Exercise-Induced Muscle Damage, Plasma Cytokines, and Markers of Neutrophil Activation

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DOI: 10.1249/01.MSS.0000161804.05399.3B

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ABSTRACT


Introduction: Unaccustomed eccentric exercise often results in muscle damage and neutrophil activation. We examined changes in plasma cytokines stress hormones, creatine kinase activity and myoglobin concentration, neutrophil surface receptor expression, degranulation, and the capacity of neutrophils to generate reactive oxygen species in response to in vitro stimulation after downhill running.

Methods: Ten well-trained male runners ran downhill on a treadmill at a gradient of −10% for 45 min at 60% VO2max. Blood was sampled immediately before (PRE) and after (POST), 1 h (1 h POST), and 24 h (24 h POST) after exercise. Results: At POST, there were significant increases (P < 0.01) in neutrophil count (32%), plasma interleukin (IL)-6 concentration (460%), myoglobin (Mb) concentration (1100%), and creatine kinase (CK) activity (40%). At 1 h POST, there were further increases above preexercise values for neutrophil count (85%), plasma Mb levels (1800%), and CK activity (56%), and plasma IL-6 concentration remained above preexercise values (410%) (P < 0.01). At 24 h POST, neutrophil counts and plasma IL-6 levels had returned to baseline, whereas plasma Mb concentration (100%) and CK activity (420%) were elevated above preexercise values (P < 0.01). There were no significant changes in neutrophil receptor expression, degranulation and respiratory burst activity, and plasma IL-8 and granulocyte-colony stimulating factor concentrations at any time after exercise. Neutrophil count correlated with plasma Mb concentration at POST (r = 0.64, P < 0.05), and with plasma CK activity at POST (r = 0.83, P < 0.01) and 1 h POST (r = 0.78, P < 0.01). Conclusion: Neutrophil activation remains unchanged after downhill running in well-trained runners, despite increases in plasma markers of muscle damage.

Key Words: ECCENTRIC EXERCISE, MYOGLOBIN, CREATINE KINASE, ADAPTATION

Eccentric exercise such as downhill running is associated with muscle damage (11,12). Typically, this muscle damage is characterized by morphological alterations within the muscle, including streaming, disruption, and dissolution of the sarcomeric Z-disk (11). This structural damage to the muscle cell is accompanied by the leakage of proteins such as creatine kinase and myoglobin out of the cell and into the circulation (11). Creatine kinase and myoglobin have been used as indirect markers of muscle damage in many different exercise studies (7,20–22,25,28,30). Exercise-induced muscle damage may also result in activation of the complement cascade, as indicated by increase in plasma complement proteins C3a and C5a (5,7).

Neutrophils are involved in tissue injury and inflammation. The expression of complement receptor 3 (CR3 or CD11b) is enhanced on the surface of neutrophils obtained from patients suffering from thermal injuries (16). Increased CD11b expression may assist the adhesion of neutrophils to the vascular endothelium and subsequent infiltration into tissues (1). Alternatively, inflammatory conditions may result in the shedding of complement receptor 1 (CR1 or CD35) and immunoglobulin G (IgG) receptors such as FcγRIII (CD16), as indicated by increased soluble concentrations of these two receptors in the synovial fluid of individuals with chronic rheumatoid arthritis (13,24). This shedding might reflect a role for CD35 and CD16 in controlling complement activation (24) and the binding of soluble immune complexes (13), respectively. The engagement of these receptors on the surface of neutrophils stimulates neutrophil phagocytosis, degranulation, and the production of reactive oxygen species (ROS). Together, these processes assist in the destruction of damaged tissue.

Eccentric exercise is accompanied by an increase in circulating neutrophils (7,20–22). This increase may possibly

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Submitted for publication December 2003.

Accepted for publication December 2004.

0195-9131/05/3705-0737


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DOI: 10.1249/01.MSS.0000161804.05399.3B
be due to an increase in complement proteins such as C3a in plasma after eccentric exercise (7). Neutrophil surface expression of CD11b and the FcγRI receptor CD64 may also increase after eccentric exercise (21,22). By promoting neutrophil adhesion, enhanced expression of CD11b could explain reports of neutrophil infiltration into damaged muscle after exercise (12). In addition, eccentric exercise increases the plasma concentrations of elastase and myeloperoxidase, which indicate neutrophil degranulation (4,8,23). Finally, eccentric exercise augments the capacity of neutrophils to generate ROS in response to in vitro stimulation (20,23).

Some of these neutrophil responses could be mediated by the systemic release of cytokines such as interleukin (IL)-6, IL-8, and granulocyte-colony stimulating factor (G-CSF) (26,28). These cytokines have been linked with mobilization of neutrophils after exercise (26,28). Moreover, they also regulate surface receptor expression, and prime neutrophils for the release of granular enzymes and ROS (2,9,15,19). Whereas exercise-induced changes in IL-6 and IL-8 are well documented, there are fewer data available on changes in the plasma levels of G-CSF. Prolonged exercise such as marathon running significantly increases the plasma level of G-CSF (26). However, it is uncertain whether increases in plasma G-CSF and IL-8 concentrations are related to exercise-induced muscle damage.

Although several studies have examined the effects of eccentric exercise on the priming and activation of neutrophils (4,6,7,20–23), there were several limitations to their findings. First, each of these studies only measured alterations in one or two aspects of neutrophil priming and activation. To assess neutrophil priming and activation more thoroughly, a better approach is to make combined measurements of neutrophil receptor expression, degranulation, and the capacity of neutrophils to generate ROS in response to in vitro stimulation. Second, although eccentric exercise may cause changes in CD11b expression on the surface of neutrophils, the effect of this type of exercise on the expression of other neutrophil receptors such as CD16 and CD35 is unknown. Third, not all of these studies measured simultaneous changes in systemic factors that are known to influence the in vitro activity of neutrophils. Lastly, some of these studies involved downhill running at 70–75% $V^\ddot{O}_2\text{max}$ (6,22). This exercise intensity could have caused a greater release of hormones and cytokines, which in turn may have influenced circulating neutrophil number and function independently of the mode of exercise.

The primary aim of this study was to examine changes in neutrophil activation, markers of muscle damage, and plasma cytokines after downhill running at a moderate exercise intensity (60% $V^\ddot{O}_2\text{max}$). The data presented here are part of a larger study that also examined the effects of exercise intensity on neutrophil activation, plasma stress hormone, and cytokine concentrations in the same group of athletes (18). Therefore, we were able to compare alterations in these parameters after downhill running with level running at the same intensity. It should be noted that the level running protocol in our other report (18) involved 60 min of exercise, whereas the downhill running in the present report was 45 min in duration. We hypothesized that downhill running would stimulate greater increases in plasma myoglobin concentration and creatine kinase activity (indicators of muscle cell damage), plasma myeloperoxidase concentration, neutrophil CD11b expression, and the capacity of neutrophils to generate ROS in response to in vitro stimulation (indicators of neutrophil activation), and plasma concentrations of IL-6, IL-8, and G-CSF (indicators of inflammation). We also hypothesized that the expression of CD16 and CD35 receptors on the surface of neutrophils would be downregulated after exercise as part of the inflammatory response to muscle damage.

METHODS

Subjects. Subjects were 10 well-trained male runners and triathletes. These athletes had participated in our other study on alterations in neutrophil activation level running at moderate and high exercise intensity (18). The subject characteristics were as follows (mean ± SD): age 28 ± 3 yr, weight 75 ± 6.5 kg, height 178 ± 5 cm, $V^\ddot{O}_2\text{max}$ 61 ± 3.2 mL·kg$^{-1}$·min$^{-1}$, and heart rate maximum 186 ± 8 beats·min$^{-1}$. The athletes had not been involved in any strenuous eccentric exercise for at least 4 wk before the session of downhill running. All participants were informed of the purpose and risks of the study before they provided written informed consent. The experimental protocol was approved by the medical research ethics committee at the University of Queensland.

$V^\ddot{O}_2\text{max}$ testing. The runners were tested initially on a treadmill (Austredex AC190, Melbourne, Australia) to determine their $V^\ddot{O}_2\text{max}$. Each athlete was tested at least twice to ensure that the $V^\ddot{O}_2\text{max}$ values were reliable. The protocol used to determine $V^\ddot{O}_2\text{max}$ started at a treadmill velocity of 10 km·h$^{-1}$ and 0% gradient; treadmill speed was increased 2 km·h$^{-1}$ every 2 min up to 16 km·h$^{-1}$, followed by 1-km·h$^{-1}$ increments each minute up to 18 km·h$^{-1}$. The gradient was then increased by 2% each minute until volitional fatigue. Expired air was analyzed in a mixing chamber for $F_tO_2$ and $F_tC02$ every 30 s during exercise (Ametek gas analyzers; SOV S-3A11 and COV CD3A, Pittsburgh, PA). Minute ventilation was recorded every 30 s using a turbine ventilometer (Morgan, Model 096, Kent, UK). The gas analyzers were calibrated immediately before and validated after each test using a certified gas mixture (Commonwealth Industrial Gas Ltd., Brisbane, Australia); the ventilometer was calibrated before and validated after each test, using a 1-L syringe according to the manufacturer’s instructions. Oxygen consumption was calculated using an online data acquisition and analysis program (South Australian Sports Institute, Adelaide, Australia). Heart rate was measured every 30 s during this test (Polar Vantage NV, Kempele, Finland).

Exercise protocol. Once $V^\ddot{O}_2\text{max}$ was determined, the athletes ran downhill on a treadmill at a −10% gradient for 45 min at 60% $V^\ddot{O}_2\text{max}$. The treadmill speed was approximately 14 km·h$^{-1}$. Similar exercise protocols have been used in previous studies to induce muscle damage (11). A
moderate intensity of 60% VO\textsubscript{2max} was chosen to minimize the effects of intensity-related increases in the release of hormones and cytokines on neutrophil number and function. During the exercise, oxygen consumption was measured as described above for 5 min at the beginning, for two 3-min periods after 20 min, and after 40 min of the trial had elapsed, to ensure that each athlete was exercising at the correct intensity. The mean intensity in the first 5 min was 59.0% ± 3.7 VO\textsubscript{2max}, and the mean intensity after 40 min was 63.7% ± 2.9 VO\textsubscript{2max}. Heart rate was recorded every 5 min throughout the trial (Polar Vantage NV heart rate monitor). All trials were conducted between 11:00 a.m. and 12:00 p.m. to minimize the effect of diurnal variation in cortisol release.

**Blood sampling.** A 20-mL blood sample was drawn from a forearm vein by venipuncture immediately preexercise (PRE) and postexercise (POST), 1 h (1 h POST), and 24 h (24 h POST) after exercise. Blood was collected in sterile tubes containing lithium heparin and EDTA (Becton Dickinson (BD), Franklin Lakes, NJ). For practical reasons, it was not possible to measure changes in neutrophil activation at more than these four time points. However, previous studies have indicated that neutrophil CD11b expression, neutrophil respiratory burst activity, plasma myoglobin concentration, and creatine kinase activity are all elevated 24 h after eccentric exercise (7,21,23). To determine diurnal effects on plasma cortisol concentration and neutrophil parameters, blood was sampled at rest from each athlete on a separate occasion, at the same time of day as the exercise test.

Immediately after collection, the blood was centrifuged for 10 min at 1000 × g to separate plasma. The lithium heparin plasma was separated into two 1-mL aliquots for the measurement of the levels of catecholamines, myoglobin, and creatine kinase, and two 500-μL aliquots for the measurement of the levels of growth hormone and cortisol. The EDTA plasma was separated into 500-μL aliquots for the measurement of complement protein C5a, cytokine, and myeloperoxidase concentrations. All plasma samples were stored at −80°C until the day of assay.

**Hematological profile.** A full blood cell count and hemoglobin and hematocrit values were obtained using an automated cell counter (Sysmex K-2000, Kobe, Japan). Plasma volume was calculated according to the methods of Dill and Costill (10). Cell counts and the plasma concentrations of stress hormones, cytokines, myeloperoxidase, C5a, myoglobin, and creatine kinase activity were adjusted to account for changes in plasma volume.

**Neutrophil separation.** Neutrophils were separated from heparinized blood by dextran sedimentation (Amer sham Pharmacia Biotech, Uppsala, Sweden), followed by layering on Histopaque-1077 (Sigma Chemicals, St. Louis, MO). Residual erythrocytes were removed by hypotonic lysis. The cells were finally resuspended in Hanks' balanced salt solution (HBSS) containing 1 mg·mL\textsuperscript{−1} glucose and 1 μg·mL\textsuperscript{−1} of 0.9 mmol·L\textsuperscript{−1} CaCl\textsubscript{2} (Sigma). The neutrophils were at least 99% pure and viable, as determined by Trypan blue exclusion. One hour was normally required to separate the neutrophils, and the cells were kept at room temperature for no more than 2 h before their activity was measured.

**Neutrophil respiratory burst activity.** Two separate assays were used to measure the capacity of neutrophils to generate ROS in response to in vitro stimulation. The first assay measured the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Approximately 3 × 10\textsuperscript{5} isolated neutrophils were added to each of three cuvettes containing HBSS and either MTT only, MTT with phorbol myristate acetate (PMA, Sigma) (100 nmol·L\textsuperscript{−1}), or MTT with 10 mg·mL\textsuperscript{−1} opsonized zymosan (OZ, Sigma). The OZ was prepared as described previously (27). MTT (Sigma) was present in all three reactions at a final concentration of 1.2 mmol·L\textsuperscript{−1}. The first of these reactions was included to measure the “spontaneous” or basal activity of neutrophils without stimulation (27). All three cuvettes were then incubated for 1 h at 37°C before the formazan crystals were dissolved in isopropl alcohol. The absorbance was then read at 570 nm on a spectrophotometer (ATI Unicam, 5625, Cambridge, UK). The absorbance from the basal activity reaction was subtracted from each of the stimulated reactions in order to ascertain the reduction of MTT after stimulation. These assays were performed in duplicate.

The second assay measured the oxidation of the chemiluminescent probe luminol. Approximately 3 × 10\textsuperscript{5} isolated neutrophils were added to each of two reaction cuvettes each containing luminol (Sigma) (0.5 mmol·L\textsuperscript{−1}), HBSS, and either 100 nmol·L\textsuperscript{−1} PMA or 10 mg·mL\textsuperscript{−1} OZ. The oxidation of luminol after stimulation of neutrophils with PMA and OZ was then measured for 15 min at 37°C on a fluorometer (Turner Model 450, Barnstead-Thermolyne, IA) and logged on a chart recorder. Data were recorded as the peak height of chemiluminescence activity.

**Neutrophil surface receptor expression.** Whole blood (100 μL) was added to polypropylene tubes and incubated for 20 min at room temperature with monoclonal antibodies for the following receptors: phycoerythrin (PE)-conjugated IgG1 (BD) and fluorescein isothiocyanate-conjugated (FITC) IgG1 (BD) (both used as a negative control); PE-conjugated CD11b (Dako, Glostrup, Denmark); FITC-conjugated CD16 (BD); and PE-conjugated CD35 (BD). After this initial incubation, 1 mL of FACStyse solution (BD) was added, and the samples were vortexed and incubated for another 9 min. Finally, the tubes were centrifuged for 3 min at 1500 × g before the supernatant was removed and the cell pellet was resuspended in 250 μL 1% formalin. Receptor expression was then read by flow cytometry. The neutrophil population was gated according to the forward and side-scatter light profile. Fluorescence was measured on the FL1 (green) and FL2 (red) channel, and the gates were adjusted to the negative control quadrant. A total of 15,000 events were collected, and the data were recorded as mean fluorescence channel number.

**Intracellular myeloperoxidase concentration.** The myeloperoxidase content of neutrophils was measured by flow cytometry. Fifty microliters of whole blood were fixed by adding 100 μL of Fix and Perm reagent A\textsuperscript{TM} (BD),
zyme used in this assay was hexokinase, and the substrate was
100 µL of Fix and Perm reagent B™ (BD) were added. The sample
was then vortexed and incubated for a further 15 min at room temperature. Finally, the sample was further washed with 4 mL of PBS, centrifuged for 3 min at 1500 × g, and the supernatant was removed before resuspending the cell pellet with 250 µL 1% formalin. The samples were then read by flow cytometry. The neutrophil population was gated according to forward and side-scatter light properties. Fluorescence was measured on the FL1 (green) channel, and the gates were adjusted to the negative control quadrant. A total of 15,000 events were collected, and the data was recorded as mean fluorescence channel number.

**Plasma myeloperoxidase concentration.** EDTA plasma was added undiluted to an ELISA kit according to the manufacturer's instructions (Bioxytech myeloperoxidase-EIA, Oxis Research, Portland, OR). The enzyme used in this kit was alkaline phosphatase, and the substrate was p-nitrophenyl phosphate. The absorbance was read spectrophotometrically on a microplate reader at 405 nm (VERSAmax, Molecular Devices, Sunnyvale, CA), and the concentration was calculated using a standard. All measurements were made in duplicate.

**Plasma creatine kinase activity and myoglobin concentration.** Plasma creatine kinase activity was determined spectrophotometrically (VP-Super, Dinabott, Tokyo, Japan) using a test kit (Dinabott, Tokyo, Japan). The enzyme used in this kit was alkaline phosphatase, and the substrate was ortho-phenylenediamine. The absorbance was read spectrophotometrically on a microplate reader at 572 nm. Both assays were performed in duplicate.

**Plasma cytokine concentrations.** IL-6 and G-CSF were measured in EDTA plasma by ELISA (R&D Systems, Quantikine HS, Minneapolis, MN, and Immuno-Biological Laboratories, Fujoka-shi, Gunma, Japan, respectively). IL-8 was measured in EDTA plasma with an enzyme amplified sensitivity immunoassay (EASIA) kit (Biosource, Nevilles, Belgium). This kit is similar to an ELISA, except that the wells of the microplate are coated with several monoclonal antibodies against distinct epitopes of the target molecule, thereby avoiding hyperspecificity and increasing sensitivity (26). The enzyme used in the IL-6 kit was alkaline phosphatase, and the substrate was l-phosphatase, and the substrate was lyophilized NADPH. The enzyme used in the IL-8 and G-CSF kits was alkaline horseradish peroxidase, and the substrate was tetramethylbenzidine. The absorbance was read spectrophotometrically on a microplate reader (VERSAmax, Molecular Devices) at 490 nm for IL-6 and IL-8, and at 450 nm for G-CSF. The concentration of each cytokine was calculated using a standard curve. IL-6 and IL-8 were measured in duplicate, and single measurements of G-CSF were made.

**Plasma complement protein C5a concentration.** Plasma concentrations of complement protein C5a were measured by ELISA (OptEIA Human C5a ELISA kit, BD). The enzyme used in this kit was avidin-horse radish peroxidase, and the substrate was tetramethylbenzidine. Absorbance was measured at 450 nm, referenced at 570 nm. This assay was performed as a single measurement.

**Plasma stress hormone concentrations.** Plasma concentrations of cortisol, growth hormone, and the catecholamines epinephrine, norepinephrine, and dopamine were measured in lithium heparin plasma. Cortisol and growth hormone levels were measured by ELISA according to the manufacturer's instructions (Neogen, Lexington, KY, and Bioclone, NSW, Australia, respectively). The enzyme used in the cortisol assay was horseradish peroxidase, and the substrate was tetramethylbenzidine. The growth hormone assay used a biotinylated antibody to bind streptavidin peroxidase, which then acted on urea peroxide with substrate ortho-phenylenediamine. Growth hormone was only measured in PRE and POST plasma samples. Samples were diluted 1:100 for cortisol, and, if required, up to 1:8 for growth hormone. The absorbance was read spectrophotometrically on a microplate reader (Titerbek Multiskan MCC450, Flow Laboratories, Helsinki, Finland) at 650 nm for cortisol and at 490 nm for growth hormone. The concentration was calculated using a standard curve. All assays were performed in duplicate. The levels of catecholamines were measured by radio enzymatic assay (Biotrak, Amsterdam Pharmacia Biotech, UK). The radioactivity of the samples was measured using a liquid scintillation counter (Aloka Systems, Liquid Scintillation Ltd., Tokyo, Japan). All assays were performed in duplicate.

**Interassay variation.** The interassay variation for all assays is presented in Table 1. Interassay variation was calculated as follows: (standard deviation of all replicate measurements divided by the mean of all replicate measurements) × 100.

It was only possible to make single measurements of plasma G-CSF and complement protein C5a concentrations. Therefore, we could not obtain interassay variation data for these two parameters.

**Statistical analysis.** Statistical significance was set at \( P < 0.05 \). Data are expressed as mean ± SD. Before performing the statistical analysis, the residuals were tested for normality using a histogram and normality plots. If the residuals were not normally distributed, then a log transformation was applied. This transformation was performed for data on plasma growth hormone concentration, PMA-stimulated MTT reduction, and CD11b expression. The statistical significance of exercise-induced changes was assessed using repeated measures ANOVA to obtain the main effect of time. The changes from PRE to POST, PRE to 1 h POST, and PRE to 24 h POST were calculated using Student’s
RESULTS

Diurnal variation. When blood was sampled from each athlete at rest on a separate occasion at the same time of day as the exercise test, there were no significant changes over time in the level of plasma cortisol or any measure of neutrophil activation (data not shown).

Neutrophil and total leukocyte counts. Immediately after the downhill run, neutrophil count was 32% higher than preexercise values (ES = 1.1; P < 0.01); there was a further increase to 86% higher than preexercise values 1 h after exercise (ES = 2.0; P < 0.01) (Fig. 1). By 24 h, neutrophil count had returned to preexercise values. Total leukocyte counts followed a similar trend (Fig. 1).

Markers of neutrophil activation. There were no statistically significant changes in neutrophil receptor expression after exercise (Table 2). There was a moderate nonsignificant decrease (ES = −0.5) in CD16 expression from preexercise to 1 h postexercise. There was also a moderate nonsignificant decrease (ES = −0.7) in CD35 expression from preexercise to 24 h postexercise. There were no statistically significant alterations in the capacity of neutrophils to generate ROS in response to in vitro stimulation (Table 3). There was a large nonsignificant increase (ES = 0.9) in PMA-stimulated MTT reduction, and a moderate nonsignificant increase (ES = 0.5) in OZ-stimulated MTT reduction from preexercise to immediately postexercise. There was a moderate nonsignificant increase (ES = 0.7) in PMA-stimulated chemiluminescence from preexercise to 24 h postexercise. There were no statistically significant alterations in plasma or intracellular myeloperoxidase concentrations (Table 4).

Plasma myoglobin and creatine kinase. Plasma myoglobin concentration increased by 1100% immediately after exercise (ES = 4.6; P < 0.01), and was 1800% above preexercise values 1 h later (ES = 4.5; P < 0.01). By 24 h postexercise, plasma myoglobin concentration had decreased, but still remained significantly higher than preexercise values (100%; ES = 1.9; P < 0.01) (Fig. 2). Plasma creatine kinase activity was slightly elevated at POST (40%; ES = 0.3; P < 0.01) and 1 h POST (56%; ES = 0.5; P < 0.01), and maximum plasma creatine kinase activity occurred at 24 h POST (420%; ES = 2.6; P < 0.01) (Fig. 2).

![Figure 1](image-url)

**FIGURE 1**—Changes in total leukocyte (A) and neutrophil (B) counts before and immediately after, and 1 h and 24 h after 45 min of running downhill (−10% gradient). Data are mean ± SD; N = 10; † significantly different from PRE, P < 0.01.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of Replicates</th>
<th>Interassay Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>40</td>
<td>5.4%</td>
</tr>
<tr>
<td>IL-8</td>
<td>40</td>
<td>16.6%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>30</td>
<td>3.0%</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>30</td>
<td>12.0%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>30</td>
<td>7.5%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>30</td>
<td>7.6%</td>
</tr>
<tr>
<td>Dopamine</td>
<td>30</td>
<td>5.9%</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>40</td>
<td>3.2%</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>40</td>
<td>4.4%</td>
</tr>
<tr>
<td>Plasma myeloperoxidase</td>
<td>40</td>
<td>13.2%</td>
</tr>
<tr>
<td>Intracellular myeloperoxidase</td>
<td>10</td>
<td>9.0%</td>
</tr>
<tr>
<td>CD11b expression</td>
<td>10</td>
<td>12.7%</td>
</tr>
<tr>
<td>CD16 expression</td>
<td>10</td>
<td>7.8%</td>
</tr>
<tr>
<td>CD35 expression</td>
<td>10</td>
<td>2.2%</td>
</tr>
<tr>
<td>PMA-stimulated chemiluminescence</td>
<td>10</td>
<td>3.8%</td>
</tr>
<tr>
<td>OZ-stimulated chemiluminescence</td>
<td>10</td>
<td>6.1%</td>
</tr>
<tr>
<td>Basal MTT reduction</td>
<td>10</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>PMA-stimulated MTT reduction</td>
<td>10</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>OZ-stimulated MTT reduction</td>
<td>10</td>
<td>&lt;2%</td>
</tr>
</tbody>
</table>

PMA, phorbol myristate acetate; OZ, opsonized zymosan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

**TABLE 1.** Interassay variation for neutrophil and plasma parameters.

<table>
<thead>
<tr>
<th>Receptor (MFI)</th>
<th>PRE</th>
<th>POST</th>
<th>1 h POST</th>
<th>24 h POST</th>
<th>Time Effect (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>4448 (1080)</td>
<td>4579 (1319)</td>
<td>3916 (1446)</td>
<td>4474 (1726)</td>
<td>0.078</td>
</tr>
<tr>
<td>CD16</td>
<td>204 (98)</td>
<td>204 (98)</td>
<td>164 (51)</td>
<td>206 (83)</td>
<td>0.195</td>
</tr>
<tr>
<td>CD35</td>
<td>262 (131)</td>
<td>239 (108)</td>
<td>200 (87)</td>
<td>192 (80)</td>
<td>0.058</td>
</tr>
</tbody>
</table>

Data are mean (SD); N = 10; MFI, mean fluorescence intensity; PRE, preexercise; POST, immediately postexercise; 1 h POST, 1 h postexercise; 24 h POST, 24 h postexercise.

**TABLE 2.** Neutrophil cell surface receptor expression.

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creases markers of muscle damage, but does not influence IL-8 and G-CSF concentrations did not change significantly. Data are mean (SD); P < 0.01) and remained elevated 1 h postexercise (410%; ES 0.78, P < 0.01). There was a large nonsignificant increase (ES = 0.8) in plasma G-CSF concentration from preexercise to immediately postexercise. A t 1 h POST, plasma G-CSF concentration remained moderately higher than preexercise values (ES = 0.7), but this difference was not significant.

**Plasma cytokines.** Plasma IL-6 concentration increased immediately after exercise (460%; ES = 2.5; P < 0.01) and remained elevated 1 h postexercise (410%; ES = 2.6; P < 0.01), but returned to preexercise values 24 h after exercise (Fig. 3). Downhill running did not cause any significant changes in plasma IL-8 and G-CSF concentrations (Fig. 3). There was a moderate nonsignificant increase (ES = 0.5) in plasma IL-8 concentration from preexercise to immediately postexercise. There was a large nonsignificant increase (ES = 0.8) in plasma G-CSF concentration from preexercise to immediately postexercise. At 1 h POST, plasma G-CSF concentration remained moderately higher than preexercise values (ES = 0.7), but this difference was not significant.

**Plasma complement protein C5a.** There was no significant alteration in the plasma concentration of complement protein C5a after exercise (Table 4).

**Plasma stress hormones.** The plasma concentrations of growth hormone (2700%; ES = 1.9; P < 0.01), epinephrine (102%; ES = 0.7; P < 0.01), and norepinephrine (82%; ES = 0.6; P < 0.01) all increased immediately after exercise, whereas plasma cortisol and dopamine levels did not change significantly (Table 5).

**Correlations.** Significant Pearson’s correlations (r) were obtained between the neutrophil count and plasma Mb concentration at POST (r = 0.64, P < 0.05), plasma CK activity at POST (r = 0.83, P < 0.01), and 1 h POST (r = 0.78, P < 0.01).

**DISCUSSION**

Downhill running increased plasma myoglobin concentration, plasma creatine kinase activity, and the number of circulating neutrophils. In contrast to previous exercise studies, there were no significant changes in neutrophil receptor expression, degranulation, or the capacity of neutrophils to generate ROS in response to in vitro stimulation. Plasma IL-6 concentration increased after exercise, whereas plasma IL-8 and G-CSF concentrations did not change significantly. Therefore, in well-trained runners, downhill running increases markers of muscle damage, but does not influence neutrophil activation.

The large increase in plasma myoglobin concentration and creatine kinase activity supports the notion that the downhill running protocol used in this study resulted in skeletal muscle injury (6,7,22). Feasson et al. (11) reported direct evidence of structural damage to muscle after a similar protocol, and this was associated with a significant increase in plasma myoglobin concentration immediately postexercise and significantly elevated plasma creatine kinase activity 1 d after exercise. The efflux of these two proteins from muscle may occur as a result of increases in the permeability of the myocellular membrane and/or the intramuscular vasculature (6). The magnitude of increase in these markers of muscle damage was much greater 1 h after downhill running than after level running at the same intensity in the same subjects (18) (17-fold higher for plasma myoglobin concentration and 3-fold higher for plasma creatine kinase activity).

The magnitude of the increase in plasma myoglobin concentration and creatine kinase activity in the present study varied from that shown in other studies (21,22,25). In the present study, plasma creatine kinase activity peaked at around 1000 U·L⁻¹ 24 h postexercise, and plasma myoglobin concentration reached a maximum of around 750 ng·mL⁻¹ 1 h postexercise. At the same time points after eccentric contraction of the elbow flexors, Pizza et al. (21) reported plasma creatine kinase values of around 900 U·L⁻¹, whereas plasma myoglobin concentration was only 70 ng·mL⁻¹. Sayers and Clarkson (25) also used eccentric forearm flexion and found similar values to Pizza et al. (21) for plasma creatine kinase activity and myoglobin concentration. In their study of downhill running, Pizza et al. (22) observed maximum plasma creatine kinase values of around 850 U·L⁻¹ 12 h after 60 min of downhill running at 70% VO₂max. The time course for changes in these markers of muscle damage may also be dependent on exercise protocol and/or training status. In two of the studies above (21,25), plasma myoglobin concentration and creatine kinase activity peaked several days postexercise at higher values than those found in the present study. This disparity could have arisen because the participants in those studies (21,25) were not accustomed to eccentric contraction of elbow flexors. In contrast, the smaller magnitude and shorter time course for

**TABLE 3. Capacity of neutrophils to generate reactive oxygen species in response to in vitro stimulation.**

<table>
<thead>
<tr>
<th>Parameter (Arbitrary Units)</th>
<th>PRE</th>
<th>POST</th>
<th>1 h POST</th>
<th>24 h POST</th>
<th>Time Effect (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal MTT reduction</td>
<td>197 (48)</td>
<td>200 (26)</td>
<td>201 (61)</td>
<td>198 (61)</td>
<td>0.636</td>
</tr>
<tr>
<td>PMA-stimulated MTT reduction</td>
<td>776 (184)</td>
<td>896 (93)</td>
<td>799 (149)</td>
<td>753 (156)</td>
<td>0.117</td>
</tr>
<tr>
<td>O2-stimulated MTT reduction</td>
<td>351 (112)</td>
<td>416 (133)</td>
<td>359 (113)</td>
<td>325 (90)</td>
<td>0.077</td>
</tr>
<tr>
<td>PMA-stimulated chemiluminescence</td>
<td>2.59 (0.82)</td>
<td>3.12 (1.45)</td>
<td>3.11 (1.46)</td>
<td>3.23 (0.96)</td>
<td>0.338</td>
</tr>
<tr>
<td>O2-stimulated chemiluminescence</td>
<td>1.85 (0.80)</td>
<td>2.19 (0.99)</td>
<td>2.12 (0.90)</td>
<td>2.08 (0.84)</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Data are mean (SD); N = 10; *absorbance for the stimulated reaction minus absorbance for the baseline reaction; MTT, tetrazolium salt; PRE, preexercise; POST, immediately postexercise; 1 h POST, 1 h postexercise; 24 h POST, 24 h postexercise; PMA, phorbol myristate acetate; OZ, opsonized zymosan.

**TABLE 4. Plasma myeloperoxidase (MPO) concentration, intracellular MPO concentration, and plasma complement protein C5a concentration.**

<table>
<thead>
<tr>
<th>Parameter (ng·mL⁻¹)</th>
<th>PRE</th>
<th>POST</th>
<th>1 h POST</th>
<th>24 h POST</th>
<th>Time Effect (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma MPO</td>
<td>14.1 (11.9)</td>
<td>15.8 (6.8)</td>
<td>14.2 (7.5)</td>
<td>8.6 (4.6)</td>
<td>0.069</td>
</tr>
<tr>
<td>Intracellular MPO</td>
<td>776 (329)</td>
<td>670 (219)</td>
<td>661 (232)</td>
<td>753 (252)</td>
<td>0.304</td>
</tr>
<tr>
<td>C5a (ng·mL⁻¹)</td>
<td>7.3 (3.0)</td>
<td>8.2 (2.4)</td>
<td>8.4 (2.2)</td>
<td>8.1 (3.0)</td>
<td>0.150</td>
</tr>
</tbody>
</table>

Data are mean (SD); N = 10; MFI, mean fluorescence intensity; PRE, preexercise; POST, immediately postexercise; 1 h POST, 1 h postexercise; 24 h POST, 24 h postexercise.
alterations in plasma myoglobin concentration and creatine kinase activity in the present study suggest that the athletes were more accustomed to the muscular exertion associated with running.

Previous exercise studies have reported that postexercise neutrophil counts correlated with plasma myoglobin concentration and creatine kinase activity (7,22,28), and these findings are supported by data in the present study. These correlations may indicate that neutrophils are mobilized in response to exercise-induced muscle damage (7,22,28). Although myoglobin and creatine kinase are not tissue fragments, they may be regarded as indicators of changes in myocellular membrane permeability (11). In turn, damaged tissue fragments leaking across the myocellular membrane are recognized by the complement protein C3b, resulting in activation of the alternative complement pathway (17). Other complement proteins generated through this pathway, such as C3a and C5a, attract neutrophils to the site of inflammation and tissue damage (14). Previously, Cannon et al. (7) reported that increases in the plasma concentration of C3a correlated with elevated neutrophil counts after 45 min downhill running at 70% \( \dot{V}O_{2\text{max}} \). Because of the lack of any significant increase in plasma C5a concentration after downhill running in the present study, it seems unlikely that C5a was involved in the mobilization of neutrophils after exercise.

We selected a moderate exercise intensity (60% \( \dot{V}O_{2\text{max}} \)) in order to minimize the influence of stress hormones and cytokines on neutrophil mobilization after exercise (26,28). The increase in neutrophil count after downhill running in the present study was similar to 60 min of level running in our other study (18). Therefore, the increase in neutrophil counts reported in other studies after downhill running at higher exercise intensities (7) might not necessarily occur in response to exercise-induced muscle damage. Future studies need to consider the potential influence of stress hormones and cytokines when interpreting changes in neutrophil count after eccentric exercise.

The lack of any significant alteration in CD11b expression is in contrast to the findings of Pizza et al. (21), who demonstrated that CD11b expression was significantly elevated 24 h after eccentric contractions of the elbow flexors. The reasons for this disparity are unclear. Differences in the respective exercise protocols could be one factor. The large increase in CD11b expression in the study of Pizza et al. (21) could have occurred because the participants were not accustomed to using the elbow flexors eccentrically. Conversely, the lack of any increase in CD11b after downhill running in the present study might have occurred because the athletes had previously experienced damage to the leg muscles through their regular training.

In our other study of 60 min of level running (18), we observed a significant reduction in the expression of CD11b, CD16, and CD35 receptors after exercise. We speculated

![Figure 2](image1.png)

**FIGURE 2**—Changes in plasma myoglobin concentrations (A) and creatine kinase activity (B) before and immediately after, and 1 h and 24 h after 45 min of running downhill (−10% gradient). Data are mean ± SD; \( N = 10 \); † significantly different from PRE, \( P < 0.01 \).

![Figure 3](image2.png)

**FIGURE 3**—Changes in the plasma concentrations of interleukin (IL)-8 (A), granulocyte-colony stimulating factor (B) and IL-6 (C) before and immediately after, and 1 h after and 24 h after 45 min of running downhill (−10% gradient). Data are mean ± SD; \( N = 10 \); ‡ significantly different from PRE, \( P < 0.01 \).
that these responses might have indicated a mild inflammatory response to exercise-induced muscle damage. However, in light of the present findings that exercise with a stronger eccentric bias did not influence the expression of CD11b, CD16, and CD35 receptors, our explanation for the decreased expression of these receptors in our other study (18) seems less tenable.

Downhill running did not stimulate an increase in plasma myeloperoxidase concentration. There was also no increase in plasma myeloperoxidase concentration after 60 min of level running in our other study (18). Previous studies have reported elevated plasma levels of myeloperoxidase after downhill running (4), eccentric contractions of the knee (8), and a marathon race (26). The disparity between the present results and the findings of these other groups (4,8,26) may have arisen for the following reasons. First, the lack of any large increase in plasma myeloperoxidase concentration in our study could be due to the small IL-6 response. IL-6 stimulates neutrophil degranulation in vitro (2). Furthermore, Suzuki et al. (26) reported a correlation (r = 0.87, P < 0.01) between urine concentrations of IL-6 and myeloperoxidase after the marathon. The increase in plasma IL-6 concentration in the present study was considerably smaller than that after the marathon (26). Therefore, IL-6 may be an important determinant of postexercise increases in plasma myeloperoxidase concentration. Second, the exercise protocol used in the study of Camus et al. (4) involved downhill running at ~20% gradient, whereas a ~10% gradient was used in our study. This extra gradient may have placed a greater mechanical strain on the leg muscles, which could possibly account for the greater increase in plasma myeloperoxidase levels reported by Camus et al. (4). Alternatively, Camus et al. (4) did not report the fitness level of the subjects in their study. If those subjects were untrained, that might also explain some of the disparity between the results of the two studies.

Downhill running did not cause any significant alterations in the capacity of neutrophils to generate ROS in response to in vitro stimulation. Furthermore, the small changes in this parameter after downhill running were similar to those changes after 60 min of level running in our other study (18). These findings contrast with previous studies involving eccentric exercise (6,20,23). This disparity might have occurred because of differences in the training status of the subjects in each of these studies. Cannon et al. (6) used untrained subjects in their investigation of the effect of downhill running. Pizza et al. (20) showed an increase in the capacity of neutrophils to generate ROS in response to in vitro stimulation after eccentric contractions of the elbow flexors. Therefore, the familiarity of study participants with eccentric exercise seems to be an important determinant of alterations in neutrophil ROS-generating capacity after this form of activity. Further evidence in support of this concept comes from the study of Suzuki et al. (28). This group investigated changes in plasma myoglobin concentration and the capacity of neutrophils to generate ROS in response to in vitro stimulation across three consecutive days of moderate-intensity cycling in untrained individuals. Over the 3 d, they observed a progressive decline in postexercise neutrophil ROS-generating capacity, and this was accompanied by a reduction in plasma myoglobin concentration. Taken together, these results suggest that regular training might attenuate postexercise alterations in the capacity of neutrophils to generate ROS in response to in vitro stimulation. Because there was only a small difference in the training status of the athletes in the study of Pyne et al. (23) compared with the athletes used in our study, it is unlikely that training status can explain the conflicting findings between these two studies. Variation in the respective assay techniques used to measure neutrophil ROS-generating capacity could possibly have contributed to the different responses.

A correlation between plasma creatine kinase activity and IL-6 concentration after eccentric cycling led Bruusgaard et al. (3) to contend that IL-6 is involved in muscle damage. However, more recent evidence suggests that IL-6 does not play a major role in the inflammatory response to exercise-induced muscle damage. Toft et al. (30) demonstrated correlations between increases in postexercise plasma IL-6 and a delayed increase in plasma myoglobin levels and creatine kinase activity. Nevertheless, they argued that IL-6 is not involved in postexercise muscle damage because the rise in postexercise levels of plasma IL-6 was relatively small compared with the increase in plasma creatine kinase activity and myoglobin concentration. In the present study, plasma IL-6 concentration increased by 460% immediately postexercise, at which time plasma myoglobin levels were 1100% above preexercise values. By 24 h postexercise, plasma IL-6 levels had returned to rest values, yet plasma myoglobin levels and creatine kinase activity remained significantly elevated above preexercise values. In addition, the increase in plasma IL-6 concentration after downhill running was similar to that after 60 min of level running in our other study (18). Furthermore, there was only a minor increase in plasma creatine kinase activity and myoglobin concentration after 60 min of level running (18). Therefore, our data suggest that exercise-induced muscle damage is not the major cause of increased plasma IL-6 concentration after eccentric exercise.

The present study appears to be the first to examine the effects of eccentric exercise on changes in plasma IL-8 and G-CSF concentrations. The lack of any significant change in the plasma concentrations of these two cytokines after exercise suggests that they are not involved in the inflammatory response to muscle damage. Relative to the increase in plasma IL-8 and G-CSF concentrations after strenuous ex-

**TABLE 5. Plasma stress hormone concentrations.**

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>POST</th>
<th>1 h POST</th>
<th>Time</th>
<th>Effect (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine (pg·mL⁻¹)</td>
<td>77 (31)</td>
<td>156 (76)</td>
<td>95 (27)</td>
<td></td>
<td>0.018</td>
</tr>
<tr>
<td>Norepinephrine (pg·mL⁻¹)</td>
<td>574 (232)</td>
<td>1057 (403)</td>
<td>558 (171)</td>
<td></td>
<td>0.019</td>
</tr>
<tr>
<td>Dopamine (pg·mL⁻¹)</td>
<td>38 (15)</td>
<td>46 (21)</td>
<td>45 (24)</td>
<td></td>
<td>0.631</td>
</tr>
<tr>
<td>Cortisol (ng·mL⁻¹)</td>
<td>93 (58)</td>
<td>138 (80)</td>
<td>72 (57)</td>
<td></td>
<td>0.137</td>
</tr>
<tr>
<td>Growth hormone (ng·mL⁻¹)</td>
<td>1.5 (0.8)</td>
<td>52 (57)</td>
<td>—</td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

Data are mean (SD); N = 10; PRE, preexercise; POST, immediately postexercise; 1 h POST, 1 h postexercise; † significantly different from PRE, P < 0.01. Growth hormone was only measured in PRE and POST samples.
exercise such as a marathon (26), the changes in the plasma levels of these two cytokines observed in our study were very small. Although marathon running might also be expected to induce muscle damage, other factors such as stress hormones might contribute to the systemic release of IL-8 and G-CSF during exercise.

In summary, the data from this study indicate that downhill running in well-trained runners caused a large increase in the number of circulating neutrophils, and the efflux of myocellular proteins such as myoglobin and creatine kinase. There were no significant changes in neutrophil receptor expression, degranulation, or the capacity of neutrophils to generate reactive oxygen species in response to in vitro stimulation. Although there was an increase in plasma IL-6 concentration after exercise, it is unlikely that this occurred in response to exercise-induced muscle damage. Furthermore, downhill running did not alter plasma concentrations of IL-8 or G-CSF. The variation between the results of the present investigation and those of previous studies with respect to alterations in neutrophil activation after eccentric exercise could be due to differences in exercise protocols and muscular adaptation to regular exercise.

The authors would like to thank the participants of this study for their time and effort, Sports Medicine Australia for funding, and Sullivan and Nickolaides Pathology for the use of their laboratory.

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15. Ichinose, Y., N. Hara, M. Ohta, et al. Recombinant granulocyte colony-stimulating factor and lipopolysaccharide maintain the phenotype of reactive oxygen species in response to in vitro stimulation. Although there was an increase in plasma IL-6 concentration after exercise, it is unlikely that this occurred in response to exercise-induced muscle damage. Furthermore, downhill running did not alter plasma concentrations of IL-8 or G-CSF. The variation between the results of the present investigation and those of previous studies with respect to alterations in neutrophil activation after eccentric exercise could be due to differences in exercise protocols and muscular adaptation to regular exercise.