Inhibitory effects of voluntary wheel exercise on apoptosis in splenic lymphocyte subsets of C57BL/6 mice

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Reddy Avula, C. P., A. R. Muthukumar, K. Zaman, R. McCarter, and G. Fernandes. Inhibitory effects of voluntary wheel exercise on apoptosis in splenic lymphocyte subsets of C57BL/6 mice. J Appl Physiol 91: 2546–2552, 2001.—Two-month-old mice were placed in cages with (Ex) or without exercise running wheels with free access to the wheel 24 h/day for 10 mo. An equal amount of food for both groups was provided daily. Ex mice ran an average of 33.67 km/wk initially, and exercise decreased gradually with age. Ex mice had gained an average of 43.5% less body weight at the end of the experiment. Although serum lipid peroxidation was not altered by exercise, superoxide dismutase and glutathione peroxidase activities in serum were significantly increased. Flow cytometric analysis of spleen cells revealed an increased percentage of CD8+ T cells and a decreased percentage of CD19+ B cells in Ex mice (P < 0.05). Exercise decreased apoptosis in total splenocytes and CD4+ cells incubated with medium alone or with H2O2, dexamethasone, tumor necrosis factor-α (TNF-α), or anti-CD3 monoclonal antibody (P < 0.05) and CD8+ cells with medium alone or with TNF-α (P < 0.05). Even though exercise did not alter the intracellular cytokines (TNF-α and interleukin-2) or Fas ligand, it did significantly lower interferon-γ in CD4+ and CD8+ cells (P < 0.05). In summary, voluntary wheel exercise appears to decrease H2O2-induced apoptosis in immune cells as well as decrease interferon-γ production.

H2O2; dexamethasone; tumor necrosis factor-α; anti-CD3; antioxidant enzymes

Moderate physical exercise is known to be beneficial in the prevention and management of certain chronic diseases (44, 60). A recent report indicates that high-intensity resistance exercise training strengthens weakened muscle and alleviates physical frailty in elderly people (15). However, several studies also showed that high-endurance exercise could increase free radical generation, causing immune suppression through enhanced stress-induced glucocorticoid production (27, 28, 61, 63). Exercise has also been reported to increase antioxidant enzymes (3, 31, 46), however, which could counteract the effects of increased free radical generation.

In recent years, increased caloric intake and decreased physical activity have been commonly observed in the US population, promoting obesity, cardiovascular disease, and diabetes (5, 66). To alter this trend, strategies for weight reduction are emphasized as one of the highest public health priorities, particularly adopting preventive measures from a young age (5, 38).

It is now well established that apoptosis (or programmed cell death) plays a central role in embryogenesis, morphogenesis, and immune cell regulation, particularly for maintaining optimal defense mechanisms to ward off infectious diseases and probably in preventing the aging process. Apart from its central role in normal immune cell regulation, inappropriate induction of apoptosis could result in a variety of pathological effects, such as development of Alzheimer’s disease, cancer, and neurodegenerative disorders, including chronic autoimmune disorders (i.e., acquired immunodeficiency syndrome and systemic lupus erythematosus) (7, 8, 29, 57, 64).

However, maintenance of physiologically regulated apoptosis could play a beneficial or protective role in the maintenance of an optimally functioning immune system because of the presence of physiologically induced triggers, which can cause apoptosis (12, 58). Some of the known triggers include glucocorticoids, oxidative stress, activation of Fas ligand (Fas-L), tumor necrosis factor-α (TNF-α), and depletion of activating cytokines (33, 65). Apoptosis is also important in maintaining lymphocyte homeostasis via the continuous process of nonleterious activation-induced cell death, in which cells that have been activated are removed by apoptosis once they have served their effector function (49).

Maintenance of regular physical activity, including strenuous physical exercise, has been shown to cause significant changes in several immunological parameters (35). Many studies have shown elevated oxygen free radical formation due to strenuous exercise training in athletes (25, 52). However, elevated free radicals have been found to induce oxidative stress, which could...
 promote much deleterious apoptosis in lymphoid cells and in various other target tissues, including the brain and kidney (6, 37). Antioxidant enzymes have been known to play an important role in protecting tissue against oxidative damage.

Over the past several years, a number of studies have revealed that acute physical exercise in both rats and mice is associated with greatly elevated apoptosis in thymocytes and splenocytes (14, 22). Even though many studies have been carried out on exercise, oxidative stress, immune function, and apoptosis, most of these studies were done with either acute or severe physical exercise (14, 22, 50). However, so far, the effects of voluntary wheel exercise (moderate) on splenic lymphocytes and particularly their susceptibility toward induced apoptosis have not been well characterized. Therefore, the present study was aimed at determining whether voluntary wheel exercise alters intracellular Th-1-like cytokine levels and the ratio of splenic lymphocyte subsets and their susceptibility to cell death induced by different stimulators compared with sedentary mice.

MATERIALS AND METHODS

Materials. Thiobarbituric acid, 1,1,3,3-tetraethoxy propane, butyl hydroxyanisole, brefeldin A, ionomycin, phytohemaglutinin, cytochrome c (oxidized), glutathione, glutathione reductase, cumene hydroperoxide, NADPH, phorbol 12-myristate 13-acetate, brefeldin, and ionomycin were obtained from Sigma Chemical (St. Louis, MO). Dexamethasone (Dex), anti-CD3, TNF-α, annexin V, propidium iodide (PI), and all FITC- and phycoerythrin (PE)-labeled antibodies and antibody isotype controls were purchased from Pharmingen (San Diego, CA). All other chemicals (analytic grade) were obtained from Sigma Chemical.

Animals and exercise protocol. Six-week-old female C57BL/6 mice (Jackson Laboratory) were provided an American Institute of Nutrition-76 formula (2) semipurified diet containing 5% corn oil prepared weekly and water ad libitum. Initially, at 6 wk of age, the equivalent of 2 g dry weight of food in a moisturized form was provided per mouse. The amount was raised slowly to provide 4 g per mouse daily, from age 3 mo on, to both Sed and Ex mice. Ex mice were thus prevented from increasing their food intake. Food intake and body weight were monitored at regular intervals for Sed and Ex mice.

After 10 mo, mice were fasted overnight, and peripheral blood was then collected by retroorbital bleeding under mild anesthesia. Mice were then killed by cervical dislocation, and spleens were aseptically removed and placed in RPMI-1640 complete medium (containing 10% heat-inactivated FCS, 100 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 M 2-mercaptoethanol). Serum was separated by centrifuging the whole blood at 1,000 g for 15 min and analyzed immediately. All animal care and procedures were approved by the Institutional Animal Care and Use Committee.

Serum lipid peroxides. Serum lipid peroxides (as thiobarbituric acid-reactive substances) were determined by using the fluorescence method previously described (3).

Antioxidant enzymes. Superoxide dismutase (SOD) activity was measured by the inhibition of cytochrome c reduction mediated via the superoxide anions that were generated by xanthine-xanthine oxidase and monitored at 550 nm (17). For each sample, a blank sample (a reaction mixture containing all components except xanthine oxidase) was measured separately, and the blank rate was subtracted from the overall reaction rate. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of cytochrome c by 50%. Glutathione peroxidase (GSH-Px) activity was determined by NADPH oxidation using a coupled reaction system consisting of glutathione and glutathione reductase, with cumene hydroperoxide as substrate (56).

Splenocyte preparation. Single-cell suspensions were prepared by disrupting the spleen between frosted glass slides in RPMI-1640 media with 2% heat-inactivated FCS. After a 5-min centrifugation at 100 g to separate cells from debris, the cells were washed twice in RPMI (13). Spleen lymphocytes were purified by layering them over Lympholyte (Cedarlane Laboratories, Hornby, Ontario), centrifuging at 1,000 rpm for 15 min at 22°C, and then washing them twice in RPMI-1640 complete medium. Cells were counted, and viability was determined by Trypan blue exclusion. The spleen from each animal was prepared and analyzed separately.

Mitochondrial membrane potential. Changes in mitochondrial membrane potential were evaluated by measuring the cellular retention of rhodamine 123 (54). Lymphocytes (1 × 10^6) were washed with isotonic NaCl-phosphate buffer containing 0.5% BSA and 0.2% NaN₃ (wash buffer). Cells (1 × 10⁶ cells) were resuspended in RPMI complete medium with or without 10 µM rhodamine 123 (stock solution of 10 mM in ethanol, kept in the dark at 0–4°C) and incubated for 1 h in a final volume of 1 ml in a 5% CO₂ incubator at 37°C. After incubation, cells were washed by centrifuging at 200 g, resuspended in PBS, and kept in the dark, on ice, until analysis. Cellular fluorescence was analyzed by flow cytometry with a Becton Dickinson FACScan flow cytometer. For visual comparison of lymphocyte population histograms, each analysis was recorded at a single amplification setting.

Apoptosis and necrosis. Lymphocytes (1 × 10⁶) were incubated for 4 h in a 5% CO₂ incubator at 37°C in a final volume of 1 ml of RPMI complete medium alone or with 25 µM H₂O₂. Alternatively, an overnight (16 h) incubation with 1 µM Dex or 2 µg anti-CD3 and or TNF-α was done. After incubation, cells were washed twice with binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), resuspended in 200 µl binding buffer in the presence of 0.2 µg FITC-annexin V and 2 µg PI, and then incubated in the dark for 10
min (9). Annexin V and PI staining, as indications of apoptosis and necrosis, respectively, were measured with the FACScan (Becton Dickinson) using the Cell Quest software as described earlier (3).

Apoptosis of splenocyte subsets. Viable spleen cells incubated with medium alone, with 25 μM H2O2, with anti-CD3, and/or with TNF-α were blocked with Fc receptor and cell aliquots incubated for 30 min with anti-CD4, anti-CD8, or anti-CD19-PE-labeled monoclonal antibody (MAb), as described earlier (46). Cells were washed twice with binding buffer and then incubated with 0.2 μg of annexin V-FITC for 15 min in the dark. Cellular fluorescence was analyzed by flow cytometry (Becton Dickinson FACScan flow cytometer) using the Cell Quest program.

Fas-L and intracellular cytokine staining of splenic lymphocytes. Cells (1 × 10⁶) were activated in the presence of soluble anti-CD3 MAb (2 μg/ml) for 2 h and, after washing, were further stimulated with brefeldin A, phorbol 12-myristate 13-acetate, and ionomycin (2 μg, 10 ng, and 1 μg/ml, respectively) for 4 h in a humidified atmosphere at 37°C with 5% CO2. The cells were then washed again and blocked with Fc receptor, and cell aliquots were incubated for 30 min with 2 μl of anti-CD4 or anti-CD8-PE MAbs and/or FITC-labeled MAb as described earlier (47). Cells were then fixed with 200 μl of 1% paraformaldehyde in wash buffer for 15 min with continuous shaking at room temperature. Lymphocytes were washed with wash buffer and then permeabilized by suspending in 200 μl of 0.33% saponin at 4°C for 45 min. Permeabilized cells were then washed and stained with either anti Fas-L-PE, anti-TNF-α-PE, interleukin (IL)-2-PE, and/or interferon (IFN)-γ-PE in 200 μl wash buffer at 4°C for 45 min. Proper isotype controls were used in all experiments.

Statistical analysis. The data shown are the means ± SE of five individual measurements. Data were statistically analyzed using the Student’s t-test or one-way ANOVA with Bonferroni’s test as appropriate, and P < 0.05 was considered significant.

RESULTS

Voluntary exercise, started from 8 wk of age, maintained a significantly reduced body weight compared with Sed controls (Fig 1). Mice on wheels initially ran an average of 122 ± 9.0 × 1,000 revolutions/wk, which is equal to 33.67 km·wk⁻¹·mouse⁻¹. The rate of running decreased gradually with age. Ex mice had gained 43.5% less body weight than the Sed mice at the end of the experiment. Because an approximately equal amount of food was provided to both Sed and Ex mice, the significant decrease in weight gain in the Ex mice is most likely due to increased energy expenditure from exercise.

**Table 1. Influence of voluntary wheel exercise on serum lipid peroxides and antioxidant enzyme activity**

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA, nmol/ml serum</th>
<th>SOD, U/ml serum</th>
<th>GSH-Px, U/ml serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>5.73 ± 0.53</td>
<td>30.57 ± 1.51</td>
<td>368 ± 32</td>
</tr>
<tr>
<td>Exercised</td>
<td>6.13 ± 0.15</td>
<td>40.74 ± 1.57*</td>
<td>516 ± 54*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 mice. MDA, malondialdehyde; SOD, sodium dismutase (see MATERIALS AND METHODS for explanation of unit of measure); GSH-Px, glutathione peroxidase (1 unit = 1 μmol NADPH oxidized/min). *Significantly different from sedentary control, P < 0.05.

Serum lipid peroxides, SOD, and GSH-Px. Serum lipid peroxides, measured as thiobarbituric acid-reactive substances (Table 1) were not altered by exercise, yet serum SOD and GSH-Px activities were increased by 33.3 and 40.4%, respectively (Table 1) in Ex mice compared with the Sed mice.

Membrane potential of splenocytes. Although antioxidant enzyme activities were increased by exercise, the mitochondrial membrane potential measured by rhodamine 123 retention was not altered by exercise (Sed 21.95 ± 4.9 vs. Ex 20.99 ± 4.8% of lymphocytes stained), indicating that voluntary wheel exercise did not cause any increased oxidative stress.

Influence of exercise on splenocyte subsets. The influence of exercise on splenic lymphocytes is summarized in Fig 2. Ex mice maintained a significantly higher percentage (133%) of CD8⁺ T cells compared with Sed mice, whereas the number of CD4⁺ T cells was increased by 17% in Ex mice compared with Sed mice. This increase was not statistically significant. Also, exercise decreased CD19⁺ cells by 16.4% compared with the Sed group.

Apoptosis. Apoptosis of splenic lymphocytes cultured in medium alone or in the presence of H2O2 was analyzed by flow cytometric detection of annexin V and PI

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**Fig. 1.** Effect of voluntary wheel exercise on body weight of mice. Each point represents the mean ± SE of 5 individual mice. Means are significantly different at each time after the initial measurement (P < 0.05). Sed, sedentary mice; Ex, exercised mice.

**Fig. 2.** Influence of voluntary wheel exercise on the relative percentage of splenocytes in different subsets. Values are means ± SE of 5 individual measurements. *Significantly different compared with the respective controls, P < 0.05.
Apoptosis (annexin V staining only) was decreased by 18.9 and 48.3% and necrosis (annexin V and PI staining) was decreased by 33.2 and 30% in cells from Ex mice compared with cells from Sed mice incubated with and without H₂O₂, respectively. Although neither apoptosis nor necrosis in cells incubated with H₂O₂ was affected significantly by exercise, apoptosis was significantly decreased by exercise in cells incubated in medium alone (Fig. 3). Interestingly, Dex, TNF-α, and anti-CD3 apoptosis were also significantly decreased by 45.9, 34.3, and 32%, respectively, in cells from Ex mice compared with cells from Sed mice (Fig. 4).

Apoptosis of splenocyte subsets. Annexin V staining for apoptosis was decreased in CD4⁺ and CD8⁺ T cell subsets from Ex mice incubated with medium alone (34.65 and 43.06% respectively, Fig. 5) and in CD4⁺ cells (33.74%) incubated with H₂O₂ or anti-CD3 (Table 2). Similarly, apoptosis was significantly decreased in CD4⁺ and CD8⁺ cells incubated for 16 h with TNF-α by 27 and 33.2%, respectively, in Ex mice compared with Sed mice (data not shown). No significant effect of exercise was observed in CD19⁺ cells incubated either with medium alone or with H₂O₂ or TNF-α. However, significantly lower anti-CD3-induced apoptosis (34%) was observed in CD19⁺ cells of Ex mice (Table 2). No effects of exercise on necrosis were observed in this study.

Expression of Fas-L and intracellular cytokines in splenic lymphocytes. Because incubation of spleen cells in vitro with anti-CD3 antibody and TNF-α revealed differences in apoptosis between cells from Sed and Ex mice, we measured both Fas-L and TNF-α expression in total splenic lymphocytes cultured with anti-CD3. Expression of these factors was not affected by exercise (Fas-L: Sed, 19.9 ± 6.16 vs. Ex, 21 ± 4.5; TNF-α: Sed, 30.03 ± 6.16 vs. Ex, 32.6 ± 4; IL-2: Sed, 3.64 ± 0.06 vs. Ex, 5.03 ± 0.85% of lymphocytes stained). We also determined the expression of Fas-L, TNF-α, and IL-2 in T-cell subsets. Again, no differences between Sed and Ex mice were observed in these cells (data not shown). Even though there was no change in total IFN-γ expression found with exercise, the IFN-γ levels in CD4⁺ and CD8⁺ cells were lower by 20 and 40%, respectively, in cells from Ex mice (Fig. 5).

**DISCUSSION**

In the present study, mice voluntarily exercising on wheels daily showed a significantly lower weight gain, which we attributed to increased energy expenditure caused by voluntary wheel exercise. Similar results were observed previously from both voluntary wheel exercise with rats and forced treadmill exercise with rats and mice (3, 28, 36, 46). Our present observations clearly show that moderate physical exercise can prevent excessive body weight gain with age from ad libitum food consumption and remaining sedentary in the cage. One primary purpose of this study is to determine whether voluntary exercise would alter the ratio of lymphocyte subsets and also the rate of spontaneous apoptosis and apoptosis induced by various activators in lymphocytes.

Several previous studies have been described using lipid peroxides as an indicator of tissue oxidative stress, resulting in free radical attack on cellular mem-
intensity exercise is associated with a transient in-
For example, changes in the proportions of subsets of
mental effects of exercise are often cited (14, 21, 32).
modulate immune function, both beneficial and detri-
apoptosis (33, 65).
in antioxidant enzymes could alter apoptosis by pre-
comparing with sedentary controls. The increases seen
lymph nodes, thymus, and spleen of swim-trained mice
significant decrease in lipid peroxidation and variable
organs, as well as differential effects on antioxidant
contrary to a number of studies investigating
chronic exercise training (involving bouts of high-in-
tence (22, 32), we find a marked resistance to H2O2-
delusive and beneficial effects on immunocompe-
cells in human blood (16). However, the
effect of low-intensity or moderate exercise training on
function in highly trained athletes (34, 41). It appears that decreased IFN-
rendering the occurrence of sudden infection in highly trained
plasma and several other tissues, including brain, of
voluntary wheel-running exercised rats compared with
sedentary rats. When rats were previously fed a high
lipid peroxide diet, total lipid peroxides per whole body
were removed significantly faster in the exercised rats
than in the sedentary controls (55). Slower training
with longer duration also reduced the basal levels of
hydroxyl radicals in plasma to a greater extent than
more intense training. Thus these results suggest that
moderate endurance exercise can increase the antiox-
idant capacity in rats (24).
In recent years, antioxidant enzymes have been
found to play an important role in the defense against
free radical damage and oxidative stress. In the
present study, voluntary wheel exercise significantly
increased the antioxidant enzyme activity in the serum.
Similarly, elevated blood levels of SOD have also been
reported in healthy volunteers after a single, acute
treadmill exercise test (31). We and others have previ-
ously shown that moderate treadmill exercise was able
to increase antioxidant enzymes and decrease lipid
peroxidation in spleen cells (3, 27, 46). Furthermore,
similar to our findings, Pereira et al. (43) reported a
significant decrease in lipid peroxidation and variable
changes in SOD, catalase, and GSH-Px in mesenteric
lymph nodes, thymus, and spleen of swim-trained mice
compared with sedentary controls. The increases seen
in antioxidant enzymes could alter apoptosis by
preventing increases in oxidant species, which trigger
apoptosis (33, 65).

In general, although exercise has been shown to
modulate immune function, both beneficial and detri-
mental effects of exercise are often cited (14, 21, 32).
For example, changes in the proportions of subsets of
immune cells in the peripheral circulation have been
related to exercise intensity and duration (21). High-

Table 2. Effect of exercise on annexin V binding to subset splenocytes incubated
with or without inducers of apoptosis

<table>
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<tr>
<th></th>
<th>Sed</th>
<th>Ex</th>
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<tr>
<td></td>
<td>± SE of percentage of splenocytes binding annexin V</td>
<td>nos. in parentheses, no. of animals</td>
</tr>
<tr>
<td>Medium</td>
<td>30.85 ± 1.63 (8)</td>
<td>20.16 ± 1.87*(8)</td>
</tr>
<tr>
<td>H2O2</td>
<td>41.36 ± 3.53†(5)</td>
<td>28.86 ± 2.83*(5)</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>39.03 ± 4.86 (4)</td>
<td>27.68 ± 5.10*(4)</td>
</tr>
</tbody>
</table>

Values are means ± SE of percentage of splenocytes binding annexin V; nos. in parentheses, no. of animals. Sed, sedentary; Ex, exercised.
Even though several recent studies have shown exhaustive exercise to cause DNA damage and increased apoptosis in thymocytes and splenocytes (14, 22, 50), others have shown that regular, less intense exercise training could help to reduce the severity of the DNA damage (20). In addition, X-ray-induced chromosomal damage was also found to be increased with endurance running in untrained groups, but again it was less in trained exercised groups. The ratio of X-ray-induced spontaneous chromosomal damage also tended to increase after running only in the untrained group (59). Thus regular exercise training of longer duration appears to provide beneficial effects against DNA damage in immune cells.

Interestingly, in the present study, voluntary wheel exercise did not alter either anti-CD3-induced Fas-L or intracellular TNF-α levels in lymphocytes compared with Sed control mice. Similarly, Venkataraman and Pendergast (62) have shown that endurance running had no effect on the level of plasma IL-2, IL-6, and TNF-α in young runners. On the other hand, others have shown increased TNF-α levels in plasma of strenuously exercised humans and also in the adipose tissue of insulin-resistant rats (40, 42).

Even though exercise did not alter the expression of Fas-L and production of TNF-α, which are well documented to induce cell death (1, 4), in our present study, it significantly lowered the anti-CD3-induced in vitro apoptosis (Fig. 4) in total splenic lymphocytes as well as in CD4+ and CD19+ cells (Table 2) compared with cells from Sed mice. The decreased anti-CD3-induced and activation-induced apoptosis observed in cells from Ex mice suggests that exercise is most likely to influence the multiple signaling pathways involved in causing apoptosis and requires further study. Interestingly, apoptosis of CD4+ cells, with or without stimulation, was significantly decreased by exercise. In contrast, although apoptosis of CD8+ cells incubated with medium alone was also decreased by exercise, apoptosis induced by H2O2 or anti-CD3 was not affected, indicating that exercise effects may vary between CD4+ and CD8+ cells. However, further studies would be needed to confirm whether exercise selectively induces resistance to apoptosis.

It is also well established that in vitro exposure of lymphocytes to glucocorticoid hormones induces apoptosis (3, 45, 58). However, voluntary wheel exercise significantly lowered Dex-induced apoptosis. These preliminary results indicate that exercise may influence in vivo regulation of hormone receptors on immune cells. With age and also with intense sports activity, glucocorticoid levels were shown to decrease in human peripheral blood mononuclear cells (18). However, new studies with intense and moderate exercise are needed to establish the precise role of glucocorticoids in regulation of lymphoid cell apoptosis, both in vivo and ex vivo.

In conclusion, our results suggest that voluntary wheel exercise in mice increases serum antioxidants without altering the lipid peroxides. Voluntary exercise also protects the immune cells against spontaneous apoptosis, as seen in the cells incubated in medium alone, as well as oxidative stress, whereas activation (anti-CD3)-induced apoptosis appears to vary between CD4+ and CD8+ cells. Further lowering of Dex-induced apoptosis indicates that exercise may influence the hormone receptors and their regulation of immune cells.

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