Exercise delays neutrophil apoptosis by a G-CSF-dependent mechanism

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1Department of Sports Medicine, Institute of Sports Sciences, Justus-Liebig-University, Giessen, Germany; 2Institute of Sports Medicine, University Hospital Münster, Münster, Germany; and 3Division and Clinic of Cardiology, Department of Cardiology and Angiology, Hospital of the Westfälische Wilhelms-University, Münster, Germany

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Mooren FC, Völker K, Klocke R, Nikol S, Waltenberger J, Krüger K. Exercise delays neutrophil apoptosis by a G-CSF-dependent mechanism. J Appl Physiol 113: 1082–1090, 2012. First published August 2, 2012; doi:10.1152/japplphysiol.00797.2012.—The aim of the study was to determine whether exercise affects neutrophil apoptosis and to characterize the underlying mechanisms. Using annexin V labeling, neutrophil apoptosis was measured using flow cytometry after various bouts of exercise (marathon run, concentric/ eccentric treadmill exercise, moderate/intensive resistance training) and in vitro conditions. Similarly, apoptosis-related markers as death receptors/ligands and mitochondrial membrane potential were detected. Furthermore, concentrations of intracellular free calcium and glutathione were measured using spectrofluorometry. After both marathon run and intensive laboratory exercise tests, neutrophil apoptosis was delayed. Furthermore, neutrophils mitochondrial membrane potential and death receptor/ligand expression were not affected by exercise. Apoptosis delay was accompanied under some exercise conditions by enhanced intracellular calcium transients and decreased glutathione levels. A delay of spontaneous apoptosis in vitro could be induced by incubation of neutrophils in postexercise serum. Heating of postexercise serum abolished the apoptosis delaying effect. In vitro stimulation of resting neutrophils with granulocyte-colony-stimulating factor (G-CSF) and C-reactive protein resulted in apoptosis delay too. Addition of anti-G-CSF antibody to postexercise serum was also effective in reversing its apoptosis-delaying effect. Exercise-induced mobilization of neutrophils is associated with a delay of apoptosis. This fundamental process seems to maintain exercise-induced neutrophilia and to contribute to the alerting and activation of the nonadaptive immune system known from other inflammatory conditions. An important extracellular trigger of apoptosis delay during exercise conditions seems to be G-CSF; intracellular processes may include calcium and redox signaling.

granulocyte colony-stimulating factor; granulocytes; calcium; programmed cell death; glutathione

Neutrophils play an important role in both the innate immune response and the clearance of exercise-induced tissue damage (25, 38). During an inflammatory response, a cytokine-dependent mobilization of neutrophils from bone marrow leads to a significant elevation of circulating neutrophils (24). Peripheral neutroctyosis is accompanied by a rapid migration to inflammatory sites, where the cells eliminate pathogens, either by phagocytosis or by releasing toxic mediators (4). Moreover, neutrophils amplify the inflammatory response and direct other immune cells to inflammatory sites by production of various cytokines and chemokines (5). Therefore, they are inflammatory effector as well as immunoregulatory cells. To maintain these roles during inflammation the usually short lifetime of neutrophils can be significantly extended by a modulation of the cell’s death program. In the absence of cytokines or other proinflammatory agents, neutrophils usually undergo spontaneous apoptosis before their removal by macrophages (1). Spontaneous apoptosis shares many features of classical apoptosis, like phosphatidylserine externalization, mitochondria depolarization, and nuclear condensation (1). The phagocytic removal of intact, apoptotic neutrophils prevents them from releasing their cytotoxic content into the extracellular milieu, which usually occurs during necrotic cell death. Apoptotic neutrophils are nonfunctional and unable to move by chemotaxis, to generate an oxidative burst, or to degranulate (36).

It is suggested from this functional inability is the result of the activity of key components of the Ca2+ signaling pathway, which controls neutrophil activation. Several inflammatory mediators, such as interleukin-8 (IL-8), tumor necrosis factor-α, and granulocyte-colony-stimulating factor (G-CSF), via alterations of cellular calcium concentration ([Ca2+]i) in neutrophils for their intracellular signal processing (22, 24). Elevated cytosolic calcium levels may result in cellular activation, enhanced generation of reactive oxygen species (ROS), and increased NF-κB activity, a transcription factor shown to critically regulate granulocyte apoptosis. Additionally, Whyte et al. (45) have shown that the transient elevation of cytosolic free calcium can retard neutrophil apoptosis (45). These data raise the interesting paradox that apoptosis in neutrophils is delayed by stimuli increasing [Ca2+]i, while these events have been shown to trigger apoptosis in other cells, e.g., of the adaptive immune system (2).

Exercise is an important stress factor, which affects neutrophil numbers and function, thereby showing some similarities to an acute inflammatory response (32, 25). Acute exercise is accompanied by an increase and activation of circulating neutrophils, depending on the type, intensity, and duration (20, 26). The neutrophil response to acute exercise has a characteristic time course with an acute mobilization during exercise and a delayed neutrophilia that peaks in the hours after exercise (26, 27). The concentration of circulating granulocytes usually returns to baseline values 24 h after exercise. Exercise-induced neutrophilia seems to be primarily the result of an intensity-dependent and stress hormone-evoked release of neutrophils from the marginal pool and the bone marrow (27). However, there is evidence from several acute and chronic inflammatory conditions that a delay in neutrophil apoptosis represents a general feature also contributing to the development of neutrophilia (28, 36). However, it remains unclear whether such a process is also operative during exercise conditions. Indeed, there are some reports about an increase of neutrophil apoptosis after exercise similar to the regulation of lymphocyte counts (40, 41).
Therefore, the first aim of the present study was to investigate the effects of different exercise protocols on neutrophil apoptosis. We hypothesized that acute exercise intervention results in divergent apoptosis regulation of both the adaptive and innate immune system. While lymphocyte apoptosis is enhanced after the exercise stimulus, neutrophil apoptosis should be delayed. Next, we aimed to investigate some apoptosis-related mechanisms, such as regulation of intracellular free calcium, cellular redox status, and mitochondrial membrane potential (MMP), and finally we evaluated the role of some exercise-associated metabolic, hormonal, and inflammatory mediators in regulating neutrophil apoptosis.

MATERIALS AND METHODS

Subjects

Thirty-five healthy male subjects were informed about the nature, purpose, and potential risks of the study and signed an informed consent statement approved by the Ethical Committee of the Justus-Liebig-University Giessen (Germany) or the University of Muenster Ethics Committee (Germany).

Marathon Run Group

To investigate the influence of a long-distance exercise on neutrophil apoptosis, 10 male participants of the Muenster marathon run were included. Their anthropometric data were as follows: age 36.1 ± 1.0 yr, weight 75.5 ± 8.6 kg, body mass index 24.5 ± 0.6 kg/m², and maximal oxygen consumption 59.2 ± 5.3 ml·min⁻¹·kg⁻¹. All subject passed through a standardized procedure consisting of signing the consent statement, general medical checkup, and endurance performance test (for details, see below). None of the subjects included into the study was involved in any exercise training for at least 2 days before the run.

Laboratory Exercise Group

Under laboratory conditions, various resistance and treadmill exercise protocols were performed (for details, see below). Therefore, 15 (resistance test group) and 10 (treadmill run group) healthy male subjects were recruited for the laboratory tests, which were preceded by a general medical checkup and an endurance performance test (treadmill group only). Their anthropometric data were as follows: resistance test group, age 26.9 ± 1.0 yr, weight 78.9 ± 2.8 kg, body mass index 24.3 ± 0.8 kg/m²; treadmill run group, age 23.8 ± 1.9 yr, weight 74.7 ± 5.3 kg, body mass index 24.7 ± 0.8 kg/m², and maximal oxygen consumption 52.8 ± 5.1 ml·min⁻¹·kg⁻¹. To measure intracellular calcium signaling after eccentric endurance test (CNC; see below), an additional 17 subjects were included. Their anthropometric data were as follows: age 24.9 ± 0.5 yr, weight 75.5 ± 1.79 kg, body mass index 23.09 ± 0.37 kg/m², maximal oxygen consumption 57.43 ± 1.96 ml·min⁻¹·kg⁻¹. The subjects of the treadmill run group were tested for their maximal oxygen uptake (V̇O₂max) during a continuous, progressive exercise test on a treadmill ergometer (Ergo XELG90 Spezial, Woodway, Weil am Rhein, Germany). The initial velocity was 8 km/h, increasing every 3 min by 2 km/h. Respiration parameters were analyzed using Quark b2 (Cosmed, Rome, Italy).

Exercise Protocols

Marathon run. Subjects of the marathon group participated in the Muenster marathon. Blood samples were taken 2 days before, directly after, 3 h after, and 24 h after the run by cannulation of the cubital vein. Resting blood samples were taken 2 days before the marathon, because the athletes refused sampling initially before the marathon. Blood sampling was performed in the morning at the same time of day to avoid diurnal bias.

Concentric/eccentric treadmill tests. As an intensive endurance exercise test, subjects performed an intensive treadmill run at an intensity corresponding to 80% of their V̇O₂max (CNC) until exhaustion. Average running time was 45 ± 3 min. The second test consisted of an eccentric treadmill-running protocol that required the subjects to run down a ~12% gradient at an intensity of 80% V̇O₂max [eccentric endurance test (ECC)]. The running time for both exercise tests was adjusted in such a way that the oxygen uptake, as determined during CNC and ECC, was the same. It has been shown previously that V̇O₂max and heart rate are lower during downhill treadmill running relative to level running at the same treadmill velocity (34). Therefore, to compare the effects of a level running protocol to a downhill running protocol without discrepancies in exercise intensity, the speed of the treadmill was adjusted to evoke the same physiological response as the intensive protocol at 80% V̇O₂max. Blood samples for the treadmill tests were taken immediately before, directly after, 3 h after, and 24 h after exercise. Between the two treadmill exercise tests, subjects obtained at least 2 wk of time for recovery.

Moderate and intensive resistance test. The moderate (MRT) and intensive resistance test (IRT) consisted of a general warm-up and a specific warm-up session consisting of 15 repetitions at 30% of one repetition maximum (1 RM) on each training devise. After warming up, the subjects performed a whole body resistance training program, including eight different exercise sets: bench press, latissimus pull-downs, seated rows, shoulder press, leg press, shoulder press, biceps curls, and leg curls. Resistance exercise intensity for the IRT was defined individually as 75% of the 1 RM. In contrast, exercise intensity for the MRT was defined as 60% of the 1 RM. Both exercise protocols were performed in three series with 2-min breaks between each session and 3-min breaks between each series. To ensure that both test programs only differ by intensity, the number of repetitions in the MRT was determined by the maximum repetitions that the subjects reached during the IRT. Total duration of the exercise tests was ~90 min. Venous blood was taken immediately before, immediately after, 3 h after, and 24 h after exercise. Again, there were at least 2 wk of recovery between both tests.

Leukocyte Counts

Blood cell counts, hemoglobin, and hematocrit determinations were performed on blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) using a semiautomated hematology analyzer (F-820, Sysmex, Norderstedt, Germany).

Cell Isolation Procedure

Leukocyte subpopulations were prepared by density gradient centrifugation. Briefly, 5 ml of a 50:50 mixture of whole blood anticoagulated with EDTA and 0.9% NaCl solution were layered upon 3 ml of Percoll-1.077 (Nycosmed, Oslo, Norway) and then centrifuged at 400 g for 30 min at room temperature. After centrifugation, the lymphocyte band between the sample layer and the Percoll solution was removed. Remaining neutrophils were washed, and erythrocytes were lysed by hypotonic shock (not more than 30 s). Cell viability of neutrophils was ~98%, as demonstrated by Trypan blue exclusion, whereas purity was ~95%, as checked by flow cytometry in the forward and sideward scatter mode. Isolated neutrophils were used for analysis apoptosis-related (surface) markers, analysis of intracellular calcium levels, and cellular redox status.

Analysis of Apoptosis-Related Cellular Markers

Cells were labeled with annexin V FITC and nuclear propidium iodide uptake for detection of apoptosis and necrosis, respectively. Apoptosis was measured either immediately after cell isolation (blood spontaneous apoptosis) or after primary cell culture for 24 h of 10^6
cells incubated in medium containing RPMI-1640 + glutamin, 5% FCS, 0.5 µl of 100 U/ml Pen/Strep, and nonessential amino acids solution at 37°C and 5% CO₂ (spontaneous apoptosis in vitro).

The MMP was analyzed using the fluorochrome DiOC6 (Invitrogen, Darmstadt, Germany). In brief, 10⁵ cells were stained in a solution with 40 nm DiOC6 for 30 min. For further detection of apoptosis signaling pathways, cells were labeled with specific antibodies against CD95 (ImmunoTools, Friesoythe, Germany) and CD95 ligand (BioLegend, San Diego, CA). Analyses were performed by flow cytometry (EPICS XL Beckman Coulter, Krefeld, Germany).

Determination of [Ca²⁺⁺]

For the determination of [Ca²⁺⁺], 1 x 10⁶ cells were labeled with the membrane-permeant derivative of the calcium-sensitive dye fura 2. Measurements were performed on a cuvette spectrofluorometer (Deltascan, PTI, Lawrenceville, NJ) at an excitation wavelength of 340 and 380 nm. During recording, cells were stimulated with N-formyl-methionyl-leucyl-phenylalanin and continuously stirred at 28°C. [Ca²⁺⁺], was calculated as previously described (29).

Analysis of Intracellular Redox Status

Intracellular redox status was analyzed using monobromobimane, which is a nonfluorescent cell-permeable bimane derivative that becomes highly fluorescent following the reaction with intracellular glutathione. Cells (5 x 10⁵) were incubated with final concentration of 50 µM monobromobimane in the dark at 37°C for 30 min, washed, and resuspended in PBS. Total fluorescence was determined using a cuvette spectrofluorometer (Deltascan, PTI, Lawrenceville, NJ) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm.

Analysis of Plasma G-CSF Concentrations

For the quantitative detection of G-CSF in serum samples, a commercially available ELISA (enzyme linked immunosorbent assay; Human G-CSF Quantikine HS ELISA, catalog no. HSTCS0) was used according to the manufacturer’s (R&D systems, Minneapolis, MN) protocol.

Effects of Exercise Serum on Neutrophils Apoptosis

To analyze the effect of serum parameters on neutrophil apoptosis, cells were isolated from nonexercised, healthy control individuals. In a first approach, we incubated 2 x 10⁷ cells for 24 h in serum taken before marathon or IRT (pre) and serum taken after marathon or IRT (post). Serum concentrations in all approaches were 50%.

Table 1. Leukocyte counts after the different exercise protocols

<table>
<thead>
<tr>
<th>Exercise Protocol</th>
<th>Subpopulation</th>
<th>Pre</th>
<th>Post</th>
<th>3 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marathon</td>
<td>Leukocytes</td>
<td>5.5 ± 0.6</td>
<td>17.4 ± 1.3*</td>
<td>15.3 ± 1.1*</td>
<td>7.1 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>2.1 ± 0.4</td>
<td>1.7 ± 0.6*</td>
<td>1.9 ± 0.4*</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>CNC</td>
<td>Leukocytes</td>
<td>3.4 ± 0.2</td>
<td>15.3 ± 1.1*</td>
<td>12.7 ± 0.9*</td>
<td>5.0 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>5.4 ± 0.2</td>
<td>7.2 ± 0.2*</td>
<td>7.7 ± 0.2*</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>ECC</td>
<td>Leukocytes</td>
<td>2.3 ± 0.7</td>
<td>3.5 ± 0.8*</td>
<td>1.8 ± 0.7*</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>3.3 ± 1.4</td>
<td>3.8 ± 1.1</td>
<td>6.0 ± 1.1*</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>IRT</td>
<td>Leukocytes</td>
<td>4.9 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>7.3 ± 0.2*</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>2.0 ± 0.5</td>
<td>2.1 ± 0.5</td>
<td>1.9 ± 0.5</td>
<td>2.1 ± 0.47</td>
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<tr>
<td></td>
<td>Neutrophils</td>
<td>2.9 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>5.4 ± 0.2*</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>MRT</td>
<td>Leukocytes</td>
<td>4.9 ± 0.4</td>
<td>6.1 ± 0.3</td>
<td>7.3 ± 1.6*</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>1.8 ± 0.5</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.6*</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>2.9 ± 0.4</td>
<td>3.7 ± 0.8</td>
<td>5.4 ± 1.3*</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Leukocytes</td>
<td>4.8 ± 0.4</td>
<td>4.7 ± 0.3</td>
<td>5.9 ± 0.4</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>1.8 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.6</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>2.9 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>3.9 ± 0.2</td>
<td>3.5 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cell counts are ×1,000/µl. CNC, concentric endurance test; ECC, eccentric endurance test; IRT, intensive resistance test; MRT, moderate resistance test; Pre, pretest values; Post, values immediately after exercise; 3 h, 3 h after exercise; 24 h, 24 h after exercise test. *Significance P < 0.05 compared with Pre values.
leukocyte counts returned to preexercise values 24 h after the other tests. Leukocytosis was mainly affected by an increase of neutrophils (Table 1). In particular, we observed maximally increased neutrophil counts immediately after the marathon, while after the laboratory exercise tests neutrophilia was observed only 3 h after cessation of exercise. After marathon, neutrophilia persisted at least 24 h, while neutrophil numbers after all other exercise tests returned to baseline at 24 h. Focusing on lymphocytes, we found a significant lymphopenia immediately after the marathon and 3 h after the CNC and IRT. In contrast, ECC did not affect lymphocyte numbers at any time point (Table 1).

**Exercise Effects on Neutrophil Apoptosis**

The marathon run was followed by a significant decrease of neutrophil spontaneous apoptosis in blood. In contrast, lymphocyte apoptosis increased significantly 3 h after exercise during identical experimental conditions. The kinetics of spontaneous apoptosis of both cell populations are shown in Fig. 1A. Similarly, after all intensive laboratory exercise tests, a significant decrease of neutrophil apoptosis was measured. After both CNC and ECC, a delayed decrease of neutrophils was observed at 3 h after exercise tests (Fig. 1C). Furthermore, IRT was accompanied by an immediate decrease of neutrophil apoptosis, while we did not observe any changes in percentage of apoptotic cells after MRT (Fig. 1E).

Additionally, cells from the same sampling time points were cultured for 24 h, and neutrophil apoptosis was measured (spontaneous apoptosis in vitro). Spontaneous apoptosis in vitro was significantly delayed after the marathon run for up to 24 h after the run (Fig. 1B). For the treadmill tests, medium spontaneous apoptosis in vitro was reduced at the same time points as for blood spontaneous apoptosis (Fig. 1D), while for IRT spontaneous apoptosis in vitro was decreased at 3 h after exercise (Fig. 1F).

**Analysis of Molecular Mechanisms of Neutrophil Apoptosis**

Neutrophils cell death is crucially related to a number of signaling mechanisms such as \([\text{Ca}^{2+}]_i\), which was addressed in the next experiments. Both basal calcium levels and calcium transients after stimulation using the synthetic chemotactic peptide formyl-Met-Leu-Phe were investigated. While basal
well as after both treadmill tests (Fig. 2, Table 2). In case of the marathon run, we performed additional measurements of MMP (Table 2). At least for the IRT, we investigated the surface expression of Fas receptor (FasR) and alterations of MMP (Table 2). At least for the IRT, we were unable to detect any changes of cellular redox status (Table 2).

![Fig. 2. A: alterations of N-formyl-methionyl-leucyl-phenylalalanin (fMLP)-induced calcium responses in neutrophils that have been isolated preexercise, postexercise, and 3 and 24 h after a marathon run. B: effect of treadmill exercise protocols (CNC, ECC) on fMLP-induced calcium responses pre- and postexercise. [Ca^{2+}], calcium concentration. Values are means ± SE. *Significant differences between preexercise and various postexercise values (P < 0.05).](image)

calcium levels were unaffected by exercise (data not shown) [Ca^{2+}], transients increased significantly after marathon, as well as after both treadmill tests (Fig. 2, A and B, respectively).

Measurements of MMP were performed after the laboratory exercise tests only. In any case, we were unable to detect any alterations of MMP (Table 2). At least for the IRT, we investigated the surface expression of Fas receptor (FasR) and Fas ligand (FasL), which were unaffected by exercise, too (Table 2). In case of the marathon run, we performed additional measurements of intracellular redox status. We found that the alterations of [Ca^{2+}], transients were accompanied by a significant decrease of intracellular glutathione levels, indicating changes of cellular redox status (Table 2).

![Fig. 2.](image)

**Table 2. MMP, expression of FasR, expression of FasL, and cellular glutathione levels of neutrophils after different exercise tests**

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Pre</th>
<th>Post</th>
<th>3 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP, units</td>
<td>CNC</td>
<td>9.96 ± 0.84</td>
<td>9.89 ± 0.81</td>
<td>9.71 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>ECC</td>
<td>9.46 ± 0.83</td>
<td>9.15 ± 0.83</td>
<td>9.12 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>IRT</td>
<td>12.91 ± 1.63</td>
<td>13.29 ± 1.65</td>
<td>10.67 ± 1.01</td>
</tr>
<tr>
<td>FasR, %positive cells</td>
<td>IRT</td>
<td>7.86 ± 0.85</td>
<td>7.18 ± 0.74</td>
<td>8.55 ± 0.87</td>
</tr>
<tr>
<td>FasL, %positive cells</td>
<td>IRT</td>
<td>10.71 ± 0.71</td>
<td>8.48 ± 0.46</td>
<td>8.83 ± 0.50</td>
</tr>
<tr>
<td>Glutathione, units</td>
<td>Marathon</td>
<td>13.515 ± 2.211</td>
<td>7.731 ± 718*</td>
<td>5.758 ± 1.067*</td>
</tr>
</tbody>
</table>

Values are means ± SE. MMP, mitochondrial membrane potential; FasR, Fas receptor; FasL, Fas ligand. *Significance P < 0.05 compared with Pre values.

**Plasma Concentrations of G-CSF**

As G-CSF is a potential mediator of neutrophil shifts during exercise, as well as a prosurvival factor for neutrophils, G-CSF plasma concentrations were assessed. After marathon run, a significant increase of plasma G-CSF was determined. Likewise, the laboratory tests were followed by significant increases of G-CSF with slightly different kinetics (Table 3).

**Analysis of the Role of Serum Factors in the Regulation of Neutrophil Apoptosis**

Next, we investigated whether postexercise serum was able to affect neutrophil apoptosis under in vitro conditions. For these experiments, neutrophils from nonexercised control individuals were incubated with marathon and IRT serum for 24 h. These approaches revealed that neutrophil apoptosis was significantly lower after incubation in postexercise serum than in preexercise serum, suggesting the involvement of some serum correlates (Fig. 3, A and B). The nature of the serum correlates was addressed by incubating neutrophils in heat-inactivated postexercise serum. Heat-inactivated serum failed to decrease neutrophil apoptosis, indicating protein factors to be involved in apoptosis delay (Fig. 3, C and D).

**DISCUSSION**

It has been shown previously demonstrated that intensive exercise is followed by an increased apoptosis of lymphocytes,
which might contribute partially to the transient loss of adaptive immunocompetence in the postexercise period (18, 31, 33). In contrast, the main finding of this study was that different modes and intensities of exercise induce a delay of neutrophil apoptosis, indicating an alerting and activation of the nonadaptive immune system. All intensive and/or long-duration exercise protocols used in this study were followed by a significant increase of peripheral neutrophil numbers, which has been shown to be attributed to the elevation of both catecholamines and cortisol so far (11, 17, 25). These hormones act predominantly through a mobilization of neutrophils from the endothelial walls and the bone marrow, respectively (15). This well-known exercise-induced leukocyte mobilization seems to be accompanied by a substantial delay of neutrophil apoptosis. Possibly, this delay might also contribute to the maintenance of postexercise neutrophilia. The decrease of blood spontaneous apoptosis might partially be related to a dilution effect by a mobilization of neutrophils of different maturation stage from bone marrow or from marginal pool. However, the results of the in vitro experiments clearly suggest that neutrophil apoptosis is delayed after exercise. While neutrophils have a life span of ~8–24 h under normal conditions, both marathon run and laboratory exercise tests seemed to prolonged neutrophil survival significantly (1). Generally, apoptosis is an essential mechanism for modulating neutrophil homeostasis during normal and pathophysiological processes. During various forms of inflammatory conditions, apoptosis delay elevates neutrophil numbers in both blood and infected
tissues, thereby enhancing the microbial competence (1, 11, 24). During resolution of inflammation, apoptosis eventually increases as an essential mechanism to prevent the release of neutrophil cytotoxic contents into the blood and surrounding tissues (24). Our results contradict to some other findings. In contrast to our results, Lagranha et al. (21) detected an increased neutrophil apoptosis after exercise in rats. The discrepancy results most likely from different experimental conditions, such as cell origin and isolation procedure. Lagranha et al. (21) used peritoneal neutrophils, which had been sampled after their migration into the intraperitoneal cavity due to an artificial stimulation. This procedure might have induced some kind of neutrophil priming, resulting in a more apoptosis-sensitive status. Contradictory findings come also from human studies. Tuan et al. (43) detected an increase of apoptosis in neutrophils after intensive short-term exercise on 3 consecutive days. However, differences in exercise protocols and in isolation method might be the reason for different results. In addition, Syu et al. (40) found a slight increase in neutrophil apoptosis. Indeed, the authors used similar isolation methods compared with our study. On the other hand, their subjects performed only an incremental exercise test of short duration of ~30 min. Furthermore, cells were isolated only immediately after exercise with various culture times. Direct labeling of cells as performed in the actual study revealed no change between exercise and sedentary conditions, pointing to the crucial role of culture conditions as a possible reason for the divergent results. Focusing in cellular mechanisms, our data suggest that apoptosis delay is mediated on a cellular level by 

\[ \text{Ca}^{2+} \]. After both marathon run and treadmill exercises, ligand-induced calcium transients were enhanced, confirming our previous results (29). Mobilization of intracellular calcium has been recognized as an important mechanism in receptor-mediated activation of neutrophils known to precede apoptosis delay. Whyte et al. (45) showed that transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils, providing a link between neutrophil activation and apoptosis delay. It can be speculated that exercise-induced alterations of intracellular redox status might affect calcium signaling (34). Beside their well-described cytotoxic properties, ROS have gained acceptance as important mediators of signal transduction, acting as and on second messengers (6, 13). Thus ROS act on membranous calcium channels as well as calcium pumps, such as sarcoplasmic reticulum and plasma membrane Ca-ATPases (35, 42).

Our data on intracellular glutathione levels, an important indicator of cellular redox status, confirm previous results by Tauler et al. (41), who observed a decreased antioxidant enzyme defense after prolonged exercise. More specifically, Syu et al. (40) also detected a shift in neutrophil redox status toward a relatively oxidative state in neutrophils after acute exercise. These shifts toward a more oxidative status seem to contradict our findings of an apoptosis delay, as many publications suggest a positive correlation between oxidative stress and apoptosis rate; for example, in lymphocytes, a more oxidized state is usually accompanied by the induction of early stages of apoptosis after exercise, but in neutrophils, there is some convincing experimental evidence that ROS have a different role in cell cycle physiology being associated with the activation of prosurvival signals (28). Neutrophils possess a specific antioxidant enzyme activity profile, probably related to the lower numbers of mitochondria and the production of high amounts of ROS during the oxidative burst (9, 10). Accordingly, Fadeel et al. (10) demonstrated that activated neutrophils showed an increased ROS production and suppressed caspases, the main executioners of the apoptotic program. Additionally, in neutrophils, increased ROS generation is followed by NF-κB activation and translocation, which is a strong anti-apoptotic signal through the expression of proteins of the Bcl-2 family (2, 44). Accordingly, we were able to show that no other part of either the intrinsic or the extrinsic apoptotic pathways was activated, supporting the data on apoptosis delay. The MMP was preserved at least after the laboratory exercise tests, which suggest no activation of the intrinsic apoptosis-inducing pathway. A decrease of MMP constitutes an obligate step in inducing apoptotic cell death. Mitochondrial membrane depolarization precedes nuclear signs of apoptosis and the activation of endonucleases and caspases that results in irreversible DNA fragmentation (16). Decreased MMP occurred in blood leukocytes, monocytes, and lymphocytes after aerobic exercise and in peritoneal rat neutrophils after 2 h of intense treadmill running (18, 12, 21). In contrast, in our study, we could exclude a decrease of MMP, which supports the observed delay in apoptosis. In addition, we found no evidence for an upregulation or activation of the extrinsic apoptosis pathway since FasR and Fasl expression was not affected. The validity of the results on apoptosis-related cellular markers, however, is limited, as these parameters have not been measured under all exercise conditions due to technical limitations.

In our in vitro approach, we used exercise serum concentrations of 50%. A dilution was chosen because 24-h incubation time means a longer contact time of cells with potentials mediators compared with all exercise protocols. Otherwise, we wanted to prevent that dilution hampers the signal effect of some mediators. The results of the in vitro approach suggest that the apoptosis delay is elicited by a soluble extracellular mediator, most likely protein-like in nature. This was demonstrated by serum effects on apoptosis and the negligible effect of serum heat inactivation on apoptosis regulation. As the concentration of numerous hormones, cytokines, etc., is affected by exercise, further steps focused specifically on some candidate molecules potentially known to be involved in apoptosis regulation (39). G-CSF and CRP were revealed to be effective in decreasing neutrophil apoptosis rate during in vitro experiments (39). G-CSF is a main player in proliferation, differentiation, survival, and bone marrow release of neutrophils (8, 46). It can affect apoptosis rate by upregulation of cellular Mcl-1 protein levels, an anti-apoptotic protein of the Bcl-2 family (8). Yamada et al. (46) showed that levels of G-CSF increased in accordance with the increase in the number of neutrophils after an intense exercise session. Our results suggest that G-CSF exerts its effects on neutrophilia partially by apoptosis delay. First, it could be confirmed that G-CSF increased after the various exercise protocols that were used throughout the study, and, second, incubating serum with a rG-CSF neutralizing antibody reversed the apoptosis delaying effect of postexercise serum. Moreover, we could confirm the apoptosis-delaying effect of CRP, which has been described previously (14). Its increment follows the rise of IL-6 plasma concentrations, which stimulates CRP synthesis in the liver. That delay makes it unlikely that CRP is responsible for the apoptosis-delaying effects early after exercise, as CRP usually...
starts to increase later after exercise. Likewise, we could demonstrate an increase of CRP not before 24 h after a marathon run (32). However, other investigations using a high-sensitive CRP assay described an early increase of CRP after marathon run, making an additional role for CRP in the exercise-induced delay of neutrophil apoptosis possible (37). Finally, glucocorticoid hormones, which are known to be released during and after intensive or prolonged exercise, are potentially anti-apoptotic for neutrophils, despite their anti-inflammatory activities (7, 23). However, we could not find any evidence that glucocorticoids are involved in neutrophil apoptosis delay under exercise conditions.

In summary, the present data indicate that both short-term intensive and long-term moderate exercise induce a delay of neutrophil spontaneous apoptosis. This effect seems to be predominantly mediated by G-CSF. On a molecular level, apoptosis delay is accompanied by alterations of [Ca2+]i, as well as the redox status.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES