Free Radical Biology & Medicine, Vol. 28, No. 1, pp. 84–90, 2000

0891-5849/00/$–see front matter

PII S0891-5849(99)00209-9

Original Contribution

EFFECT OF EXHAUSTIVE EXERCISE ON MEMBRANE ESTRADIOL CONCENTRATION, INTRACELLULAR CALCIUM, AND OXIDATIVE DAMAGE IN MOUSE THYMIC LYMPHOCYTES

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(*Received* 18 *August* 1999; *Revised* 21 *September* 1999; *Accepted* 30 *September* 1999)

Abstract—Early Ca^{2+} signaling events in cells of the immune system after exhaustive exercise challenge (8% slope, 32 m/min^{-1} speed) of female C57BL/6 mice, and their effects on oxidative reactions in thymus were studied. Intracellular Ca^{2+} and the oscillation of free extracellular Ca^{2+} were imaged with cell permeant cell and cell impermeant Fluo 3 calcium indicator in thymocytes. The role of estradiol was assessed by RIA for levels of membrane bound estradiol. Oxidative product release and membrane lipid peroxide were also evaluated. Intracellular Ca^{2+} levels were significantly higher in thymocytes of exercised compared with control mice ($p < .001$). There was a continuous flux of Ca^{2+} after exercise when cells were monitored in Ca^{2+} rich medium, with a significant influx between 160 and 200 sec ($p < .001$). Membrane bound estradiol was elevated in thymocytes of exercised compared to control mice ($p < .05$). Immediately after exercise there was a greater release of oxidative products by thymocytes in exhaustively exercised compared with control animals. There was also significant generation of lipid peroxide in thymus of exercised mice ($p < .001$). The findings suggest that exhaustive exercise may stimulate estradiol uptake by receptors on thymocytes, with a possible opening up of estradiol-receptor operated channels for Ca^{2+} entry into cells. This may have damaging effects on thymic lymphocytes by the triggering of oxidative reactions as determined by higher oxidative product release and greater generation of lipid peroxide.

Keywords—Exercise, Oxidative damage, Thymocytes, Estradiol, Intracellular Ca^{2+}, Free radical

INTRODUCTION

Calcium ions (Ca^{2+}) serve as an intracellular signal for a great variety of processes within cells and the state of free intracellular Ca^{2+} is influenced by multiple factors. For example, in smooth muscle cells, the release of free Ca^{2+} from intracellular stores results in spontaneous transient outward currents (STOCs) [1]. In many types of cells there is also evidence of spontaneous transient inward currents (STICs) in addition to STOCs. These events are, however, thought to be the outcome of local transient elevations in intracellular calcium ('Ca^{2+} sparks') [2,3]. It is interesting to note that Ca^{2+} sparks together with the biphasic Ca^{2+} currents (i.e., STOCs and STICs) do not always follow each other sequentially; for example, even when the Ca^{2+} spark is rising, STOCs decay may begin [1]. Other factors influencing free cytosolic Ca^{2+} level changes include (i) ATP-induced Ca^{2+} ion pump into the endoplasmic reticulum, (ii) the crossing of plasma membrane by Ca^{2+} as a result of hyperpolarisation in nonexcitable cells, (iii) the depletion of intracellular stores of Ca^{2+} resulting in the gating of Ca^{2+} across the plasma membrane (capacitative entry). Capacitative entry has a critical effect on Ca^{2+}-dependent physiological processes, including the opening of intracellular Ca^{2+} stores through receptor–ligand mechanisms [4] and, (iv) free cytosolic calcium is affected by other biochemical processes within cells. For example, an elevation of intracellular inositol triphosphate (IP_3) exerts pronounced effects on Ca^{2+} signaling by controlling both the release of stored Ca^{2+} and the influx of extracellular free Ca^{2+}. Both effects are dependent upon the activation of a membrane phospholipase A_2 (PLA_2)

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with the resultant production of arachidonic acid. These events are typically of short duration, lasting maximally a few minutes, after which Ca^{2+} levels return to basal values. Longer duration signals, which display oscillatory Ca^{2+} behavior due to mechanisms like Ca^{2+}-induced Ca^{2+} release (i.e., the activation of Ca^{2+} release from other intracellular compartments as a result of Ca^{2+} sparks), also occur. Such events play a role in the early signals control of cell cycle progression [5,6].

Although it is well known that exercise influences the cellular immune response [7,8], in vivo studies of early signals and their effects during and after exercise stress on lymphocytes are limited. This lack of data may be due to the time constraints within which early signals (such as Ca^{2+} oscillation) and biochemical reactions take place in lymphoid cells (i.e., events that occur within seconds or minutes and which rarely exceed 1 h after exercise). We had previously provided evidence of oxidative stress in thymocytes and splenocytes of exercised mice as expressed in changes in antioxidants enzymes levels and lipid peroxides accumulation in their membranes [9]. In order to further understand how oxidative damage occurs in lymphocytes after exercise, we measured free intracellular Ca^{2+} and Ca^{2+} oscillation in thymocytes on completion of an exhaustive exercise bout. Because exercise has been associated with elevated blood estrogen levels [10,11], and because sex steroid hormones are known to induce Ca^{2+} fluxes [12], we also measured the uptake of estradiol by estradiol receptors on thymocyte cell membranes in relationship to changes in Ca^{2+} movement after exercise. Finally, we assessed the outcome of these events on the occurrence of oxidative damage in lymphoid tissues. The underlying hypothesis for this sequence of experiments was that exercise associated estrogen binding to thymocytes was involved in the opening of calcium gates, with possible activation of oxidative processes.

MATERIALS AND METHODS

Experimental animals and exercise protocol

Female C57 BL/6 mice (Harlan Sprague Dawley, Frederick, MD, USA), 10–12 weeks of age, were randomly assigned to one of two conditions: exhaustively exercised and sacrificed immediately after cessation of the bout (exercise group); and sedentary (control group) animals that were killed without treadmill exercise. The run to exhaustion (RTE) consisted of a single treadmill challenge at 32 m/min^{-1} 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, and on a 12/12 h reversed light dark cycle. Food (Laboratory Rodent Chow, PMI Feeds, Richmond, IN, USA) 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.

Tissue collection

Mice were killed by pentobarbitol overdose. The thymus was excised, the cells dispersed through nylon mesh into small tissue culture dishes containing calcium/magnesium free phosphate-buffered saline (PBS) (pH 7.4), the cells were rapidly counted manually using a hemocytometer and cell counts adjusted noted subsequently. The experimental procedures were rapidly conducted such that the lapsed time from death of animals to (i) seeding of cells into Fluo 3 cell impermeant solution including fluorimetric reading of extracellular Ca^{2+} flux monitoring and (ii) loading cells with FcOxyBURST Green H_2HFF BSA including fluorimetric readings, was 5–7 min. Separate groups of treadmill exercised and control mice were used for each of the experimental outcome measures (i.e., intracellular calcium concentration, oxidative products, etc.)

An aliquot of thymocytes was stored frozen at −20°C and used for membrane preparation as previously described [9] for membrane bound estradiol assay. For oxidative product monitoring and lipid peroxide studies, a set of animals treated as previously described were killed thymocytes were rapidly prepared, and an aliquot of cells was used for oxidative product studies. Membrane for lipid peroxide studies was prepared from the remaining aliquot of cells.

Calcium studies

Determination of intracellular free Ca^{2+}. Isolated thymic (2×10^6 thymocytes ml^{-1}) cells were seeded into cuvettes in 1 ml volumes using Ca^{2+} free PBS as diluent and rapidly loaded with cell permeant acetoxymethyl (AM) ester Fluo 3 calcium indicator (Molecular Probes, Eugene, Oregon, USA), to a final concentration of 4 μM Fluo 3 per milliliter. Cells and Fluo 3 were incubated at 27°C (the optimum reaction temperature of Fluo 3 as recommended by manufacturer) for 30 min (a period allowing for de-esterification reaction of cell permeant acetoxymethyl ester of Fluo 3, and maximal binding to intracellular Ca^{2+}). Because this reaction was in Ca^{2+}-free PBS, there was no possibility of further influx of Ca^{2+}. Cells were washed by centrifugation, resuspended in 1 ml of Ca^{2+}-free PBS, and incubated in the dark for 30 min at 27°C [13]. Photon readings were taken using a Shimadzu (Kyoto, Japan) RF-1501 spectrofluorophotometer at an excitation wavelength of 488 nm and emission wavelength of 530 nm. The $[Ca^{2+}]i$ was com-

puted from the equation $[Ca^{2+}]i = kd \times [F - Fmin]/[Fmax - F]$, where kd is ion dissociation constant, Fmin is fluorescence intensity in absence of Ca^{2+}, Fmax is fluorescence of Ca^{2+} saturated indicator, and F is fluorescent intensity of sample. The dissociation constant for Fluo 3-Ca^{2+} complex (kd), Fmax and Fmin were determined using a commercially available calcium calibration buffer kit (Molecular Probes).

Determination of extracellular Ca^{2+} changes. Thymic (2.0×10^6 cells/ml^{-1}) lymphocytes were rapidly seeded into cuvettes and cell impermeant Fluo 3 calcium indicator was added such that a final concentration of 4 μM was obtained in 1 ml total volume, made in PBS which has a Ca^{2+} concentration of 1 μM. This was depicted as Ca^{2+}-rich medium. Ca^{2+} flux was monitored at 40 s intervals over a period of 200 s at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Oxidative products

Thymic cells (2×10^6 cells/mL^{-1}) were seeded into cuvettes containing Ca^{2+}-free PBS + 5-mM glucose. FcOxyBURST Green H_2HFF BSA assay reagent (Molecular Probes) was rapidly added such that a final concentration of 10 μg/ml^{-1} was obtained. The release of oxidative product into the extracellular milieu was monitored over a period of 120 s at 40 s intervals using Shimadzu spectrofluorophotometer at excitation wavelength 488 nm and emission wavelength of 530 nm [14].

Membrane lipid peroxide assay

Membrane lipid peroxide was measured by ferrous ion oxidation in the presence of xylenol orange as described by Jiang et al. [15] and modified by Azenabor and Mahony [16]. This assay 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 thymic cells (2.1×10^7 cells) were treated in methanol to extract the lipid peroxide at a dilution of 1:10, centrifuged at 14,000 rpm for 3 min at 4°C, and the extracted lipid peroxide (100 μl) was added to 900 μl of xylenol orange working reagent. The standard consisted of 20 μM H_2O_2 assayed along with the samples. A blank consisting of methanol in 900 μl 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.

Estradiol studies

Estradiol levels in thymus cell membranes were done using a commercially available radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Estradiol was first extracted in diethyl ether, the extract was reconstituted in zero calibrator A supplied with estradiol assay kit and 100 μL of extract, control, and zero calibrator A were directly pipetted to the bottom of estradiol Ab-coated tubes. One milliliter of [^{125}I] estradiol was added and vortexed. The mixture was incubated for 3 h at room temperature. All excess content of each tube was decanted and samples were counted for 1 min in a gamma counter. Estradiol levels were reported as fg per 10^6 cells for thymic cell membrane. The inter-assay C.V. is $< 8\%$ and the intra-assay C.V. is $< 7\%$ for the kit.

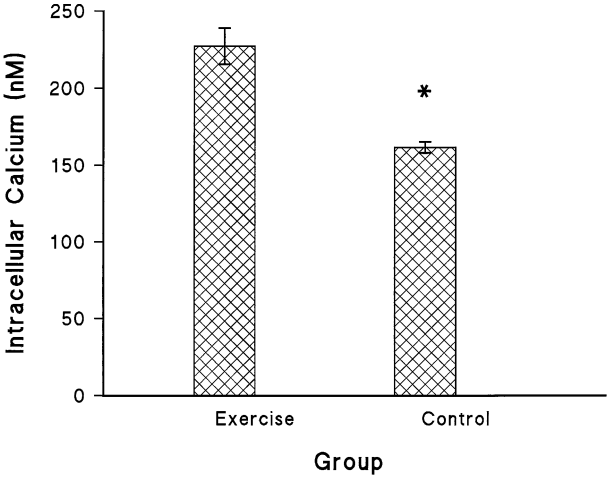

Fig. 1. Intracellular calcium (nM) in thymus cells from exhaustively exercised was significantly higher than in thymus from control mice. *$p < .001$ between exercised and control groups. All values are means ± 1 SE ($n = 10$ per group).

Statistics

Data were analyzed using a repeated measures analysis of variance (ANOVA) model for the dependent variable of time for the oxidative products and calcium flux studies. Intracellular calcium, lipid peroxide, and estradiol were analyzed by one way ANOVA. All values represent group means ± one standard error (SE).

RESULTS

Intracellular Ca^{2+} studies

Exercise was associated with an increase in intracellular Ca^{2+} of thymocytes (227 ± 11.7 nM) compared with samples obtained from nonexercised, control mice (thymocytes: 161 ± 3.6 nM) as shown in Fig. 1. The difference in intracellular calcium concentration between exercise and control mice thymocytes was significant ($p < .001$).

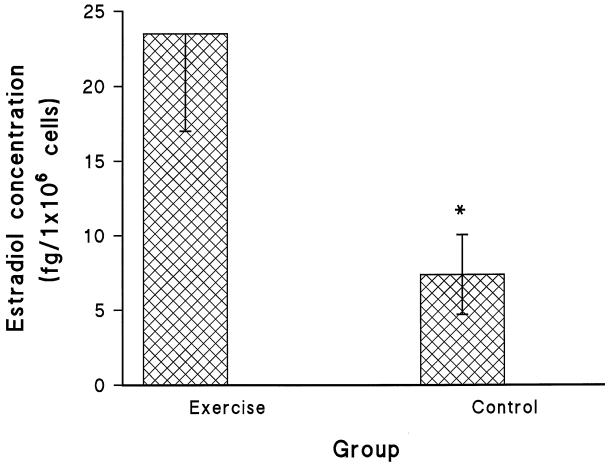

Fig. 2. Estradiol concentrations in thymus membrane (fg per 10^6 cells) in exhaustively exercised was significantly higher than in thymus from control mice. *$p < .05$ between exercised and control samples for thymus. All values are means ± 1 SE ($n = 10$ per group).

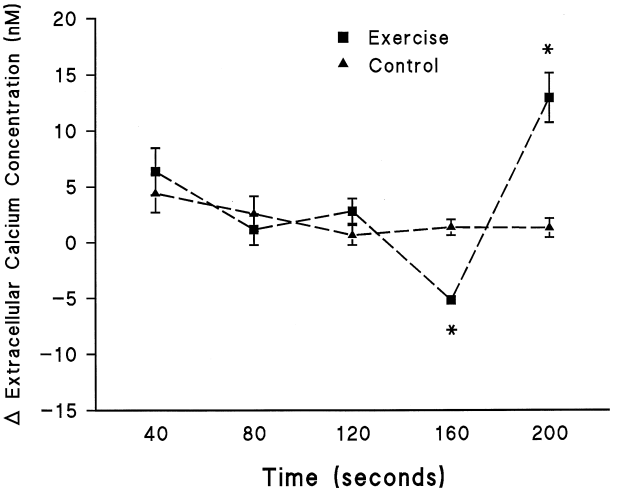

Fig. 3. Change (Δ) in extracellular calcium concentration (nM) in thymocytes was significantly different between exhaustively exercised compared with control mice at time of 160 and 200 s. Positive values on the Y-axis refer to influx of extracellular calcium ion, negative values on the Y-axis refer to efflux of extracellular calcium ion. *$p <$ .001 between exercised and control samples for thymus. All values are means ± 1 SE ($n = 10$ per group).

Estradiol studies

There was significantly higher thymic membrane estradiol concentrations in exercised relative to control animals ($p < .05$) (Fig. 2). The mean thymic estradiol concentration of 10 exhaustively treadmill exercised mice was 23.51 ± 6.49 fg/1 × 10^6 cells compared with 10 sedentary control mice of 7.38 ± 2.68 fg/1 × 10^6 cells.

Ca^{2+} flux

The higher concentration of intracellular Ca^{2+} in thymic cells from exhaustively exercised mice suggested to us that the effect of the binding of estradiol to estradiol receptors may trigger an influx of Ca^{2+} postexercise in Ca^{2+}-positive medium. To test this, calcium flux studies were performed on isolated mouse thymocytes taken from exhaustively exercised and control animals. Figure 3 shows the results of the calcium flux studies. Over the 200 s monitored, there was a greater flux of Ca^{2+} in thymocytes of exercised mice compared with values obtained in controls (interaction effect of time × treatment: $p < .001$). Ca^{2+} fluxes at 160 and 200 s for thymocytes were significantly different between the exhaustively exercised and control groups ($p < .001$ and $p < .001$, respectively).

Oxidative studies

The extracellular release of oxidative products was monitored using a sensitive fluorogenic reagent (OxyBURST Green H_2HFFBSA) rapidly added postexercise to thymocytes and monitored at 40 s intervals for 120 s. Figure 4 presents the results of the change (delta) in oxidative product release over time relative to t0 baseline. There were significant differences between exercised and control mice for the change in oxidative product release from thymocytes at t40 s ($p < .005$), t80 s ($p < .005$), and t120 s ($p < .001$) (Fig. 4).

Lipid peroxide

Lipid peroxide formation in thymocyte cell membranes is shown in Fig. 5. There was significantly greater

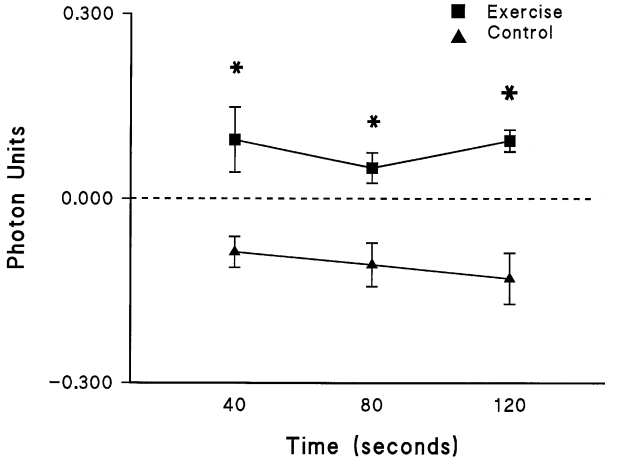

Fig. 4. Change in oxidative product release, in photon units, over time in thymocytes shows significantly greater release in exhaustively exercised mice compared with controls. *$p < .005$ at t40 and t80 s, and $p < .001$ at t120 s for differences between exercised and control samples in thymus. All values are means ± 1 SE ($n = 10$ for control and 11 for exercised groups).

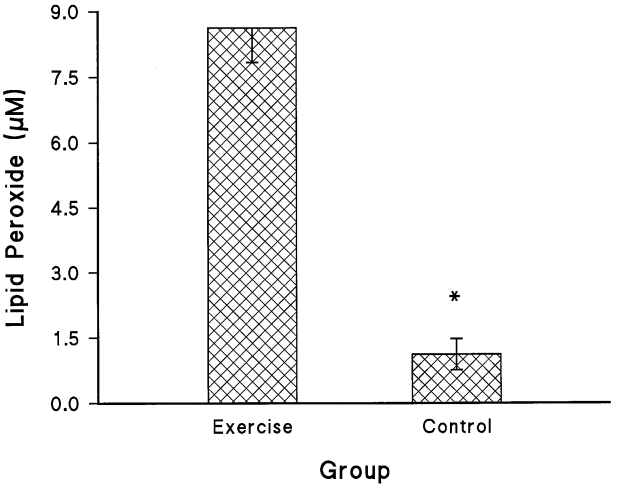

Fig. 5. Lipid peroxide (μM) in thymus (per 2.1×10^7 cells) was significantly higher in exhaustively exercised compared with control mice. *$p < .001$ for differences between exercised and control groups. All values are means $\pm$ 1 SE ($n = 10$ mice in control and 11 mice in exercised groups).

lipid peroxide in thymus cell membranes of exhaustively exercised (8.63 ± 0.79 μM) compared with control mice (1.12 ± 0.36 μM) ($p < .001$).

DISCUSSION

Considerable attention has been focused on the effect of exercise interventions on functional measures of the cellular immune system [17]. For example, it has been documented that with oxidative stress arising from intensive exercise, human leukocytes rapidly express the antioxidative stress protein heme oxygenase-1 and this has been related to proinflammatory cytokine release [18]. There are, however, few studies on the so called 'early signals' that affect immune cells after intensive exercise, i.e., the events that take place in seconds, minutes or at most tens of minutes after intensive exercise within the cells of the lymphoid tissues. Among the 'early signals' (e.g., activation of oxidative enzymes, oxidative processes, and generation of oxidative products, activation of transcription factors, gene expression), ionic events are of physiological importance. Such ionic events would include the activation of transporters, channels of the plasma membrane, and ionic redistribution between subcellular compartments. One of the ions involved in these early signals is Ca^{2+} ions. Ca^{2+} ions play a central role in the regulation of many forms of cellular activity, including muscle contraction, neurotransmitter release, synaptic plasticity, cell proliferation, and cell death.

We assessed the effect of a single exhaustive exercise stress on the surface interaction of estradiol to estradiol receptors on the plasma membranes of thymocytes. The findings suggest that one possible consequence of estradiol binding may be in an accompanying free Ca^{2+} oscillation, an increase in oxidative reactions (i.e., oxidative products release), and resultant membrane lipid peroxide generation in lymphoid tissues. Estrogen, like other steroid hormones, has major long-term effects on cell growth, cell differentiation and cell function; these effects are likely mediated through nuclear estrogen receptors which have domains for estrogen binding, nuclear localization, dimerization, DNA binding and transactivation [19]. There is evidence that $CD4^+$ and $CD8^+$ subsets of T lymphocytes have estrogen binding sites on their cell surfaces and that such membrane binding can induce both Ca^{2+} influx through Ca^{2+} channels and release of Ca^{2+} from the endoplasmic reticulum [12].

The results of this study suggest that the high levels of estrogen bound to membrane receptors in thymocytes may be involved in the observed elevations in intracellular calcium ion, and ultimately, the observed membrane lipid damage after maximal exercise stress. A possible mechanism for the higher concentration of estradiol bound to thymic membrane is through exercise-mediated hemodynamic effects, potentially resulting in greater ligand-receptor exposure in tissues. These findings may provide a mechanistic explanation for the reduced T lymphocyte proliferative responses that have been repeatedly observed after exercise [20,21].

We found elevated intracellular calcium levels in thymocytes obtained from exercised mice compared with control mice. Of the three main Ca^{2+} entry channels (voltage operated channels, receptor operated channels, and store operated channels), receptor operated channels usually provide brief high intensity bursts which are sustained for as long as the receptor ligand mechanism is in place. In this work, we have evidence of higher estrogen concentrations on the membranes of cells of the thymus following exhaustive exercise. A possible consequence of this higher estrogen concentration may be the observed increased intracellular Ca^{2+} in thymuses of exhaustively exercised mice. The suspension of cells in Ca^{2+}-rich PBS resulted in fluxes of Ca^{2+}, events which are dependent on the state of filling of Ca^{2+} stores (capacitative entry) [22]. The findings from this study suggest that at the point of adequate filling of the intracellular stores in thymus of exhaustively exercised mice, the influx of Ca^{2+} was abrogated and there was Ca^{2+} efflux (i.e., at 160 s). Minimal calcium flux (influx or efflux) was observed in thymus samples taken from control mice. It is not clear from our data at what time point the differences in calcium flux in thymic samples of exhaustively exercised and control mice resolve nor why there were no initial differences (i.e., at t40 s) between exhaustively exercised and control animals. Additional studies to determine the time course for resolu-

tion of calcium fluxes in thymus of exhaustively exercised mice would be needed.

Although a definitive causal role for estradiol in the biphasic movement is not provided by this study, the data suggest that there may be an association between uptake of estradiol and intracellular Ca^{2+} build up in thymocytes from exercised animals. The consequences of increased intracellular Ca^{2+} levels may include: (i) the induction of precipitation of phosphate resulting in the depletion of inorganic phosphate, the vital source for the replenishment of ATP (the established energy currency of cells) (ii) the activation of nucleases that cleave DNA and degrade cell chromatin; and (iii) the promotion of Ca^{2+} dependent proteases, phosphatases, and phospholipases, resulting in a loss of chromatin structural integrity [23]. Increases in Ca^{2+} influx, for instance, could potentially trigger the activation of PA_2 resulting in the production of arachidonic acid (AA). Because AA is rapidly converted to thromboxanes and leukotrines through the 5-lipoxygenase pathway, superoxides could be generated through this mechanism [24]. In this study, we were able to detect an increased release of oxidative products from thymocytes of exhaustively exercised mice; such oxidative products are normally deleterious to membrane unsaturates, leading to lipid peroxide generation as suggested by the findings here. The increase in $[Ca^{2+}]i$ may have additional implications for cellular glutathione levels. Increased $[Ca^{2+}]i$ lowers cellular glutathione content [25] resulting in compromised cellular antioxidant reserved capacity.

In conclusion, we have observed an increased presence of estradiol on membranes of thymocytes of exercised relative to control mice. We hypothesize that the estradiol-receptor binding may have opened the receptor-operated channel for Ca^{2+} entry into these cells, resulting in the observed increase in intracellular Ca^{2+} levels. The findings point to an associated release of oxidative products in the thymus as a function of exercise treatment, with consequent oxidative damage. The physiological consequences of increased estradiol levels on thymocyte membranes and the higher intracellular Ca^{2+} levels in thymocytes after exhaustive exercise for immune function remain, however, to be elucidated.

Acknowledgements — This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to L.H-G. The authors thank Andrew Aldred for technical assistance.

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ABBREVIATIONS

Ca^{2+}—calcium ions
STOCs—spontaneous transient outward currents
STICs—spontaneous transient inward currents
PLA_2—phospholipase A_2
RTE—[treadmill] run to exhaustion
PBS—phosphate buffered saline
AM—acetoxymethyl
AA—arachidonic acid
H_2O_2—hydrogen peroxide
IP_3—inositol triphosphate
ATP—adenosine triphosphate