Treadmill exercise training blunts suppression of splenic natural killer cell cytolyis after footshock

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Departments of 1Exercise Science, 2Psychology, and 4Medical Microbiology, University of Georgia, Athens, Georgia 30602-6554; and 3Division of Medical Neurosciences, Walter Reed Army Institute of Research, Washington, DC 20307-0002

Dishman, R. K., J. M. Warren, S. Hong, B. N. Bunnell, E. H. Mougey, J. L. Meyerhoff, L. J aso-Friedmann, and D. L. Evans. Treadmill exercise training blunts suppression of splenic natural killer cell cytolyis after footshock. J Appl Physiol 88: 2176–2182, 2000.—This study extended to treadmill exercise training our prior report (Dishman RK, Warren JM, Youngstedt SD, Yoo H, Bunnell BN, Mougey EH, Meyerhoff JL, aso-Friedmann L, and Evans DL. J Appl Physiol 78: 1547–1554, 1995) that activity wheel running abolished the suppression of footshock-induced natural killer (NK) cell cytolyis. Twenty-four male Fischer 344 rats were assigned to one of three groups (n = 8, all groups): 1) a home-cage control group, 2) a sedentary treatment group, or 3) a treadmill-running group (0° incline, 25 m/min, 35 min/day, 6 days/wk). After 6 wk, the treadmill and sedentary groups received 2 days of footshock. Splenic NK cytotoxicity was determined by standard 4-h51Cr release assay. Percentages of lymphocytes were determined by flow cytometry. Plasma levels of ACTH, corticosterone, and prolactin concentration were measured by radioimmunoassay. After footshock, percentage of lysis relative to home-cage controls was 40% and 80% for sedentary and treadmill-trained animals, respectively (P < 0.05). Our results indicate that the protective effect of chronic exercise on innate cellular immunity in the Fischer 344 male rat is not restricted to activity wheel running, nor is it explained by elevations in basal NK activity, increased percentages of splenic NK and cytotoxic T cells, or increased plasma levels of ACTH, corticosterone, and prolactin.

lymphocytes; ACTH; corticosterone; prolactin

NATURAL KILLER (NK) cells are important in immunosurveillance against spontaneously arising tumors, blood-borne metastasizing tumor cells, acquired immune deficiency syndrome, and certain viral, bacterial, and protozoan infections (50). Because the lysis of many microorganisms, virus-infected cells, and tumor cells, without prior exposure to or recognition of the major histocompatibility complex, is fundamental for a host’s resistance to infection, it is important to establish whether chronic exercise can influence NK cell cytolyis, as reported in human blood-sampling studies (16, 31, 38, 55). Those studies are difficult to interpret because of the migratory flux of lymphocytes between blood and lymph tissues. Moreover, the studies were restricted to measures of basal NK activity. A biologically plausible explanation of how basal NK activity is altered after moderately intense chronic exercise, as used in past studies of humans, has not been elucidated (38, 55).

Studies of rodents permit the usage of procedures that cannot be ethically performed on healthy humans, including 1) sampling of NK activity in lymphatic tissues other than blood and 2) experimental manipulations of plausible mechanisms of innate cellular immunity. Several studies of NK activity in mice and rats after chronic activity wheel running or treadmill training have found increased in vivo NK cytolyis in the lung (25, 26, 32, 33) and increased (4, 32, 33), reduced (3, 5), or unchanged (10, 17, 37) in vitro NK cytolyis in the spleen. However, those studies were limited to the analysis of basal NK activity. Another approach to the study of chronic exercise and NK activity involves the coincident study of NK suppression induced by nonexercise conditions (9, 10). This approach permits the generalization of increased NK activity after chronic exercise to be tested by exposing exercise-trained animals to a novel stressor that is known to suppress NK activity. Such an approach permits testing of the hypothesis that exercise training confers an immunoprotective effect as a result of a generalized cross-stressor adaptation in neural and/or neuroendocrine modulation of the innate immune system (45). Footshock reliably leads to a 25–50% acute suppression of in vitro splenic NK activity in rats that persists for 24 h (8–10, 41, 42, 52). Using this approach, we previously reported that 6 wk of circadian activity wheel running abolished the suppression of splenic NK activity induced by 2 days of repeated footshock without affecting basal NK cytolyis (10).

That study did not determine whether the results were explained by adaptations to chronic physical exertion rather than to a cage environment that was enriched compared with that for standard rat husbandry. Activity wheel running appears to be a motivated circadian behavior among rats (43); therefore, it might have a positive immunomodulatory effect through psychophysiological mechanisms (21, 22, 52) that is independent of physiological adaptations to physical activity. The use of treadmill running permits the
addition of a standard exercise stimulus beyond that of normal circadian physical activity, which, in our experience, does not increase physical fitness in the Fischer 344 strain, as determined by the oxidative capacity in locomotory skeletal muscle (10).

Thus the present study extended our test to treadmill exercise training. Although prior studies of mice have reported that immune responses to exercise training were similar after treadmill or activity wheel running (32, 33), those studies only examined basal NK activity. Determining whether exercise training results in a cross-stressor adaptation in innate immunity requires that responses to a nonexercise stressor be measured as well. Although treadmill exercise can confound exertion with emotional stress during novel or early sessions of exposure, rats adapt to treadmill training after 6 wk, as indicated by hypothalamic-pituitary-adrenal (HPA) cortical hormone responses after running (53).

The primary purpose of this study was to determine whether treadmill exercise training would attenuate the suppression of splenic NK cytotoxicity induced by repeated bouts of uncontrollable footshock. Because altered cytotoxicity after footshock could result from changed proportions of cytotoxic lymphocytes and NK cells (18, 28), we estimated splenic NK, B lymphocyte, and T lymphocyte cell populations using flow cytometry. Also, exercise training could affect basal NK cell percentage and activity in conditions without stress, thus confounding direct comparisons of treadmill-trained and sedentary animals after footshock. Therefore, a study replicating the treadmill exercise training and the sedentary groups without footshock was performed.

Mechanisms explaining the effects of exercise on NK activity in rats are not known. Although sympathetic nerve activity appears to modulate splenic NK activity (9, 12), evidence also suggests that splenic NK cell activity or number is modulated by HPA cortical responses to stress (1, 7, 27). Adrenal corticoids influence distribution of T lymphocytes so that the percentage of NK cells would be increased (1). Studies of humans report that glucocorticoids suppress (14, 19) or have no effect (39, 48) on blood NK activity, whereas prolactin mitigates corticosterone’s suppressive effects (2). It is plausible that the downregulation of the HPA system, a characteristic of adaptations to chronic exercise of moderate intensity (47), might lead to a cross-stressor attenuation of NK suppression. Although we previously reported that plasma levels of HPA hormones did not explain blunted NK suppression after footshock among chronic activity wheel runners (10), we reexamined those hormones in this study. We have found that the ACTH response to novel footshock is not affected after 6 wk of activity wheel running (10); however, it is augmented after 6 wk of treadmill exercise training (54).

METHODS

Subjects

Fischer 344 rats (n = 48, age ~ 45 days, mass ~ 165 g) were obtained from Charles River (Raleigh, NC) and allowed to adapt to the vivarium for 2 wk. The animals were housed in individual cages in a vivarium maintained at 22 ± 2°C with a 12-h light-dark cycle, starting at 7 am. Water and lab chow (Ralston Purina, St. Louis, MO) were available ad libitum. Animals were handled daily and weighed once a week throughout the course of the study. At an age of 60 days, animals were randomly assigned to either a sedentary group (n = 8) or a treadmill exercise training group (n = 8), each receiving uncontrollable footshock, or to a second sedentary group (n = 8) that did not receive footshock and that acted as a home-cage control. To determine the effects of treadmill exercise training on basal NK activity, another 14 rats were randomly assigned to treadmill exercise training (n = 7) or sedentary (n = 7) conditions without exposure to footshock.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205).

Experimental Procedures

A randomized factorial design (treadmill exercise vs. sedentary) was used for hypothesis testing. A randomly assigned third group was sedentary and did not receive footshock (home-cage control); it provided baseline values on the dependent variables for control comparisons. A one-way randomized design (treadmill exercise vs. sedentary) without footshock was used for the nonshock control study. The dependent measures included percent specific lysis of YAC-1 cells determined by a standard 4-h 51Cr release assay at five different effector-to-target ratios (E/T) (200, 100, 50, 25, and 12.5:1); percentages of OX6+ (B), OX8+ (Th), Thy-1.1, W3/25+ (Ts), and T lymphocytes (NK) cells were determined by flow cytometry; and plasma concentrations of ACTH, corticosterone, and prolactin were determined by radioimmunoassay.

Treadmill exercise training. After 2 wk in the vivarium, treadmill training began with familiarizing the rats with a Stanhope 2000 (Davis, CA) motor-driven treadmill. Each animal was placed in 1 of 20 running compartments for 15–20 min on each of 2 days. The rats then ran at a low speed (5–10 m/min at 0° incline) for 5 min. The running times were gradually increased from 5–10 min during 1 wk. On each of the 2 days, running performance was rated on a scale of 1–5, with 5 being the best rating (53, 54). At the end of the trial period, the animals receiving a mean rating ≥3 were randomly assigned to the experimental groups. Poor runners (n = 10) that consistently scored <3 on the running performance scale were excluded from the study to minimize dropouts during training and to optimize the group exercise training effect. Animals assigned to the treadmill exercise training condition ran at 0° incline 6 days/wk for 2 wk, during which the running time was increased from 10 to 35 min/day and treadmill speed was gradually increased from 15 to 25 m/min. The animals maintained this regimen (35 min/day, speed = 25 m/min, 6 days/wk) for an additional 5 wk. Electric shock was not used to promote running performance. The protocol elicits a significant increase in the oxidative capacity of locomotory muscle, as measured by the activities of oxidative enzymes in slow- and fast-oxidative glycolytic muscle fibers (11). In the present study, we measured the activity of citrate synthase in soleus muscle. Animals ceased treadmill running 36 h before footshock and death.

Footshock protocol. After 6 wk, animals assigned to the treadmill training and sedentary footshock groups were matched, according to mass, into pairs. Two sessions of
footshock testing, separated by 24 h, occurred. Animals that received footshock were transported from their home cages to a testing room 10 m from the vivarium immediately before footshock. They were immediately returned to their home cages after the first session of footshock. Each animal was unrestrained in an individual Skinner box (28 × 20 × 21 cm) and received repeated 2 mA of scrambled footshock, for a total of 6 min of shock delivered during a 20- to 50-min period. The duration and frequency of shocks were selected to permit comparison of results with our earlier report on footshock after activity wheel running (10). The shock duration ranged from 2–30 s, with a 35-s interval between shocks. Duration was equal for both members of each pair of treadmill-trained and sedentary rats.

Tissue collection. After the second acute session of the 2-day footshock protocol, each animal was returned to its home cage. The animal was transported to an adjacent room 30 min later and killed by decapitation using a guillotine. Suppression of NK activity was previously reported to occur 1–4 h after novel footshock (42, 52). Animals that were not footshocked were transported from their home cages immediately before decapitation. Heparinized trunk blood was chilled on crushed ice for ~1 h before being centrifuged at 2,000 g. The available plasma was pipetted into collection tubes containing 50 µl of aprotinin and stored at −70°C. Spleens were removed immediately after decapitation and were maintained in RPMI 1640 (Flow Laboratories, Rockville, MD) containing 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, NY) within polypropylene vials kept at room temperature until the cytotoxicity assay was performed 1–2 h later. Soleus muscle was removed from the left hindlimb, quick frozen in liquid nitrogen, and stored at −70°C.

Cytotoxicity and Flow Cytometry

Reagents and media. Cell lines were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum at 37°C in 6% CO2 during the cytotoxicity assay. Na251CrO4 was purchased from Amersham (Arlington Heights, IL), and the fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and IgM were purchased from Sigma Immunochemicals (St. Louis, MO). The procedure for the ACTH assay with a commercial kit (Ictstar, Stillwater, MI, no. 24310) was described previously (36). Assay sensitivity was 5 pg/ml. The within-assay coefficient of variation was 5% and 10% of 33 pg/ml.

Radioimmunoassay

A radioimmunoassay technique was used to determine the plasma concentrations of the HPA cortical hormones. Radioimmunoassay for corticosterone was performed using an antibody produced in rabbits at Walter Reed Army Institute of Research (35). The sensitivity of the assay was 2.0 µg/100 ml plasma. The intra- and interassay coefficients of variation were 5% and 10%, respectively. Materials for the assay of prolactin were provided by the National Institutes of Health, through the Rat Pituitary Hormone Distribution Program. The sensitivity for the prolactin assay was ~0.8 ng/ml. The intra- and interassay coefficients of variation were 8% and 12%, respectively.

Spectrophotometry

Citrate synthase activity was assayed to determine whether the treadmill exercise training increased the oxidative capacity of locomotor muscle. The soleus muscle was homogenized in a 100 mM K2HPO4 and 10 mM glutathione buffer (pH 7.4). Citrate synthase activity was measured spectrophotometrically (Bausch and Lomb Spectronic model 1001) at 412 nm and 30°C (46).

Statistical Analysis

We used SPSS Windows (SPSS, version 9.0, Chicago, IL) for our statistical procedures. Percentage of specific lysis was compared among groups across the five E/T, using a mixed-model ANOVA with the E/T repeated. Sphericity adjustments were made using Huynh-Feldt e. Effects for plasma hormones and percentages of splenic lymphocytes were determined using one-way ANOVA across groups for each dependent variable. Duncan’s post hoc tests were conducted at P < 0.05.
Group comparisons of percentage of NK cells in the control study, body mass, and citrate synthase activity were made using a t-test for independent samples. Missing observations and data points exceeding the criterion of Grubb and Beck (15) for outliers were ≤4% of all observations and were replaced by the cell mean for each variable. Sample size was based on an expected effect size of 1.0 standard deviation (SD). Eight animals per cell provided a power of 0.80 at an α of P < 0.05. Values are reported as means ± SD in the text and means ± SE in Figs. 1–3.

RESULTS

The suppression of NK cell cytotoxicity observed among sedentary animals after repeated footshock was blunt by treadmill exercise training. After footshock, NK activity was ~80% of control for the treadmill training group, but it was 40% of control for the sedentary group. The percentage of NK cells compared with total lymphocytes did not differ among groups. Plasma levels of ACTH, corticosterone, and prolactin were elevated above home-cage control levels after footshock but did not differ among the experimental groups. In the control study without footshock, treadmill-trained animals did not differ from sedentary controls on NK activity, percentage of NK cells compared with total lymphocytes, or plasma levels of ACTH, corticosterone, and prolactin. NK activity was ~95% of sedentary controls, indicating that 6 wk of treadmill exercise training did not elevate basal NK activity.

Cytotoxicity. Repeated-measures ANOVA of percentage of specific lysis across the E/T indicated an expected increase in NK activity with increasing E/T [F (4, 84) = 91.2, ε = 0.44, P < 0.001], a group effect, [F (2, 21) = 6.82, P = 0.005] and a group × E/T effect, [F (8, 84) = 5.35, P < 0.001]. Post hoc tests indicated that the sedentary unshocked controls and the footshocked treadmill group had higher percentages of specific lysis at each E/T compared with the sedentary footshock group, and this difference was even greater at E/T of 100:1 and 200:1 (P < 0.05; See Fig. 1).

A similar increase in the percentage of specific lysis across increasing E/T also occurred in the control study when the nonshocked treadmill-trained and sedentary groups were compared on basal cytotoxicity [F(4, 48) = 50.59, ε = 0.83, P < 0.001]; however, there was no group effect [F(1, 12) = 0.264, P = 0.772] (Fig. 2).

Lymphocyte subsets. Percentages of B, Pan T, Th, TSc, and NK cells did not differ among experimental groups (P > 0.23–0.68) (Table 1). Percentages of NK cells in NWNA cells also did not differ between the sedentary (75.29 ± 1.5%) and treadmill-trained (74.7 ± 3.5%) groups that did not receive footshock in the control study (t[12] = 0.402, P = 0.695).

Hormones. Group effects were found for plasma levels of ACTH [F(2, 20) = 4.94, P = 0.018] and corticosterone [F(2, 20) = 6.81, P = 0.006]. Post hoc tests indicated that the levels in sedentary and treadmill groups, respectively, of ACTH (103.6 ± 15.5, 106.75 ± 13.3) and corticosterone (16.2 ± 2.0, 20.85 ± 4.3) were elevated (P < 0.05) compared with levels of ACTH (60.25 ± 5.1) and corticosterone (4.0 ± 3.2) in the home-cage control group that was not shocked. Levels of prolactin in sedentary (8.3 ± 3.7) and treadmill-trained groups (9.2 ± 1.6) were not significantly higher compared with the home-cage control group (2.6 ± 0.76) [F(2, 20) = 2.59, P = 0.10]. Treadmill exercise training had no effect on plasma hormone levels after footshock (Fig. 3).

Body mass and citrate synthase activity. By week 6 of the experiment, sedentary animals were ~7% heavier.
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## DISCUSSION

Our results indicate that treadmill exercise training protects against the suppression of splenic NK activity induced by footshock. This finding extends prior reports (32, 33) of increased murine basal splenic NK cell numbers and activity after chronic activity wheel or treadmill running by demonstrating a protective crossstressor attenuation of NK suppression that is independent of changes in basal NK activity. Rats that were treadmill exercise trained for ~6 wk, but did not receive footshock, had NK activity and percentages of NK cells that did not differ from sedentary control animals. We believe the attenuation of NK suppression after footshock observed in the treadmill training group is specific to single cell activity and not to a differential migration of lymphocytes, as percentages of the lymphocyte subsets did not differ between groups. An assay of single cell activity is needed to confirm this view.

The treadmill group had lower body mass than the sedentary animals by the end of the study, but we did not assess body composition or control food intake. Thus the role of energy balance on splenic NK activity cannot be determined from our study or from prior studies in this area (32, 33). Like us, Nasrullah and Mazzeo (37) reported that 15 wk of treadmill exercise training had no effect on basal NK cytotoxicity in 8-mo-old male Fischer 344 rats, despite their lower body mass and higher citrate synthase activity in soleus muscle when compared with sedentary rats. Other investigators have reported that male mice had increases in basal splenic NK activity that were similar after chronic activity wheel running and treadmill training, but only treadmill training led to increased peak oxygen uptake (33) or increased activity of citrate synthase in soleus muscle (32).

Footshock was administered during the early diurnal photoperiod, when basal cortisol levels were low, to optimize the cortisol response to stress. Although mitogenic lymphocyte proliferation after footshock is equivalent during the early diurnal and nocturnal periods of the day in Fischer 344 male rats (29), we are unaware of studies examining whether NK activity after footshock differs according to time of day. The treadmill exercise training also was diurnal. In our experience, fitness adaptations to the protocol we used do not differ according to time of day, but, as far as we know, the interaction of the light-dark cycle with neuroendocrine and immune responses to treadmill training has not been previously reported.

The mechanisms underlying the effects of physical activity on NK cell activity are not clarified by our observation that the activity wheel and sedentary groups did not differ on plasma levels of ACTH, corticosterone, and prolactin. We reported similar results in a comparison between sedentary Fischer 344 males and those having 24-h access to activity wheels for 6 wk (10). The relation between neuroendocrine and immune responses after chronic exercise has not been well described. Our observations are consistent with past findings that intracerebral, but not intraperitoneal, injections of antibodies for corticotropin-releasing factor abolish the suppression of splenic NK activity after footshock in Wistar rats without affecting blood ACTH and corticosterone levels (22). The lack of association observed between hormone levels and NK activity in the present study is limited to one data point taken 30 min after footshock. Samples of hormonal responses taken during, immediately after, and 24 h after repeated footshock would more fully address the possible neuroendocrine effects on NK activity. For example, infusion of cortisol (5 µg/kg) for 1–5 h did not affect NK
activity in lymphocytes taken from human blood (48). In another report (39), NK activity increased 4 h after, but decreased 24 h after, a 300-mg infusion of hydrocortisone. The NK activity paralleled the decrease and increase, respectively, of the percentage of T lymphocytes, suggesting an increase in percentage of NK cells. Glucocorticoid modulation of NK activity might differ according to lymph compartment and between humans and rats. Also, studies should examine whether activity wheel training affects regulatory responses by corticotropin-releasing factor on sympathetic responses to footshock or alters the sensitivity of NK cells to glucocorticoids and prolactin (50).

Alternative explanations for a cross-stressor attenuation in NK activity after treadmill exercise training might involve brain and splenic noradrenergic and opioidergic systems. In rats, brain opioids such as β-endorphin and metenkephalin can both inhibit and increase brain noradrenergic activity (13) and modulate splenic NK activity in response to conditioned electric shock (8). NK suppression after footshock is prevented by the opiate antagonists naloxone and naltrexone (41, 42), and it is mimicked by injection of morphine into either the peripheral circulation or the lateral ventricle of the brain, in the area of the central gray (51). Neuropeptide Y (NPY), which is colocalized with peripheral noradrenergic neurons, might also influence NK cell activity. In one report, NK activity was inversely related to plasma levels of NPY (20), which is coreleased with catecholamines during exercise in men (30) and with norepinephrine during footshock in rats (6, 56). Immobilization stress also increases splenic norepinephrine release and suppresses NK activity in rats (44). We have observed that treadmill exercise training leads to augmented ACTH responses to both footshock and immobilization in female rats (53, 54), so it will be interesting to determine whether treadmill exercise training has a protective effect against NK suppression after immobilization stress that is similar to our current observation after footshock.

The effects of neuropeptides and catecholamines on NK percentage and cytotoxicity may differ according to the lymph compartment and between humans and rats. Plasma catecholamines appear to increase migration of NK cells into the blood circulation (34); thus the increase in NK activity after infusion of epinephrine in humans might be explained by increased percentages of NK in circulating blood (48, 49). In contrast, chemical sympathectomy and β-adrenoreceptor blockade abolished and blunted, respectively, the suppression of rat splenic NK activity induced by intracerebroventricular injection of corticotropin releasing factor, which has an excitatory effect on the rat brain noradrenergic system (21).

We conclude that treadmill exercise training protects against the suppression of splenic NK activity induced by footshock in young male Fischer 344 rats. Similar to our earlier report on activity wheel running (10), this protective effect was not explained by elevations in basal NK activity or increased percentages of NK or cytotoxic T cells. Whereas activity wheel running might exert a protective effect by enriching the cage environment beyond that of standard rat husbandry, the blunting of NK suppression after footshock by progressive exercise training leading to an increase in fitness level indicates that physical activity has an independently protective cross-stressor immunomodulatory effect. The hypothesized influence of HPA responses on NK activity was not supported. We recommend that responses by splenic norepinephrine and NPY to treadmill exercise running and footshock be examined as possible explanations for our observations.

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