Acute immune response in respect to exercise-induced oxidative stress

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Abstract

The relationship between exhaustive exercise, oxidative stress, the protective capacity of the antioxidant defense system and cellular immune response has been determined. Exhaustive exercise in well-trained young men (n = 19)-induced leukocytosis, decreased proportion of activated-lymphocyte subsets (CD4+ and CD8+) expressing CD69, decreased lymphocyte mitogenic response to concanavalin A (ConA) and phytohemagglutinin (PHA), increased lipid peroxidation, increased total antioxidant status (TAS) and catalase activity, immediately after exercise. Suppressed blood concentration of T-lymphocyte subsets (CD3+, CD4+, CD8+, NK), increased TAS and blood total glutathione (TGSH) in early recovery period (30 min after exercise) were found. Strong positive correlation was observed between TGSH and lymphocyte mitogenic response to ConA and PHA (r = 0.85 and 0.85, respectively) immediately after exercise. Moderate positive correlation was observed between TAS and lymphocyte mitogenic response to PHA (r = 0.59) immediately after exercise as well as between TAS and lymphocyte mitogenic response to PHA and ConA (r = 0.69 and 0.54, respectively). Moderate to weak correlation was observed between TAS and conjugated dienes with exercise (r = 0.66) as well as in 30-min recovery (r = 0.50). After a short-term bout of exhaustive exercise, immune system was characterized by acute phase response, which was accompanied with oxidative stress. Suppression of the cellular immunity 30 min after exercise shows that this period is not enough for recovery after exhaustive exercise. The results suggest the interactions between exercise-induced oxidative stress and immune response. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Exercise; Immune system; Oxidative stress

1. Introduction

One of the most actual problems of sports medicine is to prevent athletes from disease and infections during intensive training and competition periods. The risk of infection near the decisive competition is significantly elevated, especially when, during training camps, the athletes live together and infection can be spread and ruin the work of several years. Although, moderate training enhances many aspects of immune function, exhaustive exercise may impair immune responses increasing athlete’s susceptibility to infection [1–12] and, possibly, to autoimmune diseases and cancer [13,14]. Epidemiological data suggest that endurance athletes are at increased risk for upper respiratory tract infection (URTI) during periods of heavy training and up to 2-weeks period following competition [15]. Exercise-related immunological changes include signs of inflammation, such as release of cytokines, activation of immunocompetent cell lines, complement, and the induction of acute phase proteins [7,8,16–19]. However, the signs of immunosuppression, such as decreased T and B cell function and impaired cytotoxic or phagocytic activity, can also be observed [20–22]. The immunological response to exercise comprises numerous alterations within the immune system; but how these
processes are regulated is still largely unknown. There has been substantial evidence for link between the neurohormonal and the immune system [23–25]. It is thought that stress hormones like adrenaline and cortisol cause the mobilization of granulocytes from extracirculatory pool [26]. Plasma concentrations of adrenaline and noradrenaline increase almost linearly with the duration of exercise and exponentially with its intensity when expressed relative to individual maximal oxygen uptake [27,28]. The expression of β-adrenoceptors on T, B and NK cells, macrophages and neutrophiles provides the molecular basis for these cells to be targets for catecholamine signaling [29,30]. On the other hand, it has been reported that catecholamines can autooxidize and lead to free radical production [31,32] or undergo metal ion-catalyzed oxidation to free radical products [33]. Hence, they provide a potential source of free radical production during exercise.

Heavy exercise is associated with substantial increases in oxygen consumption and production of reactive oxygen species (ROS) [34–37]. Strenuous exercise is known to induce oxidative stress [35,37–40], a state where pro-oxidants overwhelm the antioxidant defense capacity [41–43]. Previously, it has been shown that physical exercise may cause depletion and oxidation of GSH, a crucial factor in the maintenance of tissue-antioxidant defenses and in the regulation of redox sensitive-signal transduction [44–48]. Likewise, reactive oxygen species promote complement activation [49], as well as facilitate vascular translocation of leukocytes by inducing expression of adhesion molecules [50]. Despite the substantial evidence indicating that strenuous exercise induces oxidative stress and acute immune response, information concerning interactions between antioxidant defense and immune system within exercise is scanty. This study was, therefore, primarily focussed on monitoring of oxidative stress markers and cellular immune response within exercise until volitional exhaustion.

Increased knowledge about the cross talk between exercise-induced oxidative stress and acute immune response is important for developing means for maximizing the health benefits of exercise and improving sports performance.

2. Material and methods

Nineteen male endurance-trained athletes (age 22.21 ± 5.58 years, VO2max 69.79 ± 8.22 ml/kg/min) were recruited into the study after obtaining an informed consent. The experimental procedures and protocol conformed to the principles of the Declaration of Helsinki and were approved by the Human Ethics Committee of the University of Tartu. Reasons for exclusion included any deviation from the criteria of good health, smoking, chronic medication or vitamin supplementation.

2.1. Protocol for exercise

Subjects performed incremental treadmill (LE 3000) exercise test until volitional exhaustion. VO2max was measured using ‘breath by breath’ gas monitoring (Oxycon Record, Erich Jaeger, Germany). Heart rate was recorded by using a PE 300 heart rate monitor (Polar Electro Ltd., Kempele, Finland). Before exercising, all subjects were instructed to refrain from intense exercise for at least 2 days before exercise testing. On the day of exercise, the subjects ate a light, carbohydrate-rich breakfast. Exercise test was carried out 2–4 h after breakfast.

2.2. Blood sample collection and preparation

Venous blood samples were collected before exercise, immediately 30 min after exercise (recovery period). At each time point, blood was drawn from antecubital vein into three different (without additives, with Na2EDTA and with heparin) evacuated collection tubes (Vacutainer, Becton Dickinson, USA).

For catalase and total antioxidant status (TAS), assays serum was frozen immediately after separation in Eppendorf tubes and kept at −80°C until analyzed. For glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) determination, the heparinized whole blood was treated, as described in the commercial kit.

For total glutathione (TGSH) determination, tridistilled water (1.8 ml) and 1-ml 500-mM perchloric acid were added to 200-μl heparinized whole blood to hemolyze RBCs and to remove protein. After 5 min procedure, protein sediment was eliminated by centrifugation for 10 min at 3000 rpm. Supernatant was frozen at −80°C until determination.

2.3. Routine analyses

Complete blood cell counts, hemoglobin (Hb) and hematocrit (Hct) were measured from EDTA-treated blood using an automated hematology analyzer (Sysmex-SE9000, Japan).

2.4. Flow cytometry for determination of lymphocyte subpopulations

Whole blood leukocytes were labeled with monoclonal antibodies against the surface determinants CD45+, CD3+, CD4+ (all FITC conjugated) and CD14+, CD19+, CD8+, anti HLA-DR, CD16+, and CD56+ (all PE conjugated). All antibodies were purchased from Becton Dickinson (San Jose, USA). Data were acquired and analyzed on FACSort flow
cytometer (Becton Dickinson) by two-color flow cytometric analysis using SimulSET.

2.5. Proliferation assay

Mononuclear cells were separated from venous blood by density-gradient centrifugation on Histopaque-1077 (Sigma Chemical Co, St Louis, USA). Mononuclear cells, removed from the interface, were washed two times in PBS with 2% pooled human serum (Sigma). Cells \((n = 5 \times 10^6)\) in a final volume of 200 ml of RPMI 1640 with 10% pooled human serum (Sigma) were cultured in flat bottom microtiter tissue-culture plates (Falcon, Becton Dickinson Labware, New Jersey, USA). The cells were incubated with the T-cell mitogens phytohemagglutinin (PHA; Sigma) 5 mg/ml and concanavalin A (ConA; Sigma) 30 mg/ml. Assays were performed in triplicate, and unstimulated control cultures were included with every assay. Cells were incubated at 37°C in humidified 5% CO\(_2\) environment for 72 h. For the last 18 h, methyl-\(^3\)H-thymidine (Amersham International plc., UK) was added. Cells were harvested on glass-fiber filters, and \(^3\)H-thymidine incorporation was determined by liquid-scintillation counter (Beckman Instruments, Fullerton, USA). The lymphocyte proliferative response was expressed as mean counts per minute (cpm).

2.6. The fast immune assay

Whole blood leukocytes were incubated with mitogen PHA (Sigma) and the fast immune activation control for 4 h at 37°C in CO\(_2\) incubator. A three-color antibody-conjugate combination (CD4FITC/CD69PE/CD3PerCP and CD8FITC/CD69PE/CD3PerCP and isotype control reagent \(\gamma_1\)FITC/\(\gamma_1\)PE/CD3PerCP; Beckton Dickinson) was added to activated and control whole blood cultures. Stained cultures were diluted in FACS lysing solution (Becton Dickinson) and were acquired and analyzed by three-color analysis on FACSort flow cytometer. Data were displayed as two-color dot plots to determine the proportion of activated lymphocyte subsets expressing CD69. Data were analyzed using CELLQuest software.

2.7. Lipid peroxidation assays

For the measurement of thiobarbituric acid reactive substances (TBARS) and diene conjugates (DC), serum (nonhemolyzed) was separated by centrifugation at 3000 rpm for 10 min at +4°C and treated with antioxidant butylated hydroxytoluene (15-\(\mu\)l BHT added to 1 ml serum) immediately to suppress artificial changes during handling and assay procedures and kept at −80°C until analyzed. Serum DC levels were measured according to methods previously described [51] with minor modification [52]. Briefly, 0.25% butylated hydroxytoluene (BHT)-treated serum samples (0.15 ml) and 0.150 ml of 0.9% NaCl were incubated at 37°C for 25 min and lipids were extracted by heptane/isopropanol (1:1). Samples were acidified by 5 mol/l hydrochloric acid and extracted with cold heptane. After centrifugation for 5 min at 3000 rpm, the absorbance of the heptane fraction was measured at the absorbance maximum between 220 and 250 nm, using isotonic saline as blanks.

Conversion of diene conjugates results in stable secondary products (aldehydes, alkenals) of lipid peroxidation. The assay for thiobarbituric acid (TBA) reactive products (TBARS), in which TBA reacts with malondialdehyde, is a sensitive but relatively non-specific method for detection of later stage lipid peroxidation. TBARS levels were measured in serum samples [53] with minor modifications to increase specificity [52]. Samples (0.25 ml) were incubated with 0.475 mM Fe\(^{2+}\) at 37°C for 30 min. After incubation, BHT (0.25%) was added to the samples. This mixture was treated with acetate buffer (pH 3.5) and heated with TBA solution (1%, 80°C, 10 min). The samples were then cooled and acidified (5 mol/l hydrochloric acid). After extraction with cold butanol, samples were centrifuged. The absorbance of the butanol fraction was measured at 534 nm. A standard plot for malondialdehyde (end product of lipid peroxidation) was prepared using 1,1,3,3-tetraethoxypropane.

2.8. Total antioxidant status assay

Total antioxidant status was measured in serum by the commercially available kit (Randox Laboratories Ltd., Ardmore, UK).

2.9. Total blood glutathione

Total blood glutathione was measured with Ellman reagent [54], as described by Beutler et al. (1963) [55]. The content of TGSH was calculated on the ground of standard plot.

2.10. Assay for antioxidant enzymes

Catalase (CAT) activity of the serum was measured according to the method described by Goth (1991) [56]. Determination of erythrocyte Cu, Zn-SOD activity was carried out using washed red-cell hemolysates with commercial available kit (Ransod, Randox Laboratories Ltd., UK). The method employs xantine and xantine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was measured by the degree of inhibition of this reaction. GSHPx activity was measured by the com-
mmercially available kits (Ransel, Randox Laboratories Ltd, Ardmore UK), based Paglia and Valentine (1967) [57].

2.11. Statistical analysis

Calculations were performed using STATISTICA software. All results are presented as mean ± S.D. ANOVA for repeated measures was used for analyzing the change of variables with exercise. Results were adjusted as necessary for hemoconcentration by using changes in Hct with exercise as a covariate. In all analyses, a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Maximal oxygen consumption

The mean $\text{VO}_{2\text{max}}$ (69.72 ± 8.73 ml/kg/min) indicated that the men were highly fit.

3.2. Hemoglobin and hematocrit

Both Hb concentration and Hct rose markedly with exercise ($P = 0.0004–0.0014$), but returned to pre-exercise level in 30-min recovery period (Table 1).

3.3. Cellular immune response

Exercise until volitional exhaustion induced leukocytosis ($P < 0.00001$) in the peripheral blood, which returned to pre-exercise level during 30-min recovery (Table 2). Neutrophilia was prominent ($P < 0.00001$) immediately after exercise, but returned to pre-exercise level in 30-min recovery period (Table 1). The proportion of PHA-stimulated lymphocytes expressing CD69 decreased ($P = 0.01$ and 0.0007, respectively) immediately after exercise, but returned to pre-exercise level in 30-min recovery (Fig. 1).

Lymphocyte mitogenic response to ConA and PHA was significantly decreased ($P = 0.0045$ and 0.0003, respectively) after exercise. However, lymphocyte mitogenic response to PHA returned to pre-exercise level in 30-min recovery, while the response to ConA still remained decreased ($P = 0.035$) (Table 2).

3.4. Lipid peroxidation

Serum DCs ($P = 0.002$) and TBARS ($P = 0.00064$) concentrations increased with exercise (Table 1). These increases were significant even after controlling for changes in Hct ($P = 0.039$ and 0.004, respectively). Serum DCs and TBARS returned to the baseline in 30-min recovery period (Table 1).

3.5. Total antioxidant status

Serum TAS increased significantly after exercise ($P = 0.0012$) and in recovery period ($P = 0.0183$) (Table 1). When controlling for change in Hct, however, the change after exercise was no longer significant; but the change in recovery period still remained significant ($P = 0.01$).

3.6. Catalase

Serum CAT activity rose with exercise ($P = 0.0267$), but returned to the baseline in 30-min recovery period until volitional exhaustion.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th>Recovery (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct (l/l)</td>
<td>0.43 ± 0.02</td>
<td>0.45 ± 0.02 ($P = 0.00136$)</td>
<td>0.42 ± 0.02 ($P = 0.351$)</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>147.68 ± 7.67</td>
<td>152.89 ± 6.42 ($P = 0.0004$)</td>
<td>146.63 ± 7.21 ($P = 0.568$)</td>
</tr>
<tr>
<td>TBA reactivity ($\mu$M MDA equivalents)</td>
<td>1.61 ± 0.38</td>
<td>1.92 ± 0.50 ($P = 0.00064$)</td>
<td>1.70 ± 0.45 ($P = 0.214$)</td>
</tr>
<tr>
<td>CD ($\mu$M)</td>
<td>57.59 ± 12.67</td>
<td>63.66 ± 15.06 ($P = 0.002$)</td>
<td>60.98 ± 16.17 ($P = 0.35$)</td>
</tr>
<tr>
<td>TAS (mmol/l)</td>
<td>1.38 ± 0.31</td>
<td>1.44 ± 0.25 ($P = 0.0012$)</td>
<td>1.47 ± 0.34 ($P = 0.0183$)</td>
</tr>
<tr>
<td>TGSH ($\mu$mol/l)</td>
<td>827.82 ± 77.7</td>
<td>944.26 ± 145.9 ($P = 0.013$)</td>
<td>880.49 ± 114.2 ($P = 0.023$)</td>
</tr>
<tr>
<td>S-CAT (u/l)</td>
<td>74.59 ± 27.85</td>
<td>95.50 ± 49.64 ($P = 0.0267$)</td>
<td>68.39 ± 24.27 ($P = 0.461$)</td>
</tr>
<tr>
<td>E-SOD (u/g) Hb</td>
<td>752.22 ± 129.39</td>
<td>753.69 ± 164.20 ($P = 0.82$)</td>
<td>749.53 ± 148.36 ($P = 0.92$)</td>
</tr>
<tr>
<td>E-GSHPx (u/g) Hb</td>
<td>50.90 ± 14.34</td>
<td>50.90 ± 13.32 ($P = 0.814$)</td>
<td>50.43 ± 11.27 ($P = 0.807$)</td>
</tr>
</tbody>
</table>

* Values are means ± S.D.
Table 2
Leukocytes, lymphocyte subsets (CD3+, CD4+, CD8+, CD19+, NK) and lymphocyte mitogenic response to ConA and PHA in highly fit 19 young men before, immediately after and 30 min after exercise test until volitional exhaustion

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th>Recovery (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (cell × 10⁶ per l)</td>
<td>5.71 ± 0.90</td>
<td>9.01 ± 1.45</td>
<td>5.44 ± 1.04</td>
</tr>
<tr>
<td>Neutrophils (cell × 10⁶ per l)</td>
<td>3.01 ± 0.64</td>
<td>3.99 ± 0.95</td>
<td>3.25 ± 0.93</td>
</tr>
<tr>
<td>B-lymphocytes (cell × 10⁶ per l)</td>
<td>0.22 ± 0.11</td>
<td>0.314 ± 0.14</td>
<td>0.179 ± 0.54</td>
</tr>
<tr>
<td>CD3+ (cell × 10⁶ per l)</td>
<td>1.30 ± 0.35</td>
<td>2.09 ± 0.63</td>
<td>1.13 ± 0.24</td>
</tr>
<tr>
<td>CD4+ (cell × 10⁶ per l)</td>
<td>0.76 ± 0.23</td>
<td>1.12 ± 0.31</td>
<td>0.65 ± 0.16</td>
</tr>
<tr>
<td>CD8+ (cell × 10⁶ per l)</td>
<td>0.79 ± 0.27</td>
<td>1.84 ± 0.57</td>
<td>0.67 ± 0.16</td>
</tr>
<tr>
<td>CD4+/CD8+ (cell × 10⁶ per l)</td>
<td>0.96 ± 0.27</td>
<td>0.64 ± 0.18</td>
<td>1.05 ± 0.36</td>
</tr>
<tr>
<td>NK (cell × 10⁶/l)</td>
<td>0.51 ± 0.16</td>
<td>1.50 ± 0.49</td>
<td>0.34 ± 0.13</td>
</tr>
</tbody>
</table>

Lymphocyte mitogenic response to
ConA (cpm) × 1000                  | 30.73 ± 16.15| 19.42 ± 8.7   | 24.82 ± 10.74     |
PHA (cpm) × 1000                   | 56.41 ± 34.74| 41.21 ± 24.83 | 51.53 ± 37.55     |

* Values are mean ± S.D.

(Table 1). However, after controlling for change in Hct, the increase after exercise was no longer significant.

3.7. Total glutathione

Total blood glutathione levels increased significantly after exercise (P < 0.013) and remained increased in recovery period (P < 0.023; Table 1). When controlling for change in Hct, the increases were still significant (P = 0.039 and 0.013, respectively).

3.8. Erythrocyte antioxidant enzymes

There were no significant changes in either GSHPx or SOD activity with exercise (Table 1).

3.9. Correlations

Strong positive correlations were observed between TGS, and lymphocyte mitogenic response to ConA and PHA (r = 0.85 and 0.85, respectively) immediately after exercise and in 30-min recovery (r = 0.72 and 0.79, respectively). Moderate positive correlation was observed between TAS and lymphocyte mitogenic response to PHA (r = 0.59) immediately after exercise as well as between TAS and lymphocyte mitogenic response to PHA and ConA (r = 0.69 and 0.54, respectively).

Moderate to weak correlation was observed between TAS and DCs with exercise (r = 0.66) as well as in 30-min recovery (r = 0.50).

4. Discussion

Exercise until volitional exhaustion caused oxidative stress as evident from the lipid peroxidation data. Our finding of elevated serum TBARS and DC in post-exercise samples is consistent with previous reports [58,59].

We found that serum total antioxidant status (ability to scavenge free radicals) increased significantly in 30-min recovery, which may indicate compensation in response to exhaustive exercise. Previously, it has been shown that half-marathon in 17 trained male runners induced increase in serum total antioxidant capacity [60]. Total blood glutathione rose with exercise and was significantly increased in 30-min recovery period. This finding is in agreement with previous reports [46,59]. A serious limitation of present study is that, due to the technical problems, oxidized glutathione was not measured. Most probably, the increase in blood TGS may be expected to be due to export from peripheral tissues, such as skeletal muscles [61].

In the present study, serum CAT and erythrocyte antioxidant enzyme activities (SOD and GSHPx) were unaffected by the exercise. Previous studies support this finding [44,62].

Exercise-induced changes in peripheral leukocyte counts observed in this study represented typical

![Fig. 1. CD69 expression of T-cell subsets (CD4+ and CD8+) stimulated by PHA in highly fit 19 young men before, immediately after and 30 min after exercise test until volitional exhaustion. **, P < 0.01; ***, P < 0.001, as compared with pre-exercise level.](image-url)
changes that are expected in response to exhaustive exercise [63–65]. Exercise until volitional exhaustion induced leukocytosis with granulocytes, contributing the greatest part. It is thought that stress hormones like adrenaline and cortisol cause the mobilization of granulocytes from extracirculatory pool. However, unlike catecholamines, cortisol exerts its effect with lag time of several hours [66,67]. It is therefore unlikely that cortisol is responsible for lymphocytopenia, observed in 30-min recovery after acute bout of exhaustive exercise. Recently, it has been shown that lymphocyte apoptosis may, in part, account for the exercise-induced lymphocytopenia and reduced immunity [68].

4.1. Lymphocyte proliferative response

The post-exercise altered percentual distribution of the subsets of blood mononuclear cells influencing the functional in vitro assays, in which fixed numbers of cells are studied. Previously, it has been shown that proliferative response per CD4+ cell did not change in relation to exercise (75% VO2max, 60 min.), but the contribution of the CD4+ subgroup to proliferation declined during exercise due to the decreased proportion of CD4+ cells [69]. In the current study, a marked increase in amount of CD4+ cells was found after exercise, but their proportion declined with exercise. Thus, probably, exercise-induced decline in ConA- and PHA-stimulated proliferative response may be explained in part by a relative fall in CD4+ cells. However, we found that proportion of activated lymphocyte subsets (CD4+ and CD8+) expressing CD69 was also significantly decreased with exercise. The CD69 antigen is one of the earliest markers expressed on activated lymphocytes, following stimulation by a variety of mitogenic agents [70]. Positive strong correlation between TGSH and lymphocyte mitogenic response to ConA and PHA after exercise makes to reflect on possible interaction between TGSH and lymphocyte proliferation activity. Moreover, there is evidence that cell proliferation in response to PHA stimulation is reduced in GSH depleted cells [71].

The mechanism underlying exercise-related changes in cellular immune response seems to be multifactorial and related not only to hormonal and metabolic changes that occur in relation to muscular work but also to the exercise-induced oxidative stress and altered gene expression. ROS may play an important role in the tangled net of factors, which are responsible for exercise-induced stress reactions. Future studies have to apply molecular-biology techniques to further clarify mechanisms by which exercise-induced oxidative stress influences immune function. Understanding of these mechanisms will not only allow to extend knowledge to clinical implications, but also to enhance general understanding of regulation and integration of immune function under a variety of conditions.

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