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17β-Estradiol Increases Ca^{2+} Influx and Down Regulates Interleukin-2 Receptor in Mouse Thymocytes

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The influx of Ca^{2+} across the T lymphocyte membrane is an essential triggering signal for activation and proliferation by an antigen. The aim of this study was to determine if Ca^{2+} influx through estradiol receptor (ER) operated channels of Ca^{2+} entry induced activation of lymphoid cells. Mouse thymocytes were incubated with 17β-estradiol (E) and in the presence or absence of the mitogen, phytohemagglutinin (PHA). Despite evidence of an enhanced binding of E to ER on thymocyte membranes, and an E dose-related influx of Ca^{2+}, there was a consistent down regulation of IL-2 receptor expression ($P < 0.001$). Incubation of thymocytes with PHA enhanced IL-2 receptor expression although the down regulatory effect of E was still evident. The results suggest that the Ca^{2+} channel activated by E may have a down regulatory effect on the IL-2 receptor in thymus cells leading to the dampening of cell activation process.

Key Words: **intracellular Ca^{2+}; estrogen; thymocytes; interleukin-2 receptor.**

Recent evidence indicates that T lymphocytes contain estrogen receptors (ER) on their plasma membranes. Ca^{2+} influx events exerted by plasma membrane impermeable estradiol conjugated to BSA (1) and direct detection of estradiol on T cell membranes (2) have been described. The binding of estrogen to membrane ER is associated with Ca^{2+} influx from the extracellular environment and Ca^{2+} release from the intracellular stores, a process that appears to be independent of the classical nuclear estrogen receptors (3). Estrogen and its metabolites have effects on cell growth, differentiation and function, events that vary with the target tissue (4). Alteration in Ca^{2+} signalling pathways, such as those associated with increases in cytosolic free Ca^{2+} concentrations in uterine endometrial cells (5), rat osteoblasts (6), and human prostate cancer cells (7), are involved in growth, differentiation, and functional events.

One of the early consequences of antigen or mitogen-induced activation of matured resting T cells is the expression of high-affinity IL-2 receptor and a subsequent decline in high-affinity receptor display which probably contribute to the normal termination of the T cell immune response (8). The early stages of T cell development may be regulated by chemical substances to which these cells have receptors. A recent demonstration of ER on the T lymphocyte membrane (1, 2) suggests that estradiol may be a possible candidate in this respect. If this is so, IL-2R may play an important pathogenic role in human lymphoid malignancies and immunodeficiency syndromes which are either enhanced or regulated by 17 β-estradiol. There are important data already on T lymphocyte activation through many traditional second messengers signal transduction events including: (i) the hydrolysis of phosphotidylinositols and the subsequent release of inositol triphosphate (IP3) (9) and IP3 evoked intracellular free calcium mobilization (10, 11) and (ii) activation of protein kinase C and the induction of guanylate or adenylate cyclase biochemical pathways (12, 13). Inhibitors and activators of calcium channels have modulatory roles on cellular function in a manner related to the activity of the calcium channel concerned (11). The role of calcium signalling events in T cell function regulation therefore cannot be overemphasized.

The rise in intracellular Ca^{2+} concentration $[Ca^{2+}]i$ constitutes an essential triggering signal for T cell differentiation and proliferation (14). $[Ca^{2+}]i$ must remain elevated for several hours to commit T cells to the activation pathway, a period during which Ca^{2+} influx is required (15). We have previously shown an increase in estradiol binding to thymocyte membrane, an increase in thymocyte intracellular calcium, and the production of lipid peroxide and oxidative products in the thymus of female mice given exhaustive exercise (2, 16). Those findings suggested that exercise associated

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TABLE 1

Free Estradiol Concentrations Detected in Whole Thymocytes and Thymocyte Plasma Membranes Exposed to Varying Concentrations of Estradiol

	17β-Estradiol concentration in culture medium (pg/ml)			
	4.2	7.2	13.2	31.2
Thymocytes (fg/10^6 cells)[a]	650 ± 16	899 ± 15	1113 ± 29	1535 ± 23
Thymocyte membrane (fg/10^6 cells)[b]	475 ± 21	786 ± 14	954 ± 22	1068 ± 19

[a] $P < 0.001$ for differences across concentrations of estradiol.
[b] $P < 0.001$ for differences across concentrations of estradiol.

increases in tissue estrogen might modulate the function of thymocytes through calcium signalling. The purpose of the present study was to further explore the relationship between estradiol exposure and intracellular Ca^{2+} changes in thymocytes with a correlate of function, i.e., IL-2 receptor expression.

MATERIALS AND METHODS

C57Bl/6 mice (Harlan Sprague Dawley, Frederick, MD), age 5–7 weeks of age, were sacrificed by pentobarbitol overdose to allow cardiac exsanguination. Thymuses were excised, cells dispersed through nylon mesh into small tissue culture dishes containing calcium and magnesium free phosphate buffered saline (PBS), rapidly counted, and seeded into sterile 12 well plates at a concentration of 1×10^6 cells/ml. Thymus cell cultures were maintained in RPMI 1640 medium (without phenol red) supplemented with 5% fetal calf serum (FCS). Four replicate trials were conducted for each *in vitro* experiment. Cell viability at the point of seeding was determined by trypan blue exclusion and was >98%.

Thymocyte cultures were maintained such that the final concentrations of 17β-estradiol in medium were 4.2, 7.2, 13.2, and 31.2 pg/ml. The lowest concentration (4.2 pg/ml) of estradiol was provided by the FCS, without exogenous estrogen; this concentration corresponded to physiological anestrus. Because cycling female rodents are not completely without estradiol *in vivo,* the lowest concentration of hormone in culture was 4.2 pg/ml and, thus, served as the "control." Other concentrations of estradiol (13.2 and 31.2 pg/ml) corresponded to diestrus and proestrus, respectively (17, 18, 19). Cells were incubated with hormone for 1 h at 37°C, removed from culture, washed rapidly in PBS, and packed for determinations of estradiol in thymocytes and thymocyte membranes (16).

Cell and membrane bound estradiol radioimmunoassays were done by first extracting the estradiol content in diethyl ether. The extract was reconstituted in zero calibrator A supplied with estradiol assay kit (Diagnostic Products Corporation, Los Angeles, CA). The extract was treated as described in the manual supplied along with kit. In brief, 100 μL of extract, control, and zero calibrator A were directly pipetted to the bottom of estradiol Ab-coated tubes. 1.0 mL of ^{125}I estradiol was added and vortexed. The mixture was incubated for 3 h at room temperature. All excess content of tube was decanted and tube was counted for 1 min in a gamma counter. Estradiol levels were reported in fg/1×10^6 cells.

Imaging of $[Ca^{2+}]$i was carried out by seeding thymocytes into 12 well plates, treating with hormones as described above, and recovered from culture at 20 min intervals, after the addition of estradiol. At each time point, cells were rapidly loaded with cell permeant acetoxymethyl (AM) ester of Fluo 3 calcium indicator (Molecular Probes, OR) at a final concentration of 4 M Fluo 3 per ml, and then incubated at 27°C for 30 min. Cells were washed by centrifugation and resuspended in 1 ml of Ca^{2+} free PBS and incubated in the dark for 30 min at 27°C. Photon readings were taken using a Shimadzu RF-1501 spectrofluorophotometer at an excitation wavelength of 488 nm and emission wavelength of 530 nm. The $[Ca^{2+}]$i was computed from the equation $[Ca^{2+}]i = kd \times [F - Fmin]/[Fmax - F]$. The dissociation constant for Fluo 3 − Ca^{2+} complex (kd), Fmax and Fmin were determined in our laboratory using calcium calibration buffer kit (Molecular Probes, OR).

For IL-2 receptor expression, thymocytes were seeded at a density of 5.0×10^5/ml into RPMI 1640 medium with 5% FBS and incubated for 16 h with varying concentrations of estradiol. After incubation, samples were pooled to a concentration of 3.0×10^6/ml and washed in PBS, resuspended, and treated with phycoerythrin (PE)-conjugated rat anti-mouse CD25 (IL-2 receptor) monoclonal antibody (Pharmingen, Burlington, Canada) at a final concentration of 1 μg per 10^6 cells. Thymocytes were incubated for 30 min at 4°C, washed, and resuspended in Ca^{2+} free PBS. Photon readings were recorded at excitation wavelength of 488 nm and an emission wavelength of 576 nm. In other experiments, where it was necessary to show the extent of 17β-estradiol inhibitory effect on T lymphocyte proliferation, PHA stimulation was adopted by treating thymocytes for 16 h with mitogen at a concentration of 2.5 μg/1.0×10^6 cells together with varying doses of estradiol. After incubation the expression of IL-2 receptor was monitored by spectrofluorometry, a technique which has been shown to give highly reproducible results and is comparable to flow cytometry for the expression of receptors on lymphocytes (20).

Data were analysed using a two way (concentration × time) analysis of variance (ANOVA) model for intracellular calcium-estradiol experiments; one-way ANOVA was used to analyze IL-2 receptor data. All values represent group means ± 1 standard error. A P value of <0.05 was indicative that results were significantly different from chance.

RESULTS

Table 1 shows that estradiol levels in thymocytes and thymocyte plasma membranes increased significantly with increasing concentrations of estradiol in culture ($P < 0.001$ for whole thymocytes and plasma membranes, respectively). There was significantly greater uptake of estradiol in whole thymocytes preparations compared with estradiol uptake on thymocyte plasma membranes ($P < 0.01$).

$[Ca^{2+}]$i levels in thymocytes treated with increasing concentrations of estradiol *in vitro* are shown in Table 2. There was a significant dose effect of estradiol concentration on $[Ca^{2+}]$i levels ($P < 0.001$). There was a significant time effect on the influx of $[Ca^{2+}]$i ($P < 0.001$), and a marginally nonsignificant interaction effect ($P = 0.09$) of estradiol concentration by time. At

TABLE 2

Intracellular Ca^{2+} Levels (nM) in Thymocytes after Exposure to Varying Concentrations of Estradiol

Estradiol concentration (pg/ml)[b]	Thymocyte intracellular calcium ion (nM) at time (min)[a]				
	20	40	60	80	100
4.2	58.2 ± 2.0	71.3 ± 2.8	89.3 ± 1.8	68.6 ± 1.1	57.9 ± 0.3
7.2	75.6 ± 1.8	109.0 ± 3.2	132.2 ± 2.2	104.5 ± 2.5	80.6 ± 1.2
13.2	152.5 ± 6.3	210.9 ± 3.0	222.7 ± 12.4	193.2 ± 1.9	169.6 ± 9.3
31.2	184.1 ± 1.8	352.6 ± 15.8	458.2 ± 16.9	377.2 ± 8.0	202.7 ± 7.7

[a] $P < 0.001$ for differences across time in minutes.
[b] $P < 0.001$ for differences across estradiol concentrations.

the lowest concentration of estradiol (at 4.2 pg/ml), there was little change in $[Ca^{2+}]i$ over time. At the intermediate (7.2, 13.2 pg/ml) and high (31.2 pg/ml) concentrations of estradiol, $[Ca^{2+}]i$ reached a maximum at 60 min, after which $[Ca^{2+}]i$ declined, with values at 100 min equivalent to the initial 20 min influx.

IL-2 receptor expression in thymocytes exposed to increasing concentrations of estradiol is shown in Fig. 1. There was a significant effect of estradiol concentration on the expression of IL-2R in thymocytes ($P < 0.001$). Values for "unstimulated" (without mitogen) thymocyte IL-2R expression were in photon units: 75.7 ± 1.2 (4.2 pg/ml residual estradiol), 63.6 ± 4.9 (7.2 pg/ml estradiol), 57.8 ± 2.5 (13.2 pg/ml estradiol) and 48.9 ± 2.4 (31.2 pg/ml estradiol). As estradiol concentration in thymocyte culture increased, IL-2R expression on thymocytes decreased.

As shown in Fig. 2, there was a significant effect on IL-2 receptor expression in thymocytes stimulated with PHA mitogen ($P < 0.001$). Estradiol appeared to down-regulate IL-2R expression as a function of increasing concentration of the hormone *in vitro.*

DISCUSSION

Calcium signalling is an intracellular communication system widely used by cells and changes in cytosolic Ca^{2+} concentration in cells have been associated with a variety of cellular processes (11). $[Ca^{2+}]i$ can be changed transiently in response to a stimulus, through a set of channels and pumps allowing Ca^{2+} fluxes across membranes. Events preceding induction of T-lymphocyte activation have been associated with increases in intracellular Ca^{2+} (21). The results of this study suggest that thymic lymphocytes have plasma membrane receptors for estrogen, a finding which is in agreement with previous findings (1). There was an increase in the binding of estrogen to plasma membrane of thymocytes in a dose-related fashion. $[Ca^{2+}]i$ in thymocytes showed a parallel increase, with increas-

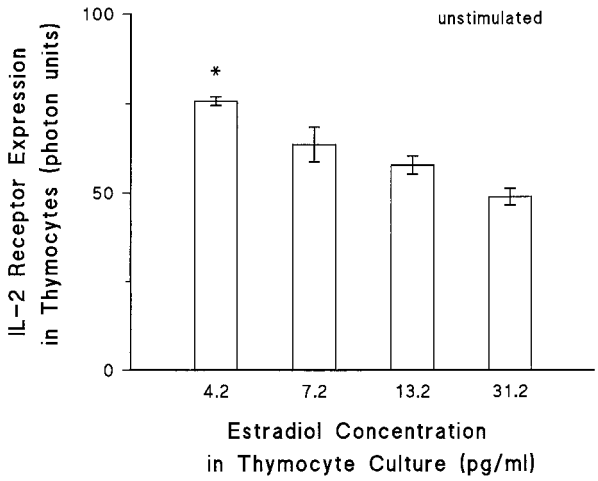

FIG. 1. Interleukin-2 receptor expression (photon units) in mouse thymocytes treated with varying concentrations of estradiol in culture. $P < 0.001$ for main effect of estradiol on IL-2R expression. See text for details of statistical analysis.

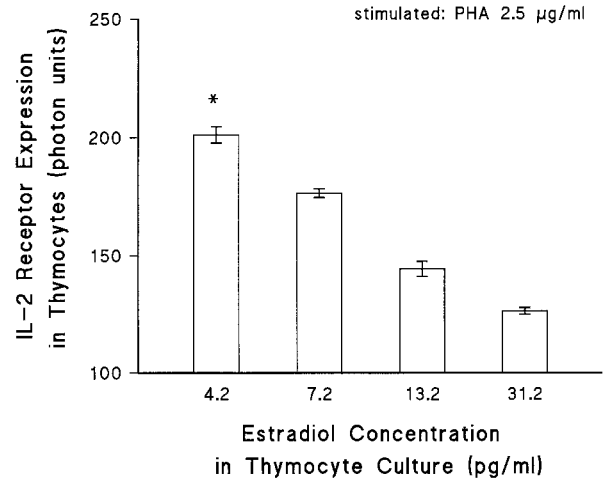

FIG. 2. Interleukin-2 receptor expression (photon units) in mouse thymocytes treated with varying concentrations of estradiol and activated with the mitogen, phytohemagglutinin (PHA). $P < 0.001$ for main effect of estradiol on IL-2R expression in the presence of mitogen. See text for details of statistical analysis.

ing estrogen concentrations. These findings suggest that estrogen can act as a calcium ionophore and may be important in cellular regulatory processes in thymocytes. However, estradiol produced a down regulation of IL-2R in a dose-related manner, an observation which was persistent despite the use of PHA mitogenic stimulation. The details of how this happened are not known but estradiol–estradiol receptor interactions induce Ca^{2+} fluxes in a dose-related manner; an increase in intracellular Ca^{2+} may activate cellular kinases, thus leading to phosphorylation processes. Cellular kinase signalling processes are known to initiate IL-2R cytoplasmic residue phosphorylation (22). This may account for the observed down regulation of Il-2R activity in estradiol treated cells. While the implications of the apparent estradiol mediated modification of Il-2R expression on thymus cells are yet to be understood, these observations imply that there is a functional link between estradiol–estradiol receptor interaction and IL-2R. The data presented here suggest that: (i) estradiol induced change in cellular redox status (2) may be related to IL-2R down-regulation by way of Ca^{2+} signalling induced phosphorylation processes explained above, and (ii) estradiol as a steroid may have a contributory role in down-regulating inappropriate proliferative signals that are elicited in disease conditions associated with IL-2R hyperfunction e.g., T-cell leukaemia. It may also have a role in the general homeostatic regulation of IL-2R cell clonal expansion.

Estrogen has a regulatory effect *in vitro* on a variety of lymphocyte functions, including cellular cytotoxicity (23). For example, Chao *et al.* (24) found that estrogen at low or at high physiological levels significantly inhibited the release of tumor necrosis factor by macrophages; among the mechanisms hypothesized for the inhibition of TNF production by macrophages were estrogen effects on arachidonate and prostaglandin metabolism. Free-radical induced increases in $[Ca^{2+}]i$ have been related to early events in lymphocytes activation (21). Estrogen-associated increases in $[Ca^{2+}]i$ and the generation of membrane lipid peroxide have been described for lymphoid cells (2). The present findings suggest that estrogen, acting as a $[Ca^{2+}]$ ionophore, reduces expression of IL-2R; this may be mediated by a pro-oxidant role of estrogen, with resultant thymocyte membrane oxidation (e.g., lipid peroxide formation).

In summary, estradiol may be a physiological moderating agent in the expression of IL-2 receptor by thymus lymphocytes, with the possibility of generating a pronounced Ca^{2+} influx. The concentrations of estradiol used were within the physiological range of cycling female rodents. This Ca^{2+} influx may provide an early signal associated with down-regulation of the IL-2R protein on thymus cell membranes. Investigations aimed at specifically defining calcium channels involved in the signalling process and the mechanisms by which estradiol mediates the IL-2R response are currently underway.

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