Intrathymic and intrasplenic oxidative stress mediates thymocyte and splenocyte damage in acutely exercised mice

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Azenabor, A. A., and L. Hoffman-Goetz. Intrathymic and intrasplenic oxidative stress mediates thymocyte and splenocyte damage in acutely exercised mice. J. Appl. Physiol. 86(6): 1823–1827, 1999.—Reactive oxygen species may contribute to apoptosis in lymphoid tissues observed after exercise. Thymic and splenic tissues excised from control mice (C) or mice immediately after (t0) or 24 h after (t24) a run to exhaustion (RTE) were assayed for biochemical indexes of oxidative stress [thymic and splenic membrane lipid peroxides, superoxide dismutase, catalase, plasma uric acid (UA), and ascorbic acid (AA)]. There were significant increases in membrane lipid peroxides in thymus (P < 0.001) and spleen (P < 0.001) in acutely exercised mice relative to controls (thymus: C = 2.74 ± 0.80 μM; t0 = 7.45 ± 0.46 μM; t24 = 9.44 ± 1.41 μM; spleen: C = 0.48 ± 0.22 μM; t0 = 1.78 ± 0.28 μM; t24 = 2.81 ± 0.34 μM). The thymic and splenic tissue antioxidant enzymes concentrations of superoxide dismutase and catalase were significantly lower in samples collected at t0 relative to C and t24 mice (P < 0.001). Plasma UA and AA levels were used to assess the impact of the RTE on the peripheral antioxidant pool. There was no significant change in UA levels and a significant reduction in plasma AA concentrations (P < 0.001); the reduction in plasma AA occurred at t24 (6.53 ± 1.64 μM) relative to t0 (13.11 ± 0.71 μM) and C (13.26 ± 1.2 μM). These results suggest that oxidative damage occurs in lymphoid tissues after RTE exercise and that such damage may contribute to lymphocyte damage observed after acute exercise.

MATERIALS AND METHODS
Experimental animals and exercise protocol. Thirty female C57BL/6 mice (Harlan Sprague-Dawley, Frederick, MD), 10–12 wk of age, were randomly assigned to one of three conditions: exhaustively exercised and killed immediately after cessation of the bout (t0 group); exhaustively exercised, returned to home cage, and killed 24 h after cessation of exercise (t24 group); and “sedentary” control animals that were killed without treadmill exercise (C group). The run to exhaustion (RTE) consisted of a single treadmill challenge at 32 m/min and a slope of 8%. Mice were run at this speed and slope until they were unable to respond to continued prodding with a soft brush. The mean time to exhaustion was 90 min (range: 65–110 min). Mice were housed in a temperature- and humidity-controlled room, at 21°C, on a 12:12-h reversed light-dark cycle. Food (Laboratory rodent chow, PMI Feeds, Richmond, IN) and tap water were provided ad libitum. All procedures involving mice were conducted in accordance with the guidelines established by the Canadian Council on Animal Care.

Tissue collection. Mice were killed by pentobarbital sodium overdose to allow cardiac exsanguination. Blood was centrifuged down to separate the plasma portion and frozen at −20°C until assayed for uric acid (UA) and ascorbic acid (AA). The spleen and thymus of each mouse were excised and cells dispersed into small tissue-culture dishes containing phosphate buffer, pH 7.4, by filtration through fine mesh (spleen) or by using an Ultra Turrax (Germany) mechanical homogenizer (thymus). Red blood cells were removed from spleen by differential centrifugation. Samples were centrifuged at 300 rpm for 5 min at 4°C, then the supernatant was frozen at −80°C for 20 min and rapidly thawed at 37°C for 2 min. This procedure lysed all thymocytes and splenocytes completely. The lysates were centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatants were collected and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were again collected and used for the tissue antioxidant enzymes, super-

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oxide dismutase (SOD), and catalase. The cell-fragment pellets were used for the membrane lipid peroxide assays.

Lipid peroxide assay. Membrane lipid peroxide was measured by ferrous ion oxidation in the presence of xylenol orange, as described by Azenabor and Mahony (1). In brief, this method is based on peroxide-mediated ferrous ion oxidation in the presence of xylenol orange, with the measurement of the ferric ion-xylenol orange complex spectrophotometrically. Membranes packed from lysed splenocytes and thymocytes were treated with methanol to extract the lipid peroxide at a dilution of 1:10 and were centrifuged at 12,000 rpm for 3 min at 4°C. Extracted lipid peroxide (100 µl) was added to 900 µl of xylenol orange working reagent. The standard consisted of 20 µM H₂O₂ assayed along with the samples. A blank consisting of 100 µl of methanol in 900 µl of xylenol orange was used to establish a zero baseline. Reactions were allowed to stand for 30 min at room temperature, and absorbance was read at 560 nm.

SOD and catalase assays. SOD was measured by using the method described by Rice-Evans et al. (22). This method is based on the use of the xanthine-xanthine oxidase system to generate superoxide, and the reduction rate of cytochrome-c by superoxide is monitored at 550 nm. The inhibition in this reduction when SOD-containing sample was added was used as a measure of the activity of SOD. The assay of catalase was also based on the method described by Rice-Evans et al. In brief, 2 ml of lysate obtained from thymocytes and splenocytes were placed in a 3-ml cuvette, and the spectrophotometer was set at 240 nm at room temperature. One milliliter of a 30% H₂O₂ solution was added, mixed rapidly by inverting, and the decrease in absorbance over a period of 7 min was noted. Standard catalase (Sigma Chemical) was treated in the same fashion as test samples.

Plasma UA and AA assays. Plasma UA was measured by the method of Rock et al. (23), which involved the reduction of NAD⁺ to NADH. The rate of reduction was assessed spectrophotometrically at 340 nm. Plasma AA was assayed by the method described by McCormick (18). This involved the initial deproteinization of plasma by using 6% metaphosphoric acid. The supernatant obtained was treated with a reagent system consisting of thiourea solution, copper sulfate solution, and 2,4-dinitrophenylhydrazine at a ratio of 1:1:20. The reaction was incubated for 3 h at 37°C, chilled in an ice bath for 10 min, and then 12 M sulfuric acid was added. After equilibration, absorbance was measured at 520 nm.

Assay of protein. Protein estimation on each sample was done by the biocinchoninic acid-copper [II] sulfate reagent system assay, as described by Smith et al. (24).

Data analysis. All data are expressed as mean ± 1 SE. Data were analyzed by using one-way ANOVA with post hoc Scheffé’s tests; a value of P < 0.05 was considered significantly different from chance.

RESULTS

Effect of RTE exercise on membrane lipid peroxide in thymus and spleen. Membrane lipid peroxide accumulation was used as a biochemical index of cell death arising from free radical reactions with the unsaturated fatty acid component of lymphocyte membranes. Figure 1 shows the accumulation of lipid peroxides in thymocytes and splenocytes from acutely exercised and C (sedentary) mice. There was a significant increase in lipid peroxides immediately after and 24 h after RTE, relative to C animals, in both thymus (P < 0.001) and spleen (P < 0.001). The amount of lipid peroxidation that occurred in thymocytes was not significantly greater at t₂₄ (9.44 ± 1.4 µM) compared with t₀ (7.45 ± 0.48 µM). In contrast, in spleen, there was a small increase in lipid peroxidation at t₂₄ (2.81 ± 0.34 µM) after RTE, compared with t₀ (1.78 ± 0.28 µM). The magnitude of lipid peroxidation after the RTE was substantially greater in thymic lymphoid tissue than in splenic lymphoid tissue.

Effect of RTE exercise on SOD and catalase in thymus and spleen. Figure 2 shows the consumption of SOD (Fig. 2A) and catalase (Fig. 2B) in thymic and splenic tissue obtained from exhaustively exercised mice. There was a significant depletion of SOD in both thymic and splenic tissue immediately after a single RTE, with rebound at 24 h after exercise (thymus: P < 0.001; spleen: P < 0.01). Similarly, catalase concentration was lower immediately after exercise in both thymus (P < 0.001) and spleen (P < 0.001), with recovery or rebound to control levels at 24 h after RTE.

Effect of RTE exercise on plasma UA and AA. Figure 3, A and B, respectively, shows the concentration of UA and AA in mouse plasma after exhaustive treadmill exercise. Plasma UA levels were not significantly lower at t₀ (699.4 ± 31.0 µM) and t₂₄ (683.5 ± 36.7 µM), relative to control values (758.2 ± 45.9 µM). In contrast, plasma AA levels were significantly lower at t₂₄, relative to the other groups (P < 0.001).

DISCUSSION

Programmed cell death is a normal physiological process that occurs during embryonic development as well as in the maintenance of tissue homeostasis, including thymocyte selection (25). Inappropriate induction of apoptosis, however, has broad-ranging pathological implications. For example, it is associated with Alzheimer’s disease (11), Hodgkin’s lymphoma (16), graft vs. host disease and transplant rejection (13), and...
acquired immune deficiency syndrome (27). A nonpathological example of an inappropriate induction of apoptosis in lymphoid tissue is that of acute exercise (4, 5). The mechanism by which lymphoid tissue apoptosis occurs is not known.

The present study suggests that one mechanism of thymocyte and splenocyte cell damage or apoptosis after acute exercise is through the generation of free radical reactions. There are several possible pathways by which intrathymic and intrasplenic free radical reactions could occur. First, acute exercise is associated with an increase in blood estrogen (E2) levels (26), which could lead to an enhanced binding of E2 to E2 receptors on thymocytes and splenic T lymphocytes and to opening up of calcium “gates” (21). Increased intracellular calcium would then activate phospholipases, liberating free fatty acids from membrane phospholipids. For instance, the cleavage of arachidonic acid from the membrane causes rapid formation of prostaglandins, leukotrienes, and thromboxanes, with superoxides produced as a by-product. Superoxides and other free radicals elaborated in the cyclooxygenase and lipoxygenase pathways could contribute to membrane lipid peroxidation (12, 15). A second possibility is that of the activation of the xanthine oxidase reaction through exercise-induced ATP degradation to produce hypoxanthine and conversion to xanthine and UA (19). Third, it has been suggested that maximal exercise induces mitochondrial uncoupling due to inner membrane trauma and hyperthermia (10), with a resultant generation of ROS. Finally, neutrophils may contribute to superoxide generation in splenic tissue through myeloperoxidase and NADPH oxidase reactions during phagocytosis (3). Which of these pathways of free radical generation occur in thymus and spleen in response to exercise was not addressed in our study.

Fig. 2. Effect of exercise on thymic and splenic homogenate superoxide dismutase (A) and catalase (B) levels in mice immediately after and 24 h after exercise to exhaustion, compared with sedentary mice. Letters that are different (within tissue) indicate significance at P < 0.05 (Scheffé’s test).

Fig. 3. Effect of exhaustive exercise on plasma uric acid (A) and ascorbic acid (B) immediately after and 24 h after exercise, compared with sedentary mice. Letters that are different indicate significance at P < 0.05 (Scheffé's test).
The formation of superoxide brings about a cascade of free radical reactions. We found that plasma UA was not dramatically reduced immediately or 24 h after an RTE. The gradual and nonsignificant reduction in plasma UA may reflect two simultaneous but opposing biochemical reactions during exercise: first, the elaboration of UA from hypoxanthine as a result of ATP degradation; and, second, the depletion of UA arising from free radicals generated in the xanthine oxidase reaction. Plasma AA was, however, significantly reduced 24 h after an RTE. The patterns of response for UA and AA are consistent with a dynamic equilibrium between oxidative damage potential and antioxidant defense capacity in peripheral circulation in response to severe exercise. Plasma AA concentrations, however, may not be reflective of changes in other compartments, including thymus and spleen.

We found evidence of oxidative damage to thymocytes and splenic lymphocytes, as indicated by the increase in membrane lipid peroxidation in exercised compared with nonexercised animals. This finding is significant, since lipid peroxides are themselves free radicals with large reaction rate constants and will, therefore, contribute to the cell death process. That ROS are likely responsible for thymocyte and splenocyte cell damage events during RTE exercise is supported by the pattern of depletion (t0) and restoration (tSO) of the antioxidant enzymes, SOD, and catalase in both thymus and spleen. The generation of ROS results in the depletion of antioxidant reserve capacity of any compartment; in this instance, since the antioxidants are inducible enzymes, a feedback mechanism results in a restoration to normal enzyme levels. In contrast to our findings, Pereira et al. (20) reported a significant decrease in lipid peroxidation in mesenteric lymph nodes, thymus, and spleen of swim-trained mice, compared with sedentary controls, and variable changes in SOD, catalase, and glutathione peroxidase. These differences may be explained by exercise protocol (single exhaustive treadmill exercise vs. chronic endurance swim training) and assay procedures (membrane lipid peroxides measured spectrophotometrically vs. nonspecific thiobarbituric acid-reacting substances).

In summary, we have shown that acute exhaustive exercise affects the balance of oxyradical production and antioxidant enzyme defenses within tissues involved in host immune reactions and that generated oxyradicals have impact on membrane unsaturated fatty acids, as expressed in enhanced lipid peroxide formation. These results provide further insight into the mechanism of cell damage occurring within lymphoid tissues after exercise. The contribution of lipid peroxidation and oxidative stress to the transient T-cell immunosuppression after acute exercise (8, 17) remains to be determined.

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