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Effect of estradiol and exercise on lymphocyte proliferation responses in female mice

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Abstract

Many North American women are encouraged to take estrogen replacement therapy and to begin (or continue) physical activity during the postmenopausal years. This study investigated the effect of estradiol exposure and physical exercise on lymphocyte proliferation responses to T and B cell mitogens in female mice. Sixty C57BL/6 mice were randomized to hormone and exercise treatment conditions: hormone treatment consisted of estradiol in vivo (71.4 μg estradiol per day for 21 days) or placebo pellet following bilateral ovariectomy, or surgical sham (mice were not ovariectomized). Exercise consisted of a single forced treadmill run (26 m/min, 6° slope, 90 min) or a sedentary control condition. Outcomes were thymic and splenic lymphocyte proliferation responses to the mitogens concanavalin A (ConA, 1.0 and 5.0 μg/mL concentrations) and lipopolysaccharide (LPS, 0.5, 1.0, and 2.0 μg/mL concentrations). In the thymus, there was a significant reduction of proliferation to ConA in the Ovx + E2 animals relative to the other conditions at both concentrations of mitogen. At 1.0 μg/mL concentration, there was a significant interaction of hormone and exercise treatments. Sham (control) mice given exercise had a higher proliferation response relative to sedentary counterparts, whereas E2 mice did not differ in proliferation responses, irrespective of exercise condition. In the spleen, exposure to high concentrations of estradiol was associated with reduced proliferation responses to both mitogens; there were, however, no main or interaction effects of exercise. These results suggest that high levels of estradiol exposure following ovariectomy in mice significantly reduces lymphocyte blastogenesis responses, and that thymic immunomodulation after acute exercise is masked by the hormonal effect.

Keywords: Estrogen; Treadmill running; Thymus; Spleen; Mitogenesis

1. Introduction

There is a growing body of epidemiological and clinical evidence that suggests that female sex hormones influence immune responses, and may contribute to the etiology of immune-mediated diseases [1–3]. However, experimental evidence on the modulation of functional cellular immune responses by female hormones has been inconsistent. Reports of the in vivo effects of estrogen and T lymphocytes indicate suppression and enhancement of functions. For example, Bonello and colleagues [4] reported lower peripheral blood lymphocyte (PBL) responses to the T cell mitogen, phytohemagglutinin (PHA), in postmenopausal women taking estrogen therapy compared with those not taking estrogen. In contrast, hormone replacement therapy (HRT) significantly enhanced PBL responses to PHA and concanavalin A (ConA) in postmenopausal women [5]. The percentage of CD4+/CD45RO+ T lymphocytes of 10 healthy postmenopausal women on HRT was lower compared to controls [6].

Results of studies on the in vitro effects of estradiol on lymphocyte function have been equally problematic. Athreya et al. [7] assessed physiological to pharmacological concentrations of estrogen on PBL responses of healthy males to PHA; estrogen did not have any consistent effect on the response to mitogens. In contrast, in vitro pharmacologic doses of 17-β-estradiol, and the metabolite 2-OH estrone, suppressed lymphocyte proliferation to PHA; in vivo exposure (8 mg/kg estrogens in female mice) did not affect splenic proliferative responses to PHA relative to untreated controls [8]. Pharmacological concentrations, but not physiological concentrations, of estradiol inhibited mixed lymphocyte reactions in rat cell cultures [9]. These differences in cellular immune outcomes likely reflect a number of variables including species differences, estrogen dose effects, varying duration of estrogen exposure, in vitro compared with in vivo effects, and differences by primary and secondary lymphoid compartment effects.

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There is also a body of literature linking acute exercise with transient alterations in T cellular proliferation response (e.g., [10]). However, the direction, magnitude and timing of the effects vary from study to study. Blood lymphocyte proliferation responses to T cell mitogens were decreased in men who exercised at 90 and 120% of $\dot{V}O_{2max}$ [11]. No effect of physical activity relative to sedentary condition on splenic lympocyte proliferation responses to ConA or lipopolysaccharide (LPS) in hamsters; PBL response to ConA was significantly elevated proliferative responses in regularly physically active versus sedentary hamsters [12]. Tchórzewski et al. [13] found no change in ConA-induced lymphocyte suppressor function immediately after maximal bicycle ergometry exercise in untrained men, although a reduction from baseline was observed 2 h later. Lin et al. [14] reported an increase (between 100 and 50 μg/mL) and a decrease (between 50 and 10 μg/mL) in blastogenesis to staphylococcal enterotoxin B of rat splenocytes immediately after acute exercise. Response of pure T-lymphocyte cultures to PHA were not different during exercise and postrecovery relative to baseline for runners given an acute intensive interval training protocol [15]. ConA-stimulated lymphocyte proliferation responses were elevated above preexercise conditions in adults engaging in a single bout of weight training exercise [16].

Regular physical activity is routinely advised as a component of healthy lifestyle for postmenopausal women. Exercise during the postmenopausal years is encouraged because of associated increased muscle strength, improved cardiorespiratory fitness, and increased bone mass (or at least attenuation of bone loss). Many women who take estrogen replacement therapy to reduce climacteric symptoms also engage in physical activity. Nevertheless, the effects of both estrogen replacement and exercise on lymphocytes have been largely unexplored.

This study was designed as exploratory, because there is virtually no evidence on the interaction between estradiol supplementation and physical exercise on immune parameters. The provisional hypothesis was that high levels of estrogen exposure in vivo would suppress lymphocyte proliferation responses in vitro, and that this suppression would be exacerbated by acute exercise stress.

2. Method

2.1. Subjects

Sixty adult female C57BL/6 mice, aged 6–7 weeks, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Upon arrival, all mice were randomly assigned to one of three hormone treatment conditions, and ear notched for identification. Animals were acclimated to the vivarium for 1 week before treatment began. Mice were housed in a temperature- and humidity-controlled chamber, at 21°C, on a 12/12-h reversed light/dark cycle. Mice had ad lib access to food (Laboratory Rodent Chow, PMI Feeds, Richmond, IN) and tap water. All animals were handled in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2. Ovariectomy

Mice were anesthetized with avertin (tribomoethanol, 0.016 mL per g body wt), a midline dorsal incision was made in the lumbar region to reveal the dorsal fat pads containing the ovaries, the ovaries were pinched off, and mice were implanted with either estrogen (17β-estradiol pellet releasing 71.4 μg estradiol (E2) per day for 21 days, Innovative Research Corporation of America, Toledo, OH), or placebo (containing only inert vehicle) pellets. Mice were given temsgesic (subcutaneous, 0.05–0.1 mg/kg), recovered on a heating pad, then returned to home cages for 14 days, at which time they were given the exercise intervention (described below). Surgical incisions were initially closed with wound clips. Sham mice received the same surgical treatment (anesthesia and recovery) as the ovariectomized mice without removal of ovaries.

2.3. Exercise treatment

The exercise protocol was administered 14 days after the surgical intervention. Mice were randomly assigned within hormone condition (Ovx, Ovx + E2, sham) to either an exercise condition (EX) or a control (CONTROL) condition. The exercise protocol consisted of a single treadmill challenge at 26 m/min, 6° slope, for 90 min. Electric shock was not used to motivate the animals to run; mice were gently prodded with a soft brush and/or a gloved hand. Mice were prodded to run for the 90 min, after which they were removed from the treadmill, and sacrificed 24 h later for lymphoid tissues. Control mice were exposed to the treadmill environment without exercise.

2.4. Tissue collection

Mice were rapidly sacrificed by cardiac exsangination under sodium pentobarbital (0.07 mg/g body wt) anesthesia. Cardiac blood was centrifuged at 500 × g, plasma collected, and stored at −20°C until use. Plasma was used for a subsample of mice to verify plasma 17β-estradiol concentrations.The thymus and spleen were rapidly removed, weighed, and placed in small culture dishes containing RPMI-1640 with heat-inactivated 10% fetal bovine serum (FBS). The splenocytes were dissociated from the connective tissue capsule by gently pressing the organ through fine, sterile nytox mesh. The mesh and culture dish were rinsed with medium, and the suspension collected in sterile 15 mL conical tubes. The resulting single cell suspension was layered onto a Percoll density gradient (Lympholyte-M®, Cedarlane, Hornby, ON). The thymus was placed in a small culture dish containing RPMI-1640 + heat-inactivated 10% FBS, gently teased apart, and layered onto Percoll. The preparation of lymphoid tissues has been described elsewhere [17].

2.5. Blastogenesis assay

Multiple concentrations of mitogens were used to provide results on proliferation responses to suboptimal, optimal, and supraoptimal concentrations. The mitogens were T cell (concanavalin A or ConA) and B cell (lipopolysaccharide or LPS) stimulants.The concentrations used were 1.0 and 5.0 μg/mL for ConA and 0.5, 1.0, and 2.0 μg/mL for LPS, as determined in previous studies [17,18]. One hundred microliters of 5×10^6 cells/mL of each tissue (splenocytes, thymocytes) were seeded into sterile, 96-well flat bottom plates to which 20 μL of medium and 20 μL of either ConA or LPS were added. Control wells (background) received 100 μL of cell suspension (for each tissue), and 40 μL of medium. Plates were then incubated for 66 h at 37°C, 5% CO_2. After incubation, all wells except for background were pulsed with 20 μL of [^{3}H]thymidine (1 μCi/mL) except for background plates. Plates were incubated for an additional 6 h; cell suspensions were harvested onto glass filter mats (Skatron, Mandel Scientific) and the mats were air dried for 24 h. The filter disks were placed in 8 mL of ICN cytoscint cocktail, and counted in a Beckman β counter. Data were expressed as total counts per minute (cpm).

2.6. Plasma estradiol concentration

Plasma estradiol was measured in a subsample of mice using a commerically available ^{125}I double antibody RIA kit (Inter-Medico). Estradiol was first extracted under diethyl ether. The inter assay coefficient of variation (CV) and the intra-assay CV for this kit were <8% and <7%, respectively. Plasma estradiol concentrations were 2.8 ± 1.1 pg/mL (sham, $n = 5$), 7.6 ± 5.4 pg/mL (Ovx, $n = 6$), and 620.7 ± 71.1 pg/mL (Ovx + E2, $n = 6$). The normal physiological range of plasma estradiol in the mouse is reported between 5–50 pg/mL [19].

2.7. Statistical analyses

Data were analyzed using a 3 × 2 ANOVA design separately for each organ and mitogen concentration. Post hoc analyses were conducted using Tukey's test. A value of $p < 0.05$ was considered as being significantly different from chance. All values are reported means ± 1 standard error. SPSS was used as the statistical analysis program.

3. Results

3.1. Concanavalin A-induced blastogenesis

In the thymus, there was a significant main effect of hormone treatment on ConA-induced blastogenesis at 1.0 and 5.0 μg/mL concentrations, ($F(2, 53) = 3.497$, $p < 0.05$, and $F(2, 53) = 5.101$, $p < 0.005$, respectively) where Ovx + E2 < Ovx or sham. These results are presented in Fig. 1 (top panel).

Exercise intervention had little impact on thymocyte blastogenesis at 1.0 and 5.0 μg/mL concentrations of ConA

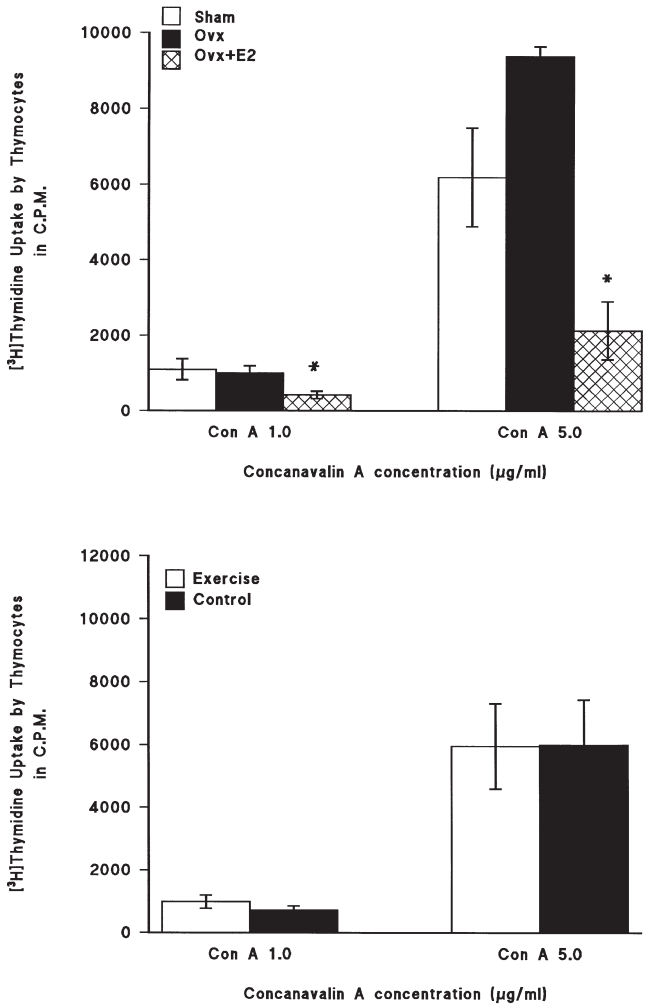

Fig. 1. Thymic lymphocyte proliferation responses in counts per minute (c.p.m.) for two concentrations of the mitogen Concanavalin A (ConA). The top panel indicates the main effect of hormone (estradiol) treatment; the bottom panel indicates the main effect of exercise treatment. *$p < 0.05$ by Tukey's test (Ovx + E2 relative to other hormone conditions). Values are ±1 standard error.

(Fig. 1, bottom panel). There was, however, a significant interaction effect of hormone and exercise treatment, ($F(2, 53) = 3.508$, $p < 0.05$), at the 1.0 μg/mL concentration of ConA. The sham (control) mice that were exercised had higher blastogenesis responses compared with sham sedentary mice. The Ovx + E2 mice given exercise had lower responses irrespective of exercise treatment; these results are shown in Fig. 2. There was no significant interaction effect of hormone and exercise treatment at the 5.0 μg/mL concentration of ConA-induced blastogenesis in the thymus.

In the spleen, the main effect of hormone was only significant at the 5 μg/mL concentration of ConA tested, ($F(2, 53) = 5.878$, $p < 0.005$). This effect of supplementation with estradiol is shown in Fig. 3 (top panel). There was no difference in the ConA-induced blastogenesis response of splenocytes between exercised and sedentary mice (Fig. 3, bottom panel). There were no significant interactions be-

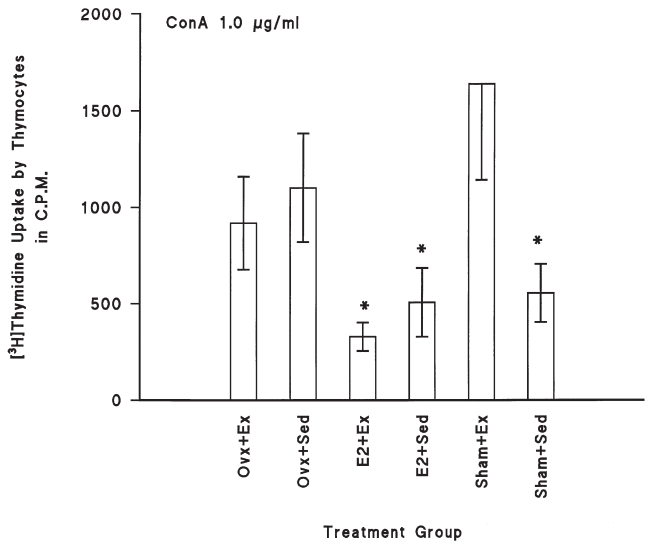

Fig. 2. Thymic lymphocyte proliferation responses in counts per minute (c.p.m.) to the mitogen ConA (1.0 µg/mL) in hormone treatment × exercise interaction conditions. *$p < 0.05$ relative to Sham + EX group. Values are ±1 standard error.

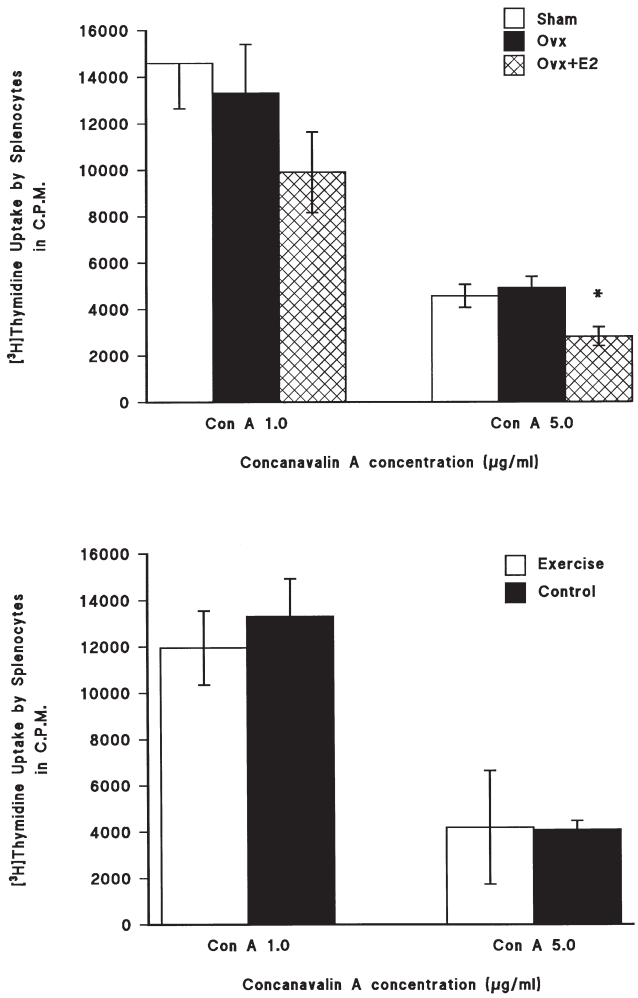

Fig. 3. Splenic lymphocyte proliferation responses in counts per minute (c.p.m.) for two concentrations of the mitogen Concanavalin A (ConA). The top panel indicates the main effect of hormone (estradiol) treatment; the bottom panel indicates the main effect of exercise treatment. *$p < 0.05$ relative to Ovx group at ConA of 5.0 µg/mL concentration. Values are ±1 standard error.

tween hormone and exercise intervention for blastogenesis response to concanavalin A in splenic lymphocytes.

Figure 4 shows the effect of estradiol supplementation on the response of splenocytes to the B cell mitogen, lipopolysaccharide. Mice that were ovariectomized and replaced with high-dose estradiol had significantly lower blastogenesis responses to LPS at the 0.5 µg/mL concentration, $F(2, 53) = 4.119$, $p < 0.05$, 1.0 µg/mL concentration, $F(2, 53) = 3.603$, $p < 0.05$, and 2.0 µg/mL concentration, $F(2, 53) = 4.504$, $p < 0.02$. Neither the main effect of exercise nor the interaction of exercise with estradiol supplementation was significant for splenic lymphocyte proliferation responses to LPS.

There were no hormone or exercise main or interaction effects for background or for media control counts per minute (proliferation responses) (data not shown).

4. Discussion

The results of this study demonstrate that exposure to high levels of estradiol in vivo result in reduced in vitro C57BL/6 mouse thymocyte and splenocyte proliferation responses to the T and B cell mitogens, ConA and LPS. These findings are consistent with reports of in vivo exposure to high physiological levels of estradiol suppressing splenic lymphocyte proliferative responses to concanavalin A in male castrated, lupus (MRL lpr/lpr) prone mice [20]. Further, the reduced immunoproliferative effect was not differentially dependent on the lymphoid tissue sampled. Forsberg [21] found that timing of exposure to the synthetic estrogen, diethylstilbestrol (DES), in female NMRI mice was critical in determining thymic weight, cellularity, and percentage of thymic cells in the S and G_2 M phases of the cell cycle. The younger the animal when exposed to DES, the more long lasting and significant the impact on thymus weight, structure, and function. Whether using mice younger or older than those used in the present study results in greater reduction in T cell proliferation responses with in vivo estradiol exposure remains to be elucicated.

In the spleen, a single bout of treadmill exercise was not associated with altered lymphocyte blastogenesis responses compared with nonexercised controls. In the thymus, there was an interaction effect at the lowest concentration of ConA (1.0 µg/mL) for thymocytes: the control exercised mice (sham group) had a higher proliferation response than control sedentary mice, whereas the estradiol-treated animals did not differ from each other (irrespective of exercise). The present ConA results confirm the work of Moyna and colleagues [22] of both an increase and no change in blood lymphocyte proliferation responses to ConA at 1.0 µg/mL

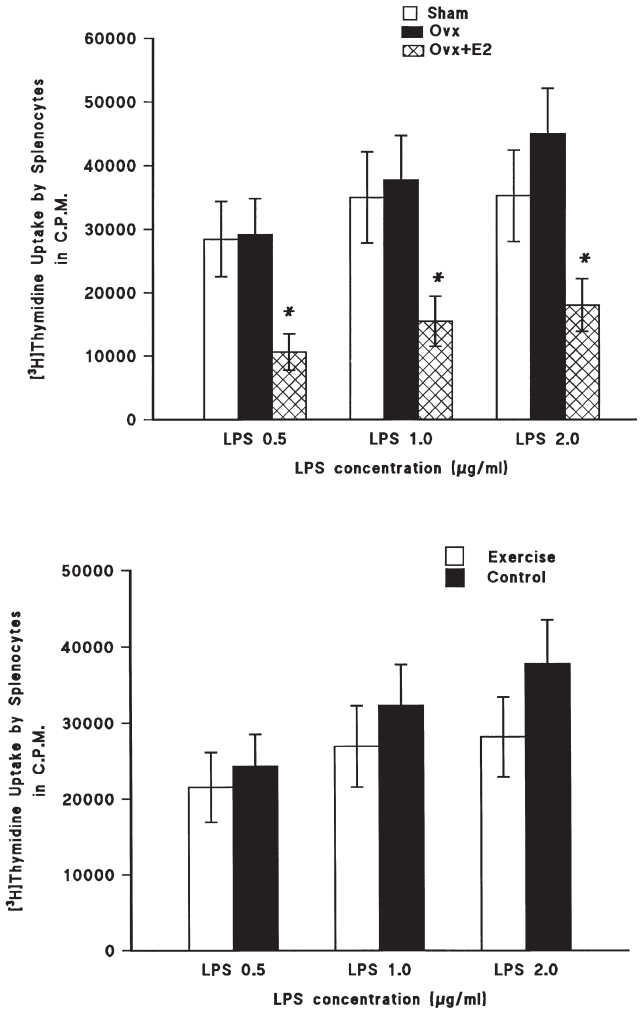

Fig. 4. Splenic lymphocyte proliferation responses in counts per minute (c.p.m.) for three concentrations of the lipopolysaccharide (LPS). The top panel indicates the main effect of hormone (estradiol) treatment; the bottom panel indicates the main effect of exercise treatment. *$p < 0.05$ Ovx + E2 group relative to other hormone treatment conditions. Values are ±1 standard error.

and 5.0 μg/mL concentrations in male and female subjects given incremental exercise up to 85% of $\dot{V}O_2$ peak relative to sedentary controls. Although others have reported blood lymphocyte immune suppression to T cell mitogens (reviewed in [23]), differences between studies may reflect the proportion and maturity of T and B cells of the lymphoid compartments. Ferry et al. [24] found that the composition (percentage distribution) of rat blood lymphocyte subsets, but not splenic or lymph node lymphocyte subsets, were affected by an acute exercise. The splenic lymphocyte responses to LPS agree with our earlier work showing an acute exhaustive exercise bout in untrained male C57BL mice did not significantly alter splenocyte functional responses to LPS relative to control mice [25].

As expected, the ovariectomized mice supplemented with exogenous 17β-estradiol had significantly higher levels of free hormone compared with the ovariectomized and not supplemented mice and sham controls. That the 17β-estradiol levels in Ovx and Sham groups did not differ was also not unexpected. The low levels of 17β-estradiol in the Ovx and Sham groups may reflect the residual, peripheral conversion of androgens to estrogens via enzymatic pathways in adipose tissue in the Ovx females and/or the production of anestrous conditions in the Sham females by socially induced corpus luteum formation. Ryan and Schwartz [19,26] showed that group housing of sexually mature female mice tends to increase the incidence of pseudopregnancy and anestrous episodes, resulting in low estradiol levels.

This study is, to our knowledge, the first to describe the effect of a single exercise bout on lymphocyte proliferation responses in estrogen replaced and nonreplaced ovariectomized mice. At the lower concentration of ConA tested, exercise differentially influenced thymocytes from estradiol supplemented mice compared with controls. Estradiol masked or abolished the small exercise effect that was observed in nonestradiol supplemented animals. The higher proliferative response in exercised (sham) control mice was not observed in the Ovx+ animals. Although the Sham and Ovx mice had similar estradiol levels, Ovx also removes progesterone, which affects immune cells. It is unclear from the present data whether differences in progesterone exposure contributed to the observed pattern of thymocyte proliferation responses in Ovx mice compared with the Sham animals.

There are limitations of this study. Mice were sacrificed 24 h after completion of an acute exercise bout. It is possible that this time point missed the “window of opportunity” to detect significant alterations in lymphocyte proliferation responses to mitogens. We [27] and others [28] have shown that intense exercise is associated with extensive cell death in thymocytes 24 h after completion of the bout; Hartmann and colleagues [29] described DNA damage in circulating lymphocytes of untrained humans after an exhaustive exercise session. It was not unreasonable to speculate that DNA damage should produce disruptions in lymphocyte DNA blastogenesis responses, including decreased lymphocyte activation to mitogens. The present study failed to demonstrate a decrease in lymphocyte proliferation 24 h after exhaustive exercise stress. Indeed, a small increase in lymphocyte proliferation was observed in the Sham group that had received exercise challenge. Additional studies at varying times in relation to exercise stress are clearly necessary to determine the relationship (if any) between DNA damage and lymphocyte proliferative responses of lymphoid cells. It is also possible that the relative short chronicity for estrogen replacement (i.e., 15 days of estrogen replacement exposure) influenced the mitogen responses to acute exercise. For example, Brunelli and colleagues [6] found that the timing of exposure of estradiol and medroxyprogesterone significantly influenced immune functions in healthy postmenopausal women: natural killer cell and CD8+ lymphocyte responses were reduced on Day 8, but returned to basal at Day 28 of hormone replacement therapy.

Hormone replacement therapies (HRT) are widely prescribed to peri- and postmenopausal women to reduce the severity of the climacteric, and as a preventive measure to reduce the risk of osteoporosis and cardiovascular disease. Many women will also continue or begin exercise in combination with HRT. The results from the present study suggest that (1) in vivo exposure to high concentrations of estradiol in female mice reduce in vitro lymphocyte proliferation responses to mitogenic stimuli, and (2) high levels of estrogen masks or abolishes small effects of exercise on thymocyte proliferation observed in the hormone control mice receiving exercise relative to sedentary controls. The physiological significance of the estrogen effects on immune function for women on HRT remains to be determined.

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