N-acetyl-l-cysteine protects intestinal lymphocytes from apoptotic death after acute exercise in adrenalectomized mice

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N-acetyl-l-cysteine protects intestinal lymphocytes from apoptotic death after acute exercise in adrenalectomized mice. Am J Physiol Regul Integr Comp Physiol 288: R1664–R1672, 2005; doi:10.1152/ajpregu.00843.2004.—Lymphocyte apoptosis has been observed after strenuous exercise. Both glucocorticoids (GC) and reactive oxygen species (ROS) have been suggested to contribute to exercise-induced lymphocyte apoptosis. The aims of this study were to 1) examine the direct contribution of GC during exercise-induced intestinal lymphocyte (IL) apoptosis and 2) determine the contribution of oxidative stress, in the absence of GC, to exercise-induced IL apoptosis. Mice were bilaterally adrenalectomized (ADX) and randomly assigned to receive saline (SAL) or N-acetyl-l-cysteine (NAC) 30 min before treadmill exercise (EX). EX consisted of 90 min of continuous running at a 2° slope (30 min at 22 m/min, 30 min at 25 m/min; and 30 min at 28 m/min), and then killed immediately (1 mm) or 24 h (24h) postexercise. Control mice were exposed to a nonexercised (NonEX) condition consisting of treadmill noise and vibration without running. ILs were isolated and measured for apoptotic (phosphatidylserine externalization, mitochondrial membrane depolarization, Bcl-2, caspase 3, and cytosolic cytochrome c) and oxidative stress (H2O2 and glutathione) markers. Plasma was analyzed for corticosterone (CORT) by radioimmunoassay. ADX eliminated the exercise-induced elevation in CORT but did not prevent IL apoptosis and cell loss relative to NonEX mice. In contrast, administration of NAC to ADX mice protected ILs from apoptotic cell death and inhibited post-exercise cell loss. These findings suggest that GC are not responsible for exercise-induced apoptosis and cell loss of ILs. The protective effect provided by the antioxidant NAC strongly suggest that oxidative stress is the primary pathway for IL apoptosis and cell loss after strenuous exercise.

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METHODS

Experimental animals. Female C57BL/6 mice (n = 85; body wt = 21.6 ± 0.1 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were group-housed and maintained in our vivarium at 21 ± 1°C and 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 guidelines established by the Canadian Council on Animal Care and were approved by the University of Waterloo Animal Care Committee.

Adrenalectomy. Mice received bilateral adrenalectomy (ADX; n = 73) or sham surgery (sham; n = 12). Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (128 mg/kg body wt and 8.5 mg/kg body wt, respectively) and buprenorphine (Temgesic; 0.003 mg/100 g body wt sc) was given for analgesia. ADX mice were provided drinking water containing 0.9% sodium chloride. Control mice (sham-operated) underwent the same drug (i.e., anesthetic and analgesic) and surgical procedure as the ADX group without adrenal excision. All animals were given 2–3 wk to recover from surgery.

NAC treatment. Mice were injected intraperitoneally with 1 g/kg body wt of NAC (Sigma Chemical, St. Louis, MO) dissolved in saline at 3°C and 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 guidelines established by the Canadian Council on Animal Care and were approved by the University of Waterloo Animal Care Committee.

Exercise protocol. After acclimation (15 min at 15 m/min, 2° slope, 2 days before experiments; 20 min at 20 m/min, 2° slope, 1 day before experiments) to rodent treadmill (Omni-max metabolic treadmill, Omni Tech Electronics, Columbus, OH) ADX mice were given treadmill exercise (EX) or exposed to treadmill noise and vibration without running (NonEX). EX consisted of a 10-min warmup, 90 min of continuous exercise (30 min at 22 m/min and 2° slope; 30 min at 25 m/min and 2° slope; and 30 min at 28 m/min and 2° slope) and 5 min deceleration. All mice completed the 90-min treadmill run. Although the mice were not excused from this protocol, they were clearly fatigued, as some prodding with a soft test-tube brush was necessary at the end of exercise. Experiments using a different group of mice of age and weight-matched mice (n = 10) were conducted with estimates that running at 22 m/min, 25 m/min, and 28 m/min at 0° grade corresponded to ~68%, 74%, and 80% peak oxygen consumption (\(V_{\text{O}_2}\)peak), respectively. Given that a 2° grade was used in the experimental treadmill protocol, running intensity was likely more intense than the estimates of \(V_{\text{O}_2}\)peak obtained separately using the 0° grade. ADX mice were killed immediately (Imm) or 24 h (24h) after exercise which resulted in five experimental groups: NonEX (n = 11), EX + SAL+Imm (n = 16), EX + NAC + Imm (n = 16), EX + SAL + 24h (n = 15), and EX + NAC + 24h (n = 15). SHAM mice (n = 12) served as a surgical control and were exposed to the NonEX condition. Because Sham NONEX mice were not different from ADX NONEX mice in any of the IL-dependent measures, data were pooled for analysis and presented collectively as the NONEX group (n = 23).

Lymphocyte isolation and plasma collection. Mice were killed by pentobarbital sodium overdose (0.06–0.08 g/kg ip). After removal of the heart, standard immunological procedure (34) was used to isolate ILs as performed previously (47). Blood, collected by cardiac puncture in a heparinized syringe, was centrifuged for 6 min at 1,500 g. Plasma was frozen at −20°C and stored for analysis of corticosterone.

Flow cytometry analysis and cell surface antigen expression. Analysis was performed by flow cytometry (Epics XL Flow Cytometer, Beckman Coulter, Hialeah, FL) equipped with a 488-nm excitation argon laser and emission detection filters at 525 nm (green fluorescence) and 575 nm (red fluorescence). ILs were identified by gating on the forward and sidescatter properties of lymphocytes and verified using a CD45 (common leukocyte antigen) FITC-conjugated monoclonal antibody (mAb). Only lymphocyte-gated data were used for final analysis of CD3αβ, CD3γδ, CD4, CD8αα, CD8ββ, and CD19+ cell surface antigen expression, PS externalization, propidium iodide (PI) uptake, mitochondria membrane depolarization, and intracellular hydrogen peroxide (H2O2) production. To determine cell surface antigen expression, cells (5 × 106) were resuspended in 50 μl of PBS and incubated with 0.5 μg of FITC conjugated CD45 (clone: YW62.3; Cedarlane Laboratories, Hornby, ON, Canada), CD3 (clone: GL3; PharMingen, San Diego, CA), or CD8β (clone: 53–5–8; PharMingen) mAb and PE-conjugated CD3β (clone: H57–597; PharMingen), CD4 (clone: GK1.5; PharMingen), CD8α (clone: 53–67; PharMingen), or CD19 (clone: 1D3; PharMingen) mAb in the dark at 4 °C for 45 min. After incubation, cells were washed with 500 μl PBS, centrifuged for 5 min at 450 g, and resuspended in 500 μl PBS for analysis by flow cytometry.

Phosphatidylserine externalization and propidium iodide uptake. During apoptosis PS is translocated from the inner to the outer leaflet of the plasma membrane. Since Annexin V has a high affinity for PS, fluorochrome-labeled Annexin V can be used to identify apoptotic cells (15). Annexin V+PI− cells are early apoptotic cells, whereas Annexin V−PI− cells are in a late stage of apoptosis (33). Briefly, 1 × 105 cells were suspended in 100 μl of binding buffer (PharMingen) and were incubated with 2.5 μl of Annexin V FITC-conjugated antibody (PharMingen) and 2.5 μl of 10 μg/ml PI (Sigma Chemical) in the dark for 15 min at RT. Binding buffer (400 μl) was added, vortexed, and analyzed by flow cytometry.

Mitochondrial membrane depolarization. Mitochondrial membrane depolarization in living cells can be determined using the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrachlorobenzimidacarbocyanine iodide (JC-1) lipophilic cation fluorochrome (54). Increased JC-1 green fluorescence is indicative of mitochondrial injury and decreased membrane potential and precedes morphological changes of apoptosis (19). Cells (5 × 105) were resuspended in 500 μl of PBS and incubated with 1.5 μM JC-1 dye (Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were washed, resuspended in 500 μl of PBS, and analyzed by flow cytometry. Increased JC-1 green fluorescence during apoptosis was confirmed by incubation of lymphocytes with anti-Fas (clone: Jo2) inducing antibody (PharMingen).

Intracellular hydrogen peroxide production. Intracellular hydrogen peroxide (H2O2) production was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Within living cells DCFH-DA undergoes deacetylation by cellular esterases to form 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH). After reaction with intracellular H2O2, DCFH is converted to the fluorescent 2′,7′-dichlorofluorescein (DCFH).

Intracellular glutathione determination. Intracellular glutathione (GSH) was determined using monochloroacine [MBC; 3-(CICH2CH3)-1,5-diazabicyclo[3.3.0]acta-3,6,-diene-2,8-dione]. MBC is a nonfluorescent cell-permeable bimane derivative that becomes highly fluorescent after reaction with intracellular GSH (49). Cells (5 × 105) were suspended in PBS, incubated with 50 μM MBC (Molecular Probes) in the dark at 37°C for 20 min, washed, and resuspended in PBS, and analyzed by flow cytometry.
Western blot analysis of Bcl-2, caspase 3, and cytosolic cytochrome c. ILs were gently lysed with lysis buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 0.1 mM phenylmethysulfonyl fluoride] on ice for 45 min. Cell lysates were centrifuged at 10,000 g for 15 min, supernatant was obtained and protein was determined by the BCA assay. Eighty micrograms of protein were electrophoresed on a 12% (Bcl-2 and caspase 3) or 15% (cytochrome c) SDS-PAGE gel and transferred onto a PVDF membrane (Sigma Chemical) and blocked overnight in 10% milk-TBST at 4–8°C. Membranes were incubated for 1 h with anti-Bcl-2 (clone: C2), anti-caspase 3, (clone: H-277) or anti-cytochrome c (clone: 6H2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:200 in 10% milk-TBST. Membranes were then incubated for 1 h in horseradish peroxidase-conjugated anti-mouse (Bcl-2 and cytochrome c) or anti-rabbit (caspase 3) IgG (Santa Cruz Biotechnology) at a concentration of 1:2,000 in 10% milk-TBST. Protein detection was performed using ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, U.K.) and the ChemiGenius 2 Bio Imaging System (Cambridge, U.K.). Samples from each experimental group were run simultaneously on each membrane and band densities were normalized relative to NonEX samples.

Corticosterone analysis. Plasma corticosterone (CORT) was determined using a commercially available Gamma-B 125I Corticosterone radioimmunoassay (IDS, Boldon, UK) with the recommended 1:10 dilution for rodent plasma and extraction under methylene chloride. Radioactivity was counted on a Gamma 5500 counter (Beckman).

Statistical analysis. IL data were analyzed using a one-way ANOVA by group (NonEX, EX+SAL+Imm, EX+NAC+Imm, EX+SAL+24h, EX+NAC+24h). A subset (n = 40) of intact animals (distinct from the sham-operated mice), which did not undergo the surgical procedure but underwent the same exercise condition (Intact NonEX and Intact EX) were used to compare the postexercise CORT levels to ADX mice (ADX NonEX and ADX EX). CORT data were analyzed using a 2 (ADX vs. intact) × 2 (NonEX vs. EX) factorial ANOVA. Statistical analysis was performed using SPSS (Version 11; Chicago, IL) statistical software with P = 0.05. Post hoc analysis used Tukey’s HSD test to determine difference between groups. Results are presented as group means ± SE.

RESULTS

Phosphatidylserine externalization (Annexin V) and loss of cell viability (PI). The percent Annexin V+/PI− and Annexin V+/PI+ ILs were not affected by treatment condition. The percent Annexin V+/PI− cells ranged from 7.0 ± 0.5% to 8.4 ± 0.7%, while the percent Annexin V−/PI+ cells ranged from 0.5 ± 0.1% to 0.8 ± 0.2%. However, the percent of ILs coexpressing both markers (Annexin V+/PI+) suggestive of a late stage of apoptosis, was significantly (F4,84 = 7.111, P < 0.001) affected by exercise and drug treatment. The percent of double-positive (Annexin V+/PI+) ILs was significantly (P < 0.005) higher in the EX+SAL+Imm group (28.0 ± 2.3%) compared with the NonEX group (17.7 ± 1.7%). The EX+SAL+Imm group also differed from the other treatment groups (Fig. 1A).

Mitochondrial membrane depolarization. IL mitochondrial membrane depolarization was significantly (F4,86 = 5.199, P < 0.001) affected by treatment condition (Fig. 1B). Immediately after exercise in mice receiving saline (EX+SAL+Imm), there was significantly (P < 0.05) greater mitochondrial membrane depolarization (99.8 ± 12.6 AU) compared with the NonEX (55.3 ± 9.1 AU) and the other groups. No significant differences in IL mitochondrial membrane depolarization was observed in NAC-treated mice immediately after exercise or SAL and NAC mice 24 h postexercise relative to NonEX animals. Figure 2 shows flow cytometry histograms of JC-1 green fluorescence from representative NonEX, EX+SAL+Imm, EX+NAC+Imm, EX+SAL+24h, and EX+NAC+24h animals.

Intracellular H2O2 and GSH. Intracellular H2O2 in ILs was not affected by exercise or drug condition (data not shown). IL GSH levels were significantly (F4,84 = 14.211, P < 0.001) reduced after exercise in mice receiving saline (EX+SAL+Imm) compared with all other groups. This reduction in IL GSH was not observed immediately after exercise in NAC treated or at 24 h postexercise in either drug condition (Fig. 1C).

Fig. 1. Annexin V+/PI− intestinal lymphocytes, mitochondrial membrane depolarization, and intracellular glutathione by exercise and drug condition. *Significant (P < 0.005) difference compared with all other groups (A). §Significant (P < 0.05) difference compared with all other groups (B). ¶Significant (P < 0.001) difference compared with all other groups (C). All values are means ± SE. Differences determined by Tukey’s post hoc test.
Western blot analysis. Protein levels of IL caspase 3 (F_{4,49} = 17.199, P < 0.001), cytosolic cytochrome c (F_{4,49} = 13.084, P < 0.001), and Bcl-2 (F_{4,49} = 8.528, P < 0.001) were significantly affected by treatment condition. IL protein levels of proapoptotic caspase 3 were significantly (P < 0.005) elevated immediately after exercise in mice receiving saline (EX+SAL+Imm) relative to all other conditions (Fig. 3A). Similarly, IL protein levels of cytosolic cytochrome c were significantly higher (P < 0.001) in the EX+SAL+Imm group relative to the other treatment groups (Fig. 3B). Levels of antiapoptotic protein Bcl-2 were significantly lower (P < 0.05) in the EX+SAL+Imm group relative to all other groups (Fig. 3C). Alterations in protein levels in all three apoptotic indices were not observed immediately after exercise in NAC-treated (EX+NAC+Imm) or 24 h postexercise (EX+SAL+24h and EX+NAC+24h) animals.

Intestinal lymphocyte counts and phenotype distribution. The percentage of CD3\(\alpha\beta\)^+ cells was significantly affected by treatment condition (data not shown). A significantly decreased percentage of CD3\(\gamma\delta\)^+ (F_{4,80} = 2.919, P < 0.05) and increased percentage of CD4^+ (F_{4,80} = 2.493, P < 0.05) ILS occurred as a function of drug and exercise. The percentage of CD3\(\gamma\delta\)^+ ILS was significantly lower (P < 0.05) in the EX+SAL+Imm group (24.5 ± 2.3%) compared with EX+NAC+24h group (31.6 ± 1.9%). The percentage of CD4^+ ILS was significant (P < 0.05) higher (16.6 ± 2.0%) compared with EX+SAL+24h group (10.8 ± 0.9%).

Total IL cell count was significantly (F_{4,84} = 3.754, P < 0.01) affected by exercise condition (Fig. 4A). Significantly fewer ILS were isolated 24 h after exercise in mice receiving saline (EX+SAL+24hr; 26.6 ± 2.1 × 10^6) compared with the NonEX animals (45.1 ± 3.3 × 10^6). The significant decrease in total IL cell numbers 24 h postexercise was not observed in mice receiving NAC (EX+NAC+24h) nor immediately after exercise compared with the NonEX group. Similarly, there was a significant reduction in the absolute number of CD3\(\alpha\beta\)^+ (F_{3,80} = 3.790, P < 0.01; Fig. 4B), CD3\(\gamma\delta\)^+ (F_{3,80} = 2.705, P < 0.05; Fig. 4C), CD8^+ (F_{3,80} = 4.003, P < 0.01; Fig. 5A), CD8^+ (F_{3,80} = 3.925, P < 0.01; Fig. 5B), and CD4^+ (F_{3,80} = 3.361, P < 0.05; Fig. 6A) ILS 24 h after exercise in mice receiving saline (EX+SAL+24h) compared with the NonEX mice. A decrease in phenotype-specific numbers was not observed 24 h postexercise in mice receiving NAC (EX+NAC+24h) nor immediately after exercise compared with NonEX mice. The absolute number of CD19^+ ILS was not affected by treatment condition (Fig. 6B).

Corticosterone analysis. ADX NonEX and ADX EX mice had significantly (P < 0.01) reduced circulating CORT levels relative to the Intact NonEX comparison group. Furthermore, the postexercise elevation (P < 0.001) in plasma CORT observed in intact mice after exercise (Intact EX) was completely abolished in ADX EX mice (Fig. 7).

DISCUSSION

Several hallmark features of apoptosis were observed in ILS after 90 min of strenuous treadmill exercise in ADX mice suggesting that GC are not directly responsible for postexercise IL cell death and cell loss. These findings support previous results that mifepristone (48) and ADX (24) do not inhibit exercise-induced IL cell loss. Further, because of the depletion of intracellular GSH and the protection provided by NAC, this study also shows that oxidative stress plays a major role in exercise-induced IL apoptosis and cell loss. The protective effect of antioxidants on exercise-induced apoptosis has been reported in mouse intestinal lymphocytes (47), mouse thymocytes (35), and human peripheral blood leukocytes (21).
Results from this study indicate 1) a significant increase in IL mitochondrial membrane depolarization, 2) a significant cytochrome c release into the cytosol, 3) an elevated proapoptosis caspase 3 protein level, 4) a decreased antiapoptotic Bcl-2 protein level, and 5) a greater PS externalization immediately after exercise with an ensuing reduction in total, CD3αβ⁺, CD3γδ⁺, CD8αβ⁺, CD8αα⁺, and CD4⁺ ILs 24 h postexercise. We and others reported that exercise stress increases caspase 3 activity (45), intracellular Ca²⁺ (4, 42), PS externalization (41, 55, 23), and chromatin condensation (32), and it induces DNA fragmentation (13, 35) in human and rodent leukocytes. Increased Fas receptor density and FasL expression has also been observed in poorly trained athletes after a marathon, as well as during an exhaustive treadmill bout (43). A common feature of immune cell apoptosis after exercise is alterations in mitochondrial function, as demonstrated by increased mitochondrial membrane depolarization, increased proapoptotic cytosolic cytochrome c and decreased antiapoptotic Bcl-2 protein levels immediately after exercise. Decreased mitochondrial membrane potential occurred in human peripheral blood leukocytes, monocytes, and lymphocytes after repeated aerobic exercise (27) and rat neutrophils after 1 h of

Fig. 3. Protein levels of intestinal lymphocyte caspase 3, cytosolic cytochrome c, and Bcl-2. §Significant ($P < 0.005$) difference compared with all groups (A). ¶Significant ($P < 0.001$) difference compared with all groups (B). *Significant ($P < 0.05$) difference compared with all groups (C). All values are presented as group means ± SE. Differences determined by Tukey’s post hoc test.
Intense treadmill running (32). Intense exercise also increased the expression of the proapoptotic genes Bax and Bcl-x{s}, and decreased the expression of the antiapoptotic gene Bcl-x{L}(32). GC contribute to apoptosis in various lymphoid compartments (14), including the intestine. Brunner et al. (7) found similar levels of apoptosis in both CD8{αβ} and CD8{αα} ILs following ex vivo culture, an effect enhanced by in vivo dexamethasone exposure or inhibited by ADX or mifepristone (RU-486) treatment. CD8{αα} IL apoptosis and cell loss caused by burn injury in mice was reversed by pretreatment with mifepristone (17). In vitro and in vivo dexamethasone treatment, as well as water immersion stress sufficient to cause a 10-fold increase in plasma corticosterone caused IL DNA fragmentation and cell loss. Although all IL subsets were susceptible to apoptotic cell death, the CD8{αα} ILs were more resistant (44). In contrast, GC treatment in mice had no effect on either IL numbers or IL apoptosis but dramatically increased thymocyte cell death (60, 61); this was hypothesized to be due to the higher expression of the antiapoptotic protein, Bcl-2, in ILs relative to thymocytes.

GC may mediate lymphocyte apoptosis and cell loss after strenuous exercise. For example, pretreatment of rats with a steroid receptor antagonist (mifepristone) decreased thymocyte DNA fragmentation after 2.5 h of exercise at 20 m/min and 10% incline (13). In contrast, ADX does not inhibit exercise-induced IL cell loss in mice after intense exercise (24). The present study supports these earlier findings that IL loss and several hallmark features of apoptosis were not inhibited by ADX. The lack of involvement of GC during the exercise-induced lymphocyte apoptosis and cell loss may reflect the low but still significant increase in plasma GC (~100 ng/ml) induced by our exercise protocol compared with other more traumatic protocols, which result in extreme plasma GC levels (~600–700 ng/ml) such as water immersion stress and burn injury (18, 44).

Several lines of evidence suggest that oxidative stress is a key pathway for IL apoptosis after exercise. Firstly, strenuous exercise leads to significant decreases in IL GSH levels that occur concurrently with increased mitochondrial membrane depolarization and PS externalization; these effects were inhibited by provision of the antioxidant NAC (47). Secondly, polyethylene glycol, which inhibits ROS generation and lipid peroxidation (36), also inhibited IL cell loss after strenuous exercise (48). Finally, plasma 8-iso-prostaglandin F{2α} levels (a measure of oxidative stress), are correlated to IL loss after exercise (23). Moreover, oxidative stress during exercise is known to affect apoptosis and cell loss of immune cells from intense treadmill running (32). Intense exercise also increased the expression of the proapoptotic genes Bax and Bcl-x{s}, and decreased the expression of the antiapoptotic gene Bcl-x{L}(32). GC contribute to apoptosis in various lymphoid compartments (14), including the intestine. Brunner et al. (7) found similar levels of apoptosis in both CD8{αβ} and CD8{αα} ILs following ex vivo culture, an effect enhanced by in vivo dexamethasone exposure or inhibited by ADX or mifepristone (RU-486) treatment. CD8{αα} IL apoptosis and cell loss caused by burn injury in mice was reversed by pretreatment with mifepristone (17). In vitro and in vivo dexamethasone treatment, as well as water immersion stress sufficient to cause a 10-fold increase in plasma corticosterone caused IL DNA fragmentation and cell loss. Although all IL subsets were susceptible to apoptotic cell death, the CD8{αα} ILs were more resistant (44). In contrast, GC treatment in mice had no effect on either IL numbers or IL apoptosis but dramatically increased thymocyte cell death (60, 61); this was hypothesized to be due to the higher expression of the antiapoptotic protein, Bcl-2, in ILs relative to thymocytes.

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other compartments. Postexercise leukocytopenia in control rats was not evident in rats given NAC or GSH before exercise (3). Plasma nitric oxide metabolites and lipid peroxidation produced by marathon running was significantly correlated to human peripheral leukocyte DNA base oxidation (59). Similarly, Steensberg et al. (55) found increased levels of 8-isoprostaglandin F$_{2\alpha}$ and blood lymphocyte PS externalization after 2.5 h of treadmill running. Exercise-induced thymocyte DNA fragmentation was prevented by the antioxidant butylated hydroxyanisole (35), whereas human peripheral leukocyte DNA damage observed after exhaustive treadmill running was blocked by vitamin E (21). Thus these studies provide evidence in support of the hypothesis that oxidative stress arising from intense exercise may be the common pathway for lymphocyte apoptosis and necrosis, and, possibly, this may contribute to the well-documented leukocytopenia after exercise.

Our results also showed that many features of apoptosis (i.e., caspase activation, downregulation of Bcl-2, cytochrome c release, mitochondrial membrane depolarization, and PS externalization) coincided with decreases in intracellular GSH levels immediately after exercise. Decreased GSH stores and these cellular indicators of apoptosis were reversed by NAC treatment. NAC is hydrolyzed to release cysteine, the rate limiting substrate in GSH synthesis (52). Since GSH is the major determinant of intracellular redox potential, maintenance of intracellular GSH is vital during apoptosis (20). Administration of NAC maintains thymocyte GSH levels and inhibits loss of mitochondrial membrane potential, PS externalization, cytochrome c release, and caspase 3 activity (37, 57) as well as maintaining lymphocyte Bcl-2 levels (50) after induction of apoptosis. The lack of H$_2$O$_2$ production in our results likely reflects the fact that ROS are short-lived and difficult to measure (30). Moreover, most apoptotic cells were in a late stage of apoptosis (i.e., double positive; Annexin V$^+$/PI$^+$) and rodent thymocytes in a late stage of apoptosis do not produce ROS probably due to reduced metabolism (58). These results indicate that intracellular antioxidant status plays an important role during lymphocyte cell death, including exercise-induced apoptosis. Moreover, the results support the conclusion that oxidative stress and not GC are responsible for exercise-induced IL apoptosis.

It has been established that dysregulation of intestinal T lymphocyte apoptosis can lead to disease. Accumulation of reactive lymphocytes due to insufficient apoptosis results in chronic intestinal inflammation, a common feature of Crohn’s disease and ulcerative colitis (28). In contrast, intestinal mucosal barrier function may be compromised by excessive lymphocyte apoptosis (17), allowing endotoxin translocation from the gut into circulation (26). Endotoxemia has been reported after strenuous exercise (10, 29), and antioxidant supplementation inhibited the postexercise endotoxemia, possibly by maintaining luminal membrane integrity from free radical damage (2). It remains to be determined whether exercise-induced IL apoptosis compromises intestinal barrier functions and/or promotes endotoxin translocation after strenuous exercise. Maintenance of intracellular antioxidant levels by exogenous antioxidant supplementation may protect against postexercise apoptosis and lead to recovery of mucosal barrier function from the potential damaging effect of strenuous exercise. Strategies to enhance the mucosal immunity in the gastrointestinal tract could be especially important during the “open window” period of immune dysregulation experienced by some athletes after intense exercise.

In conclusion, the major finding of this study was that GC are not responsible for exercise-induced apoptosis and loss of ILs. If GC were the primary pathway involved, we would have expected no apoptosis or cell loss after exercise in ADX mice. We did not observe a protective effect by ADX and found that the antioxidant NAC inhibited IL GSH depletion and apoptosis after exercise. These findings strongly suggest that oxidative stress is responsible for exercise-induced IL apoptosis and cell loss.
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