Lymphocyte apoptosis after exhaustive and moderate exercise

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—Apoptosis or programmed cell death is a process of fundamental importance for regulation of the immune response. Several reasons suggest that apoptosis is involved in exercise-induced alterations of the immune system such as postexercise lymphocytopenia. Healthy volunteers performed two treadmill exercise tests; the first was performed at 80% maximal oxygen uptake until exhaustion (exhaustive exercise) and the second 2 wk later at 60% maximal oxygen uptake with the identical running time (moderate exercise). Blood samples were taken before, immediately after, and 1 h after the test. Lymphocytes were analyzed for apoptotic and necrotic cells by using FITC-labeled annexin V antibodies and nuclear propidium iodide uptake, respectively. In addition, apoptotic/necrotic cells were measured after a 24-h incubation of lymphocytes in the presence of camptothecin, but not medium alone or in the presence of camptothecin or phytohemagglutinin. Finally, plasma membrane expression of CD95-receptor and CD95-receptor ligand was investigated. Immediately after the exhaustive exercise, the percentage of apoptotic cells increased significantly, whereas it remained unchanged after the moderate exercise. Similar results were obtained after 24-h incubation of lymphocytes in medium alone or in the presence of camptothecin, but not with phytohemagglutinin. We found an upregulation of CD95-receptor expression after both exercise tests. However, only after exhaustive exercise a characteristic shift in CD95 expression profile toward cells with a high receptor density was observed. Expression of the CD95-receptor ligand remained unchanged after both exhaustive and moderate exercise. These results suggest that apoptosis may contribute to the regulation of the immune response after exhaustive exercise. Whether this mechanism can be regarded either as beneficial, i.e., deletion of autoreactive cells, or harmful, i.e., suppression of the immune response, awaits further investigations.

intra- cellular signaling; calcium; annexin; CD95 receptor

Programmed cell death (PCD) or apoptosis is an important mechanism for the regulation of the immune response (1, 14). After exposure to antigens, the immune system becomes activated and immune cells start to proliferate and secrete cytokines and antibodies to orchestrate the intercellular communication and antimicrobial defense, respectively (5). After the pathogen is eliminated and the disease is warded off, the immunological response needs to be terminated. Apoptosis of immune cells plays an important role in this regulatory process and has been referred to as activation-induced cell death (12). A failure in the apoptotic off-switch of the activated immune cells has been proposed to result in chronic inflammation or a self-reactive immune response. Exercise has been shown to induce inflammatory-like changes in immune cell counts and acute-phase protein release (29, 34, 36). As reported previously, all leukocyte subsets increase during exhaustive exercise (11). When exercise ends, leukocyte subsets show a nonsynchronous behavior. Whereas granulocytes can remain increased for several hours after the test, lymphocytes decrease rapidly to a level well below the preexercise value (11, 30). Depending on the intensity and type of exercise, this decline in lymphocyte count may last from several hours up to days and may, at least in part, account for postexercise immune suppression (35). The mechanisms responsible for exercise-induced lymphocytopenia remain to be elucidated. Some evidence suggests that homing to lymphoreticular structures may play a role in lymphocytopenia (43). Moreover, exercise-accompanied hormonal changes may affect lymphocyte mobilization. The potential role of apoptosis in exercise-induced lymphocytopenia has not yet been studied in detail (26). Strenuous exercise modulates several factors, such as reactive oxygen species (ROS), DNA damage, and hormone and cytokine levels, which are involved in the regulation of apoptosis in various cell types (8, 15, 27, 33). ROS that increase in correspondence with the enhanced oxygen uptake during exercise are able to induce apoptosis via different mechanisms such as a decrease in intracellular glutathione levels or an alteration of mitochondrial proteins or by directly damaging cellular DNA (13, 33). Recently, two studies in humans have confirmed that exercise is able to induce DNA damage (32, 40). Likewise, in a recent study, an exercise-induced increase in apoptotic cells was reported (26). In rats, it has been shown that exercise-induced...
changes in glucocorticoids are sufficient to induce lymphocyte apoptosis (17). Application of a glucocorticoid receptor antagonist decreased the DNA damage in thymocytes of rats that experienced mild physical stress compared with control animals (10). Another major trigger of apoptosis is changes in the intracellular free calcium concentration (28, 44, 45). Our laboratory has demonstrated recently (30) that exhaustive exercise is associated with alterations in intracellular calcium signaling of lymphocytes, and this would support the hypothesis that 1) apoptosis could be involved in exercise-induced lymphocytopenia and 2) this effect depends on exercise intensity. The aim of the present study was therefore to characterize the effect of exercise on the apoptosis rate of human peripheral blood lymphocytes under exercise conditions of defined intensity.

MATERIALS AND METHODS

Reagents. Camptothecin was obtained from Calbiochem (Bad Soden, Germany), and RPMI 1640 was purchased from Biochrom (Berlin, Germany). All other chemicals were of the highest chemical grade available and were obtained from Sigma Chemical (St. Louis, MO).

Subjects and experimental design. Twelve healthy volunteers (7 men, 5 women) between 21 and 30 yr of age were recruited from the University of Muenster student population. Their mean age was 24.3 ± 0.6 yr, weight 72.9 ± 2.6 kg, height 181.3 ± 2.1 cm, and maximal oxygen consumption 50.0 ± 2.0 ml·min\(^{-1}\)·kg\(^{-1}\). None of them was on any kind of medication. After being informed about the nature, purpose, and potential risks of the study, each subject signed an informed consent statement approved by the University of Muenster ethics committee. The number of participants in the second part of the study (moderate exercise) was seven (4 men, 3 women) for different reasons (injury, disease, etc.). Their anthropometric data did not change substantially: mean age was 24.0 ± 0.6 yr, weight 73.1 ± 2.1 kg, height 183.0 ± 2.0 cm, and maximal oxygen consumption 48.0 ± 2.0 ml·min\(^{-1}\)·kg\(^{-1}\).

After a general medical checkup, the subjects were first tested on day 1 for maximal oxygen uptake (V\(_{O_2\text{max}}\)) 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 by use of Quark b\(^2\) (Cosmed, Rome, Italy).

The experimental design of the study consisted of two parts. First, participants performed an exhaustive exercise test (ET) at an intensity corresponding to ~80% of the V\(_{O_2\text{max}}\) early in the morning. The mean running time was ~29.6 ± 4.4 min. Second, 2 wk later, the same volunteers performed a moderate exercise test (MT) at 60% of the V\(_{O_2\text{max}}\) at the same time of day. The running time for each subject during the moderate test was identical to the running time achieved in the initial exhaustive test.

Blood sampling intervals were identical for both tests. After cannulation of the cubital vein, blood samples were taken before exercise, immediately after, and 1 h after the end of exercise. Subjects were not allowed any strenuous physical activity or exercise 2 days before the exercise tests.

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

Cell isolation procedure. Lymphocytes were prepared by density-gradient centrifugation as previously reported (7). Briefly, 5 ml of a 50:50 mixture of whole blood anticoagulated with EDTA and 0.9% NaCl solution were carefully layered on 3 ml of Lymphoprep (Nycomed, 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 Lymphoprep solution was removed. Cells were washed twice with buffer A containing (in mM) 118 NaCl, 5.4 KCl, 10 H-HEPES, 0.4 Na2HPO4, 0.44 KH2PO4, 5.5 glucose, adjusted to pH 7.4. Finally cells were resuspended in buffer B containing (in mM) 140 NaCl, 3 KCl, 10 H-HEPES, 0.4 Na2HPO4, 1 MgCl2, 0.8 CaCl2, and 5.5 glucose, adjusted to pH 7.4.

Cell viability was ~98% as demonstrated by trypan blue exclusion, whereas purity was ~95% as determined by flow cytometry in the forward and sideward scatter mode.

Measurement of apoptosis. Cell death was determined either immediately after cell isolation (direct assay) or after a 24-h incubation period (incubation assay). In the incubation assay, cells isolated before and after exercise were incubated for 24 h either in culture medium alone (RPMI 1640, 5% FCS, 1% l-glutamine, 1% penicillin/streptomycin) or in the presence of the topoisomerase inhibitor camptothecin (32 μg/ml) or the lectin phytohemagglutinin (PHA; 3.75 μg/ml; 21).

Cell death in both assays was measured by flow cytometry (Coulter-Immunotech, Miami, FL) using annexin-V FITC and nuclear propidium iodide uptake for detection of apoptosis and necrosis, respectively (Roche Diagnostics, Mannheim, Germany). Lymphocytes (10⁶) in 295 μl buffer B were incubated for 15 min at room temperature with 2.5 μl of each stock solution prepared according to the manufacturer's instructions.

Analysis of CD95-receptor and CD95-receptor ligand expression. Isolated lymphocytes (0.5 × 10⁶) were labeled with either FITC-conjugated mouse anti-human CD95 monoclonal antibody (clone ANC95.1/5E2, Ancell, Bayport, MN) or phycoerythrin-conjugated mouse anti-human CD95 ligand monoclonal antibody (clone Alf-2.1a, Ancell) for 45 min at 4°C. Stock solutions of both antibodies were used according to the manufacturer's instructions in a final working dilution of 1:50. CD95 and CD95L expression on the cell surface were analyzed by flow cytometry (Epics XL, Coulter).

Statistical analysis. Data are means ± SE unless indicated otherwise in the figure legends. Differences between preexercise values and values at the two postexercise time points were compared with repeated-measures ANOVA. Statistical significance was set at the P < 0.05 level.

RESULTS

The ET initially induced significant granulocytosis and lymphocytosis. One hour after the end of the test, granulocytes were still found to be increased whereas lymphocytes declined to a level significantly below the baseline. The MT induced similar early changes in leucocytes and granulocytes, but changes were not as prominent as during exhaustive exercise. Moreover, the postexercise changes in lymphocyte count were not different from preexercise values (Table 1).

Labeling of apoptotic cells with annexin V antibodies after isolation (direct assay) demonstrated a nearly 50% increase in the percentage of apoptotic cells immediately after exhaustive exercise. This value returned to preexercise levels 1 h after the test. The return to baseline level was incomplete in two subjects.
who had a delayed (>1 h) decline in annexin V-positive cells (ANOVA at 1 h after test not significant). The percentage of necrotic cells remained constant (data not shown). After moderate exercise, no change in the percentage of annexin V-positive cells was detectable (Fig. 1). No gender differences were detected in this and the following assays.

The apoptosis of isolated lymphocytes was measured after a 24-h incubation period (incubation assay), and the susceptibility of cells toward apoptosis-inducing substances such as camptothecin and PHA was investigated. The pattern of annexin V-positive cells after 24 h incubation without stimulation was similar to the annexin labeling of freshly isolated cells. The exercise-induced apoptosis rate after the 24-h incubation period was still higher in lymphocytes isolated immediately after exhaustive exercise as indicated by a 50% increase in annexin V-positive cells (Fig. 2). Moderate exercise, on the other hand, did not affect the apoptosis rate of incubated lymphocytes (Fig. 2, top left). Again, the amount of necrotic cells was not affected under both conditions (data not shown).

Incubation of lymphocytes for 24 h with camptothecin resulted in an increase of annexin V-positive cells of ~200% compared with unstimulated cells. This effect was significantly higher in cells isolated and incubated immediately after exhaustive exercise. After camptothecin treatment, the amount of necrotic cells increased slightly from 1.8 ± 0.4 (baseline) to 2.5 ± 0.3 (immediately after exercise; P < 0.01) and 2.2 ± 0.3% (1 h after exercise; not significantly different from baseline). Camptothecin-induced apoptosis and necrosis in cells before and after the MT remained unchanged (Fig. 2). After PHA treatment for 24 h, the apoptosis rate increased ~600% compared with unstimulated conditions. After this stimulus, no change in the apoptosis rate of cells isolated before or after exhaustive or moderate exercise was detectable (data not shown).

When we investigated the expression of the CD95 (Fas; Apo-1) death receptor, the percentage of CD95-positive cells increased significantly (P < 0.01) after exhaustive exercise and returned to baseline levels 1 h after the test. Also after moderate exercise, a significant increase of CD95-positive cells was detected immediately after the test (P < 0.05). However, the preexercise values in the moderate exercising group were considerable lower than before exhaustive exercise (Fig. 3A). Gating of CD95-positive cells resulted in a CD95-receptor density distribution that revealed the presence of two different cell populations, one population with low CD95-receptor expression and another cell population with an ~10-fold higher CD95-receptor expression. Under resting conditions, the relation of both populations was 70 to 30% (Fig. 3D). The CD95-receptor density distribution was characteristically affected by exhaustive exercise. Immediately after exhaustive exercise, the CD 5 receptor low-density population decreased whereas the high receptor population increased. This effect was fully reversible 1 h after the end of exercise (Fig. 3, B, D–F). Moreover, this change in the CD95-receptor density distribution was not found after moderate exercise (Fig. 3C).

Finally, we investigated the expression of the CD95-receptor ligand (Fas ligand) on lymphocytes. The percentage of CD95 ligand-positive cells was not affected either by exhaustive or moderate exercise (Fig. 4).

**DISCUSSION**

Apoptosis serves a key role in the hematopoetic system and represents a mechanism for the removal of damaged, infected, or redundant cells (1). Cell damage and especially damage to cellular DNA can result from different stimuli and agents. Several recent studies have shown that physical exercise causes DNA damage in leukocytes (15, 32, 40). By using single-cell gel electrophoresis, a sensitive test of DNA-strand damage and alkali-labile damage, an exercise-induced appearance of DNA damage in leukocytes after exhaustive

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**Table 1. Changes in leukocyte, granulocyte, and lymphocyte counts after exhaustive and moderate exercise tests**

<table>
<thead>
<tr>
<th></th>
<th>Exhaustive Test</th>
<th>Moderate Test</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>5,960 ± 320</td>
<td>8,700 ± 500†</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>4,090 ± 280</td>
<td>5,530 ± 390†</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,870 ± 150</td>
<td>3,170 ± 310†</td>
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</tbody>
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Values are means ± SE. †Significant difference, P < 0.05; ‡significant difference, P < 0.01. Symbols in the after column are vs. before values; symbols in 1 h after column are vs. after values.
exercise has been reported (15). Niess et al. (32) found DNA damage in leukocytes after a half-marathon race. Immune cells that have undergone irreparable damage are generally eliminated by apoptosis, and our own results demonstrated that, indeed, after exhaustive exercise the percentage of apoptotic lymphocytes increases immediately after the test. Apoptotic cells were detected by labeling phosphatidylserine groups with annexin V. Annexin V has been shown to be a sensitive and early marker of apoptotic cells because these cells lose membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine at the cell surface (24). Annexin V labeling was enhanced after the exhaustive test in both freshly isolated cells and cells incubated for a 24-h period. Apoptosis induced by a 24-h stimulation with the topoisomerase I inhibitor camptothecin was also found to be enhanced after exhaustive exercise. In contrast, the percentage of apoptotic cells after moderate exercise remained constant. The latter result is in agreement with data of Hoffman-Goetz et al. (16), who reported that low-intensity, voluntary activity did not increase spontaneous lymphocyte apoptosis in mice.

In a previous study using the Tunel method, Mars et al. (26) found that lymphocyte apoptosis occurs in ~63% of lymphocytes immediately after high-intensity exercise. At 24 h after exercise, 86% of cells still showed an apoptotic pattern of DNA distribution. In our study, the percentage of apoptotic cells was considerably lower. This discrepancy between the two results can probably be attributed to the different methods used and different numbers of subjects. In the study by Mars et al., the results of only three subjects were reported with a high variation in even the pretest values of apoptotic cells. In some animal studies, an exercise-induced increase of apoptotic cells in various lymphoid compartments was observed (18). In rats an increase of apoptotic thymocytes was found; however, this effect was independent of the physical stress level (10). Similar results were obtained after chronic exercise in mice splenocytes (2).

The mechanisms responsible for exercise-related apoptosis of lymphocytes are unknown. During exercise, metabolic and hormonal changes have been reported that can damage cells or induce apoptosis in in vitro experiments. Hoffmann-Goetz and co-workers (17) investigated whether exercise-induced changes in glucocorticoid levels were responsible for the apoptosis induction. Their data suggest that in vitro exposure to corticosterone at the physiological concentrations observed after moderate exercise were already effective in inducing apoptosis in thymocytes. Likewise, catecholamines were found to induce apoptosis in peripheral blood lymphocytes in a time- and concentration-dependent manner (9). Although we did not determine either catecholamines or cortisol levels in the present investigation, their levels are known to correlate to exercise intensity.

Another possible intracellular trigger of apoptosis has been demonstrated in studies from our own group. We recently observed that exercise induces alterations in cytosolic calcium. The cytosolic calcium concentration is an important intracellular signal that has been shown to be involved in apoptotic processes, and increases in cytosolic calcium have been found to precede the onset of apoptosis in a number of different cell types (19, 20, 28, 45).

Finally, changes in the cellular redox status may be another important apoptosis trigger. The generation of free radicals and ROS is markedly enhanced during exercise (27). ROS are well known to induce damage of cellular structures and DNA (4). Therefore, in a number of cell types, ROS are involved as initiators and mediators of apoptosis (39). Interestingly, there seems
to be cross talk between ROS release and calcium signaling (see above), which integrates both pathways in apoptotic signaling (13, 23). The application of antioxidants has been shown to reduce the exercise-induced DNA damage in thymocytes and suggests a role of ROS in mediating these effects (25). Training that is accompanied by enhanced expression of radical-depleting mechanisms has been found to improve exercise-induced cell damage (40). The protective role of an enhanced serum antioxidant capacity in lymphocyte apoptosis has been demonstrated recently in an animal study. Spontaneous as well as H$_2$O$_2$-induced apoptosis in immune cells was decreased in exercised mice after performance of voluntary exercise during a 10-mo period compared with sedentary controls (3).

Our data, however, provide evidence for an alternative pathway by which apoptosis can be induced. Sig-
naling through the CD95 (Fas/APO-1) receptor induces apoptosis independently of the presence of ROS. The CD95 (Fas/APO-1) receptor is a member of the tumor necrosis factor receptor family (41). It is expressed on the surface of a variety of cells, and some cells, such as lymphocytes, coexpress the CD95 ligand, a homotrimer and type II transmembrane protein (31). Binding of the CD95 ligand to the CD95 receptor induces receptor trimerization and transduces an apoptotic signal (38, 41). During lymphocyte activation, both surface molecules, the CD95 receptor and the CD95 ligand, are upregulated (42). Their expression pattern is regulated by several cytokines such as interleukin-2, transforming growth factor-β, and interferon-α and -γ (22). Environmental factors such as smoking and fasting have also been shown to increase CD95-receptor expression (6, 37). Our data demonstrate that CD95-receptor expression is upregulated in a distinct percentage of lymphocytes after strenuous exercise. Unexpectedly, we also found a small increase in CD95-receptor-positive cells after moderate exercise. A certain activation of cells can therefore not be excluded and would also be indicated by the small changes in total leukocyte counts. This suggests that the CD95 receptor is a very sensitive marker of lymphocyte activation (12). However, an upregulation alone did not seem to be sufficient to induce apoptosis, and this suggests that co-stimulatory signals that are present only after exhaustive exercise are an additional requirement. At baseline, we found two different populations of CD95-receptor-expressing lymphocytes, one with low and one with high receptor density. Exhaustive exercise induced a shift to the high-density population that was not observed after moderate exercise, making the cells probably more sensitive to soluble apoptotic stimuli because CD95-receptor ligand expression on the cell surface remained unchanged.

In summary, we have shown that exhaustive exercise induces apoptosis in peripheral blood lymphocytes whereas moderate exercise does not. The changes in apoptotic cells are small and should therefore only partially account for the exercise-induced lymphocytopenia. There is evidence that changes in the CD95-receptor expression are involved in apoptosis. Further studies will have to elucidate the molecular mechanisms of exercise-induced apoptosis in lymphocytes and their subsets.

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REFERENCES


