319.9 ± 8.4</td>
</tr>
<tr>
<td>Exhaustion time, min</td>
<td>67.5 ± 2.9</td>
<td>62.3 ± 6.1</td>
<td>158.0 ± 9.8*</td>
<td>152.0 ± 13.1*</td>
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<tr>
<td>Hematocrit, %</td>
<td>39.3 ± 0.5</td>
<td>39.3 ± 0.5</td>
<td>38.0 ± 0.4</td>
<td>40.0 ± 0.6</td>
<td>39.8 ± 0.5</td>
<td>38.8 ± 0.5</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>12.3 ± 0.4</td>
<td>12.2 ± 0.2</td>
<td>11.9 ± 0.2</td>
<td>12.6 ± 0.2</td>
<td>12.7 ± 0.1</td>
<td>12.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sed, sedentary; ET, exercise trained; C, control; Exh, exhausted; T, treated. *Significant difference from corresponding Sed groups, P < 0.05.
significantly different in ET groups compared with Sed groups. However, exhaustion in the ET group (ET-Exh) resulted in significantly increased SOD activities compared with that in the ET-C group. The antioxidant treatment not only prevented the enhancement in SOD activity, but resulted in a decrement.

DISCUSSION

The results of this study show that exercise affects erythrocyte properties and leads to hemolysis by an oxidant-mediated mechanism in Sed animals but not in ET animals. It is well known that regular physical activity triggers accommodative processes. Therefore, the results of our study must be discussed separately on the basis of responses obtained from ET and Sed rats.

Responses of Sed Groups

Free radicals are known to play a vital role in tissue damage, and they have adverse effects on erythrocytes (3, 23). In addition to the emergence of free radicals from mitochondrial leakage owing to enhanced oxygen consumption, ischemia-reperfusion process and leukocyte activation may also contribute to oxidative stress during exercise (13, 14), especially in extramuscular tissues (heart, liver, brain) and erythrocytes. We hereby show that erythrocyte TBARS values increased 2- to 2.5-fold in the Sed-Exh group, which was exhausted after nearly 1 h of running. Smith et al. (21) showed that a single episode of submaximal exercise enhanced the exposures of erythrocytes to oxidative and osmotic stresses, resulting in erythrocyte deterioration. We show that fortifying antioxidant defense mechanisms with 1 mo of treatment with vitamins E and C results in reduced erythrocyte deterioration in relation to decreased oxidant stress after exhaustive running episodes.

Significantly higher plasma hemoglobin levels were found in samples obtained 24 h after exhaustive running episode in the Sed-Exh group, indicating erythrocyte destruction. This alteration is a well-known consequence of exercise (20, 24, 27). The higher levels of these parameters maintained 24 h after exercise indicate the long-lasting adverse effects on erythrocytes. Increments in plasma hemoglobin concentration by hemolysis might be the result of increased osmotic fragility and/or decreased deformability of erythrocytes under exhaustive conditions. In a previous study by our group, the persistence of altered erythrocyte deformability was demonstrated even 24 h after an exhaustive swimming exercise (28). Change in blood pH,
rise in temperature, dehydration hemoconcentration, changes in erythrocyte morphology, and increments in intracellular Ca\(^{2+}\) due to \(\beta\)-adrenergic stimulation after acute exposure to exhaustive exercise might be responsible for decreased deformability and increased osmotic fragility (20, 24). Exercise-induced oxidative stress may also contribute to the deterioration of erythrocyte properties, because there was a marked reduction in resistance of erythrocytes of Sed-Exh rats against exogenous \(H_2O_2\) increased, thereby reducing the \(H_2O_2\)-induced hemolysis and TBARS levels. It is reasonable to assume that the administration of vitamins C and E can reduce the intensity of oxidant stress by enhancing the antioxidant defense mechanism. Suppression of exertional oxidant stress had greatly minimized the destruction of erythrocytes. There were no significant differences in values of plasma hemoglobin levels and erythrocyte deformability in Sed-Exh and Sed-C groups. Similarly GSH, CAT, and SOD values were not different among the Sed groups. These results suggest that antioxidant therapy may play a major role in preventing exercise-induced oxidative stress.

Several studies have demonstrated that exercise-induced oxidant stress occurring in various tissues and blood of experimental animals and humans might be prevented by antioxidant interventions (13, 19). Increased osmotic fragility of erythrocytes due to nonexertional oxidant stress was prevented with vitamin E and/or C supplementation, and the resistance of erythrocytes was enhanced (6, 9, 12, 18). According to our results, prevention of exertional oxidant stress associated with decreased rate of erythrocyte destruction demonstrated the validity of this mechanism among Sed rats undergoing physical activity.

Responses of Trained Groups

It is generally accepted that oxidant stress during and after exercise might be reduced by augmenting activities of antioxidant enzymes and facilitating electron transport from mitochondria, and this can be achieved by regular exercise (2, 13, 14, 19). Lipid peroxidation in the ET group (ET-C group) was not different from that in the Sed group. TBARS increment in trained animals increased significantly after exhaustive exercise (ET-Exh), although the change was lower than in the Sed-Exh group. On the other hand, changes in methemoglobin levels were different from TBARS levels in ET groups. A possible training-induced increment in methemoglobin levels were different from TBARS levels in ET groups. A possible training-induced increment in methemoglobin reductase activity may be responsible for the observed differences in methemoglobin levels between Sed and ET groups. Although we did not analyze the GSH peroxidase (GPX) activity in the present study, it is well known that regular physical exercise increases GPX activity (13, 14). Thus the prevention of increment in \(H_2O_2\)-induced TBARS level may be explained by the enhanced antioxidant status due to increased GPX activity in ET groups.
Increments in oxidative stress in the ET-Exh group with exhaustion have also been prevented by antioxidant vitamin therapy in ET rats (ET-T-Exh). Although similar responses to exercise and antioxidant vitamin therapy with respect to the level of lipid peroxidation were obtained from ET and Sed groups, the erythrocyte destructive responses differed widely. Plasma hemoglobin and erythrocyte osmotic fragility and deformability were not different from those of the ET-C group, although oxidative stress in the ET-Exh group was increased.

The difference in response of erythrocytes to exhaustion in Sed and ET rats indicates that the erythrocyte properties in these animals may differ. In individuals undergoing a regular physical activity program, the life span of erythrocytes is shorter, yielding a younger erythrocyte population in trained individuals (20, 21, 24, 27). Because younger erythrocytes are more resistant to oxidative stress (8), trained rats might be more resistant. Because younger erythrocytes are more deformable and have higher 2,3-diphosphoglycerate content, these cells may contribute to increased athletic performance (20). Therefore, oxidative stress observed during exercise might be accepted as an adaptive mechanism aimed at ensuring the prevalence of a younger erythrocyte population in trained animals (20, 21, 24, 27). Because younger erythrocytes are more resistant, these cells may contribute to increased athletic performance (20). Therefore, oxidative stress observed during exercise might be accepted as an adaptive mechanism aimed at ensuring the prevalence of a younger erythrocyte population in trained individuals.

In conclusion, a single, exhaustive exercise period resulted in elevation of oxidant stress and deterioration of erythrocyte structure and function in Sed rats that was prevented by 1 mo of antioxidant vitamin treatment. However, this impact was not demonstrated in trained rats. In Sed rats, oxidant stress is an important factor responsible for destruction of erythrocytes seen after an acute, single-exertional bout and/or at the beginning of the training period. Because sports anemia is seen in trained people, exercise-induced oxidative stress might not be a causative factor for sports anemia.

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REFERENCES