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Assessment of oxidative stress in lymphocytes with exercise

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The aim of this study was to examine whether the aforementioned exercise-induced changes in the cellular composition of blood could affect measurements and, therefore, the interpretation of exercise-induced oxidative stress in total lymphocytes.

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METHODS

Participants. Sixteen healthy nonsmoking men took part in the main part of this study, having abstained from taking vitamin supplements for at least 6 wk (see Table 1). Participants provided informed consent, and the study was approved by the School of Sport and Exercise Sciences research ethics committee of the University of Birmingham.

Procedures. Participants undertook two treadmill-based graded exercise tests on the same day to accurately determine running speed for the exercise trial as previously described (54). Briefly, in the first test, participants ran at incremental speeds until exhaustion to measure maximal oxygen consumption (VO_{2\max}) (58). After a 10-min rest, the relationship between oxygen consumption and four submaximal running speeds (range 8.2–11.6 km/h, gradient 2%) was assessed to calculate the speed to elicit 80% of VO_{2\max} using linear regression. In addition, participants were asked to report leisure time physical activity using the international physical activity questionnaire (IPAQ) (14).

Exercise trial. One week after preliminary tests, participants returned to the laboratory between 0600 and 0700 following an overnight fast. Participants were instructed to refrain from exercising and drinking alcohol or caffeine during the day before the trial. Following a 15-min rest, a baseline blood sample (Pre) was collected from an antecubital vein by an indwelling catheter, which was kept patent by flushing with saline. Participants then undertook an exercise trial, which consisted of treadmill running at a speed to elicit 80% of VO_{2\max} for 60 min. Treadmill gradient was maintained at a 2% incline. Blood was collected at the end of exercise (Ex60), and again 15 min (Post15) and 60 min (Post60) postexercise. Pilot work showed that these time points allowed for the observation of peak lymphocytosis (Ex60), a return to baseline (Post15), and the nadir of lymphocytopenia postexercise (Post60), respectively (data not shown).

Blood sample processing. Blood was collected into EDTA vacutainer tubes (Becton-Dickinson, Oxford, UK) and placed at 4°C until processing. Plasma was obtained by centrifugation at 1,350 g for 15 min at 4°C and stored at −80°C. Peripheral blood lymphocytes were isolated using density gradient centrifugation. Briefly, whole blood was diluted 1:1 with PBS (Invitrogen, Paisley, UK) and carefully layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsal, Sweden) before centrifuging at 300 g for 30 min. The lymphocyte layer was aspirated, and cells were washed in PBS at 1,000 g for 10 min. The pellet was re-suspended in either sodium carbonate buffer (50 mM, pH 7.4) and stored at −80°C for assessment of protein carbonyl concentration or re-suspended in PBS and processed for flow cytometric measurements.

General analytical procedures. Total lymphocyte protein concentration was determined using the bicinchoninic acid method as described by Smith et al. (48). Biochemical parameters were expressed relative to protein concentration or were corrected for changes in plasma volume according to Dill and Costill (16) as appropriate. Hemoglobin and hematocrit were assessed using a Coulter ACTdiff hematology analyzer (Beckman-Coulter, High Wycombe, UK). Chemicals were obtained from Sigma-Aldrich, UK, unless otherwise stated.

Table 1. Characteristics of participants in the main study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>23.6 ± 13.2</td>
</tr>
<tr>
<td>BMI, kg/m</td>
<td>23.8 ± 2.5</td>
</tr>
<tr>
<td>Measured VO_{2\max}, ml·kg⁻¹·min⁻¹</td>
<td>59.7 ± 5.2</td>
</tr>
<tr>
<td>Maximum heart rate at VO_{2\max}, beats/min</td>
<td>187 ± 9</td>
</tr>
<tr>
<td>LTPA, median MET·min/wk*</td>
<td>3.497 (720–7,290)</td>
</tr>
</tbody>
</table>

Data are means ± SD. LTPA, leisure time physical activity, as assessed by the international physical activity questionnaire. *Data are medians (min-max).

Protein carbonylation. Total lymphocyte protein carbonyl concentration was measured by enzyme-linked-immunosorbent-assay (ELISA) described by Buss et al. (10). Lymphocytes were lysed with RIPA buffer containing a protease inhibitor cocktail, and the supernatant was used for analysis. Samples and standards (0.5 mg/ml in sodium carbonate buffer; 50 mM, pH 7.4, 50 µl) were allowed to bind to 96-well multi-sorb plates for 60 min at 37°C in triplicate (Nunc, Fisher Thermo Scientific). 2,4-Dintrophenylhydrazine (DNP, 1 mM, 50 µl) in 2 M hydrochloric acid was added, and plates were incubated at room temperature for 60 min. Plates were blocked overnight at 4°C with 0.1% TBS-TWEEN-20. Mouse anti-DNP antibody (1:1,000, 50 µl) was incubated with samples for 120 min at 37°C, followed by a peroxidase-labeled rat anti-mouse antibody (1:5,000, 50 µl), incubated for 60 min at 37°C. A citrate phosphate-based substrate (0.15 M, pH 5) was added, and plates were left to develop in the dark for 30 min at 37°C. The reaction was stopped with 2 M sulphuric acid, and plates were read at 490 nm. Values were expressed as nmol/ml of protein.

Lipid peroxidation. Lipid peroxides were assayed in plasma using a modification of the method by el-Saadani et al. (19). Samples and positive and negative controls (1.1·10⁹ hydro peroxide and distilled water, respectively) were added to 96-well plates in triplicate. Working reagent (0.2 M potassium phosphate, 0.12 M potassium iodide, 0.15 mM sodium azide, 2 g/l Triton-X, 0.1 g/l alkylbenzylideneaminolimonium, 10 µM ammonium molybdate; 100 µl) was added, and plates were incubated at room temperature for 30 min. Plates were read at 340 nm. Lipid peroxide concentration was calculated using the Beer-Lambert-Law with an extinction coefficient of 24,600. Values were expressed as nmol/ml plasma.

Total antioxidant capacity. Total antioxidant capacity was assessed in plasma using the ferric reducing ability of plasma (FRAP) assay (8). Briefly, standards (0–1,000 µM ascorbic acid) and samples (10 µl/well) were added to 96-well flat-bottomed cell culture plates in triplicate. Working reagent (20 mM ferric chloride, 160 mM 2,4,6-tripryridyltriazine, 300 mM acetate buffer; 300 µl) was added to each well, and plates were read at room temperature after 8 min. Values were determined by linear regression from a seven-point standard curve (36) and expressed as µM of antioxidant power relative to ascorbic acid.

Flow cytometry. To examine the mobilization of T lymphocyte subpopulations with exercise, fresh lysed whole blood was prepared as described previously (54). Fixed cell preparations were read within 36 h of exercise on a multi-parameter flow cytometer (BD FACSCanto II, BD Biosciences). Lymphocytes were enumerated using a Coulter ACT ¿ hematology analyzer (Beckman-Coulter).

In a separate investigation, blood was collected from an antecubital vein at rest from eight healthy men (means ± SD: age, 31.0 ± 10.4 yr; body mass index 23.5 ± 2.1 kg/m², VO_{2\max} 56.2 ± 5.0 ml·kg⁻¹·m⁻¹). Two of the men took part in the main investigation, and the additional six subjects did not differ from those included in the main study. Samples were collected at least 2 h after consuming food or fluids. Intracellular GSH was measured in lymphocyte subpopulations using a modification of the procedure described by Cossarizza et al. (13). Monobromobimane (MBB) is a nonfluorescent probe that binds to intracellular thiols emitting light at 490 nm when excited with a 405-nm violet laser. GSH is the most abundant intracellular thiol; therefore, median fluorescence intensity (MFI) of MBB can be considered a measure of intracellular GSH concentration (13, 43).

Approximately 2 × 10⁶ peripheral blood lymphocytes were incubated with monoclonal antibody panels for 20 min at room temperature. The first antibody panel included anti CD19 FITC, anti CD56 PE (Pharmingen, San Diego, CA), and anti CD3 PerCP (BD Biosciences, San Jose, CA). The second antibody panel included anti CD45RA FITC, CD27 PE (Pharmingen), CD3 PerCP, CD28 PE-cy7, CD4 APC, and CD8 APC-cy7 (BD Biosciences). Samples were washed in PBS by centrifugation at 300 g. Cells were re-suspended in 1 ml of 37°C PBS, and a sample from each individual was incubated for 20 min at 37°C with 100 µM N-ethylmaleimide. Intracellular thiol concentrations were determined using a coupler ACT ¿ hematology analyzer (Beckman-Coulter).
content is depleted by N-ethylmaleimide; therefore, these preparations served as negative controls. Negative controls were washed and re-suspended in 1 ml of 37°C PBS. Samples and negative controls were incubated for 10 min at 37°C with 50 μM MBB (Invitrogen Molecular Probes, Paisley, UK). Cells were washed and re-suspended in PBS, and read within 30 min of collecting 50,000 lymphocytes. Lymphocytes were gated on the forward vs. side-scatter. T cells were identified by expression of CD3 in combination with CD4 or CD8. T-cell subpopulations were identified on the basis of CD27 and CD45RA expression (26) or CD27 and CD28 expression (3). B cells (CD3−CD19+) and Natural killer cells (CD3−CD56+) were also examined.

Statistical analysis. Data were inspected for normal distribution using the Kolmogrov Smirnov test. Non-normally distributed data were transformed logarithmically. Responses to exercise were examined with repeated-measures ANOVAs. Differences compared with baseline were examined using post hoc paired samples t-tests. Statistical significance was accepted at the P < 0.05 level. Intracellular thiol content between lymphocyte subpopulations was examined using paired samples t-tests. Effect sizes are presented as η². Data are presented as means ± SE unless otherwise stated. Data were analyzed using SPSS statistical package version 18.0 for Windows (SPSS).

RESULTS

Lymphocytes. A detailed analysis of lymphocyte responses to this bout of exercise has been undertaken by the authors and described previously (54). Briefly, exercise mobilized CD8+ T cells with a late-differentiated/effector memory phenotype (54). For illustrative purposes relevant to this investigation, alterations in the proportions of CD8+ T lymphocytes at each extreme of the differentiation continuum are presented (i.e., naive vs. EMRA) (see Fig. 1A). Compared with rest, the most differentiated EMRA cells constituted a larger proportion of total lymphocytes, whereas the least differentiated naive cells were much smaller in proportion during exercise [F(3,36) = 9.6, P < 0.001, η² = 0.443; see Fig. 1A]. CM and EM populations, and cells that did not express CD28, showed similar increases to EMRA with exercise, and an identical pattern was observed for CD4+ T cell subsets (data not shown).

Protein carbonylation. Figure 1B shows total lymphocyte protein carbonyl concentration in response to exercise. Protein carbonyls increased with exercise and returned to baseline within 15 min [F(3,39) = 16.7, P < 0.001, η² = 0.562; see Fig. 1B]. Correlation analyses showed that lymphocyte protein carbonyl content was unrelated to the cell subpopulations that constitute total lymphocytes: No correlations were observed between the numbers or proportions of any lymphocyte subset and the level of protein carbonyls measured in total lymphocytes (r < 0.12, P < 0.7). For illustrative purposes, the relationship between protein carbonyl concentration in total lymphocytes and the number of peripheral blood CD8+ T cells at each extreme of the differentiation continuum (i.e., naive and EMRA) are presented in supplementary information (see supplementary figure available online at the Journal of Applied Physiology website).

Lipid peroxidation. Figure 1C shows plasma lipid peroxide concentrations in response to exercise. Lipid peroxidation increased with exercise, and, following completion of the exercise bout, lipid peroxides fell below baseline levels within 15 min. Sixty minutes after exercise, lipid peroxide concentra-

Fig. 1. Immunological and oxidative responses to exercise. Data are means ± SE. A: revertant effector memory CD8+ T cells, which have re-expressed CD45RA (EMRA; CD27−CD45RA+) and naive CD8+ T cells (CD27+CD45RA+). Cells expressed as a proportion (%) of total lymphocytes. B: lymphocyte protein carbonyl concentration. C: plasma lipid peroxide concentration. D: plasma antioxidant capacity. Significant difference compared with baseline (paired samples t-tests): *P < 0.05; **P < 0.01; ***P < 0.001.
This was accompanied by increases in plasma lipid peroxides and elevated plasma antioxidant capacity. We hypothesized that the cells that increase the most in peripheral blood with exercise (e.g., late-differentiated/effecter memory cells or natural killer cells) may carry a greater oxidative load (i.e., increased markers of oxidative stress) compared with naive/early differentiated cells. Thus the present study investigated whether the observed increase in lymphocyte protein carbonyl groups was likely a result of exercise-induced oxidative stress or whether this was partly attributable to changes in the cellular composition of peripheral blood.

Our measurements of protein carbonyls were unrelated to the cell subpopulations that constitute total lymphocytes. No correlations were observed between the numbers or proportions of any lymphocyte subset and protein carbonyl level in total lymphocytes. For example, a higher number of late-differentiated/effecter memory cells within total lymphocytes had no bearing on the degree of protein carbonylation in the total lymphocyte pool. In support, our further investigations showed that the cells responsible for lymphocytosis (e.g., late-differentiated/effecter memory cells and natural killer cells) had a higher intracellular thiol content compared with early differentiated/naive cells and B cells (the latter populations show minimal mobilization with exercise). Cells with high thiol levels are usually associated with low levels of protein carbonylation and lipid peroxidation (27, 42). Although we did not measure protein carbonyl content of individual lymphocyte subsets, on the basis of these results and previous literature, it seems unlikely that cell populations entering peripheral blood due to exercise would carry an increased protein carbonyl load. We suggest, therefore, that the observed increase in oxidative stress to lymphocytes is likely to be a direct effect of exercise.

The results presented here show that cells selectively mobilized with exercise, carry high intracellular thiol content. The most abundant intracellular thiol is reduced GSH (37, 53). GSH is a tripeptide synthesized from the amino acids cysteine, glycine, and glutamic acid by the enzymes γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (37, 53). The actions of γ-GCS and GSH synthetase are regulated through cellular signaling pathways and can be upregulated to increase synthesis of GSH (37, 53). Thus it is possible that cells that have undergone previous oxidative insults might upregulate GSH synthesis (37), providing further protection against oxidative stress (24). Interestingly, and in addition, the role of intracellular thiols in lymphocytes is not limited to providing protection against pro-oxidants. Late-differentiated/effecter memory cells and natural killer cells are highly functional; they are able to divide rapidly on exposure to antigen, home to inflamed tissue, and employ rapid cytotoxic effecter functions without further need for differentiation (3, 4, 55). The finding that these cells exhibit increased thiol levels is not surprising since intracellular thiol groups are essential for lymphocyte activation, proliferation, and cytotoxicity (17, 33, 49, 51, 60). Intracellular thiol content might therefore be upregulated due to the memory/differentiation status of the lymphocyte, or as a result of oxidative stress, or as a result of both of these factors.

The present study showed that plasma antioxidant capacity was increased above preexercise levels for at least 1 h following the exercise protocol. This antioxidant response with exercise is well documented (28, 29, 45), and the present findings...
are in accordance with observations following similar exercise protocols (1, 38). The most likely mechanism behind antioxidant responses to exercise is ascorbic acid efflux from the adrenal glands (24). However, although speculative, it is also possible that cells in contact with plasma (e.g., lymphocytes, erythrocytes, endothelial cells) directly influence the concentration of plasma antioxidant molecules (6). This hypothesis is appealing considering the findings presented here, that cells that occupy peripheral blood with exercise show increased intracellular thiol content. In addition, the extracellular transport of lymphocyte-derived GSH is proportional to intracellular thiol level (37). Apoptosis also results in the release of GSH into the extracellular space (5), and up to 10% of lymphocytes undergo programmed cell death following exercise (34, 40, 41). It is therefore possible that the increased plasma antioxidant capacity observed in the present study was partly mediated by an influx of cells, which have an increased antioxidant capability, into the blood. In addition to these factors influencing antioxidant capacity, rapid alterations may also be present at the gene transcription level. For example, redox-sensitive signaling leading to gene expression for enzymatic antioxidants is upregulated within 15 min of exercise (28). It is argued that at least some of the benefits of regular physical activity are gained through redox-sensitive signaling (29, 44, 45).

In the present study, intense exercise caused a transient increase in lipid peroxides measured in plasma, which was followed by a fall in oxidized lipids to below baseline level 15 min after exercise cessation. Similar decreases in plasma lipid peroxides have been reported previously following exercise (23, 31), and it is argued that this might be one mechanism by which exercise exerts athero-protective effects (23, 30). Thus our observation of a bi-phasic change in plasma lipid peroxide concentration in response to this bout of exercise is not surprising.

A limitation of the present investigation is that we did not assess protein carbonyl content in individual lymphocyte subpopulations. In principle, this might be possible using fluorescence-activated cell sorting (FACS) or a combination of positive and negative selection using magnetic beads, assaying the isolated lymphocyte subpopulations for protein carbonyls. However, in reality, isolation of cells using FACS is very likely to cause oxidative stress. Second, there are very few late-differentiated effector memory cells per microliter of blood, often in the region of just 10 or 20 cells. This would make it almost impossible to obtain enough cells for assessment of protein oxidation. Techniques for the assessment of oxidative stress on a per cell basis in specific lymphocyte populations are lacking. We believe our chosen strategy is the most robust measure available for our particular application.

In conclusion, the results of the present study show that an acute bout of intense exercise caused a transient oxidative stress that could be detected by the measurement of protein adducts in lymphocytes. We suggest that the observed lymphocyte protein carbonylation following exercise is not caused by lymphocyte subpopulations carrying differing amounts of protein oxidation. We have shown that lymphocyte subpopulations show differential intracellular thiol concentrations. We propose that intracellular thiol content is related to the functional capacity and differentiation/memory stage of the lymphocyte subset.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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