

Effect of 17 β -estradiol on tumor necrosis factor- α -induced cytotoxicity in the human peripheral T lymphocytes

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Abstract

We determined the effect of 17 β -estradiol on tumor necrosis factor α (TNF- α)-induced cytotoxicity in human peripheral T lymphocytes (T cells) using lactate dehydrogenase assay. Treatment with 17 β -estradiol (1–100 nM) for 24 h showed dose-dependent reduction of TNF- α -induced cytotoxicity in T cells. To further evaluate the mechanism of 17 β -estradiol on TNF- α -induced cytotoxicity in T cells, we identified estrogen receptor (ER) protein in T cells using immunocytochemistry and used the pure ER antagonist ICI 172,780. ER α immunoreactivity was clearly observed in T cells. ER β immunoreactivity was also detected in some T cells. ICI 172,780 (10^{-7} M) alone did not affect cytotoxicity in T cells, however, ICI 172,780 (10^{-7} M) completely abolished 17 β -estradiol cytoprotective effects in T cells. TNF- α tended to increase nuclear factor κ B (NF- κ B) protein

levels in nuclear extracts but it did not reach statistical significance by Western blotting. In contrast, NF- κ B protein levels in nuclear extracts followed by TNF- α with 17 β -estradiol treatment were significantly increased compared with NF- κ B protein levels in untreated group. NF- κ B blocker pyrrolidinedithiocarbamate (PDTC) (10^{-4} M) alone did not affect cytotoxicity in T cells. In contrast, PDTC (10^{-4} M) completely abolished 17 β -estradiol cytoprotective effects in T cells. Caspase -3/-7 activity was significantly increased followed by TNF- α , and 17 β -estradiol treatment significantly reduced the increment. The present studies suggest the protective effect of 17 β -estradiol on TNF- α -induced cytotoxicity through ERs in T cells and that NF- κ B activation and caspase suppression may be involved in the mechanism.

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Introduction

It has become apparent over the past few decades that estrogens can affect cells of the immune system and may play a role in modulating lymphocyte development and function (Smithson *et al.* 1998, Grimaldi *et al.* 2002). It appears that premenopausal women are protected against atherosclerosis compared with men, and it is this gender difference that initially led investigators to propose that estrogens may well be protective against cardiovascular disease (Bush 1990). Although favorable effects on lipid profiles have been thought to account for a significant portion of estrogen's protective action, recent studies suggest that estrogens may have important actions at other steps in the atherogenic process (Nathan & Chaudhuri 1997). *In vitro* studies showed that the addition of estradiol to cultures of human lymphocytes causes a decrease in CD4⁺/CD8⁺ T-cell-subset ratios (Athreya *et al.* 1993), and enhances immunoglobulin secretion (Kanda & Tamaki 1999). However, the precise mechanism by which estrogens may be exerting this protective action remains unclear.

Survival and death in lymphoid cells are tightly regulated processes, and alteration of the delicate balance between the two can lead to a variety of autoimmune and inflammatory diseases (He & Ting 2002). Members of the tumor necrosis factor (TNF) superfamily are critical regulators of the balance between the two opposing processes, and binding to their cognate receptors can trigger signaling pathways leading to either survival or death (Locksley *et al.* 2001). Indeed, in the case of TNF receptor 1 (TNFR1), ligation of a single class of receptor can trigger either cell survival or apoptosis, depending on which signaling pathway predominates in the cell type examined. Upon binding of TNF- α to TNFR1, the death domain present in the cytoplasmic tail of the receptor is able to recruit and associate with two death-domain-containing signaling molecules known as TNFR1-associated death domain (TRADD) and receptor-interacting protein (RIP; Hsu *et al.* 1995, 1996a, 1996b). Both TRADD and RIP are required for signaling to nuclear factor κ B (NF- κ B), as well as the apoptosis pathway (Hsu H. *et al.* 1995, 1996b, Ting *et al.* 1996, Pimentel-Muinos & Seed 1999). It is at this juncture that the NF- κ B and apoptotic signals are

thought to bifurcate: TNF receptor-associated factor (TRAF) 2 recruitment leads to the activation of downstream kinases and NF- κ B activation, whereas Fas-associated death domain (FADD) recruitment leads to the activation of caspase-8 and apoptosis (Hsu H. *et al.* 1996a, Liu *et al.* 1996). A recent report demonstrated that the enhancing role of estrogens on the immune/inflammatory response was exerted by activating the NF- κ B complex (Cutolo *et al.* 2003).

In order to investigate the effect of estrogens on TNF- α -induced cytotoxicity in human peripheral T lymphocytes (T cells), we first determined the effect of 17 β -estradiol on TNF- α -induced cytotoxicity in T cells using lactate dehydrogenase (LDH) assay. Next, estrogen receptor (ER) protein in T cells was identified using immunocytochemistry and we investigated the involvement of ER for 17 β -estradiol activity on TNF- α -induced cytotoxicity in T cells. Third, NF- κ B protein levels after TNF- α with 17 β -estradiol treatment were examined and we used a NF- κ B blocker, pyrrolidinedithiocarbamate (PDTC) to evaluate whether the NF- κ B cascade was involved in the intracellular mechanism of 17 β -estradiol on TNF- α -induced cytotoxicity in T cells. Finally, caspase-3/-7 activity was investigated after TNF- α with 17 β -estradiol treatment.

Materials and Methods

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood samples from healthy women (28.2 \pm 1.1 years, $n=6$) by density-gradient centrifugation with Ficoll-Paque and washed three times with PBS. For isolation of T cells, PBMCs were cultured in plastic dishes for 1 h at 37 °C with 5% CO₂ and non-adherent cells were collected. The purity of CD3-positive cells was about 95% as determined by flow cytometry. The obtained cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin (100 U/ml)/streptomycin (100 mg/ml).

TNF- α and 17 β -estradiol treatment

T cells were plated on 96-well plates (10 \times 10³ cells/well; Beckton Dickinson Laboratories, Franklin Lakes, NJ, USA) for cytotoxicity studies. 2 days later, the media in 96-well plates were washed and replaced with fresh RPMI 1640 media without fetal bovine serum. Various doses of water-soluble 17 β -estradiol (Sigma Chemical Co.) with TNF- α (10 nM; Dainippon Pharmaceutical Co., Osaka, Japan) were added in the media 24 h prior to the LDH assay. TNF- α concentration (10 nM) was determined as described in a previous paper (Manna *et al.* 2000). In some experiments, 17 β -estradiol/TNF- α were added to the

culture 24 h prior to the LDH assay with pure ER antagonist ICI 172,780 (Tocris Cookson, Bristol, UK) or the NF- κ B blocker PDTC (Sigma Chemical Co.).

LDH assay

Cytotoxicity was assessed as a function of LDH release in 96-well plates after 24 h 17 β -estradiol/TNF- α treatment. Prior to each assay, the cells were lysed with 1% Triton X-100 in culture media for 10 min at 37 °C to obtain a representative maximal LDH release for that particular culture as the positive control with 100% toxicity. Cellular supernatant (50 μ l) were removed from each well and transferred to another 96-well plate to determine LDH release of the culture. 50 μ l reconstituted substrate mix (Promega) was added to each well for 10 min and measured on a microplate reader at 490 nm. Total LDH released by culture (i.e. 100% toxicity) in all plates tested had an absorbance in the range of 1.0–1.5 at 490 nm. The spontaneous release of LDH in untreated cells (without estradiol and TNF- α , i.e. low control) was significantly higher than that of culture media alone. As a consequence, the percentage of cytotoxicity was determined using following formula (Bursztajn *et al.* 2000):

$$\text{Cytotoxicity} = (\text{experimental value} - \text{low control}) / (\text{positive control} - \text{low control}) \times 100$$

Immunocytochemistry

T lymphocytes (5 \times 10³ cells/well) were plated on two-well culture slides (Asahi Technoglass Corp., Tokyo, Japan). The cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100, 0.01% sodium azide and 1% BSA in PBS and blocked with 10% goat serum in PBS for 15 min. The cells were incubated overnight at 4 °C with the following antibodies; anti-CD3 (Calbiochem-Novabiochem Corp., San Diego, CA, USA), anti-CD14 (Zymed Laboratories, San Francisco, CA), anti-ER α (mouse monoclonal IgG1, 1:200; Stress-Gen Biotechnologies, Victoria, Canada), anti-ER β (rabbit polyclonal IgG, 1:200; Affinity BioReagents, Golden, CO, USA), anti-type I TNF- α receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-NF- κ B (p65; Santa Cruz Biotechnology). For detection of primary antibodies, secondary antibodies (Dako A/S, Glostrup, Denmark) were used at a dilution of 1:200. Negative controls for each experimental condition consisted of appropriate dilutions of secondary antibodies alone. The coverslips mounted with Eukitt medium (O. Kindler, Freiburg, Germany) were examined under an Olympus microscope and digitized using the appropriate software (Lumina Vision, Mitani-Corp., Fukui, Japan).

Immunoblotting

The nuclear extracts (Dudek *et al.* 2001) for NF- κ B from control (medium only) or treated cells (TNF- α or

estradiol/TNF- α ; 10 μ g protein) were mixed in electrophoresis sample buffer and were boiled for 5 min and electrophoresed by SDS/PAGE using 10% linear gel. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes followed by blocking with 5% skimmed milk for 60 min at room temperature. The membrane was incubated with anti-NF- κ B at a final concentration of 1:200 in 1 \times TBS solution (0.15 M NaCl/20 mM Tris-HCl, pH 7.6) overnight at 4 $^{\circ}$ C. Subsequently, the membranes were washed for 5 min, incubated with biotinylated F(ab')₂ fragment of rabbit anti-mouse or swine anti-rabbit immunoglobulin as secondary antibodies (Dako A/S) for 30 min at room temperature, and then washed again. The membranes were incubated with streptavidin-horseradish peroxidase conjugate (Gibco BRL, Life Technologies) for 20 min at room temperature and washed in TTBS (TBS+Tween-20) for 5 min and TBS for 10 min. The bands were visualized by an enhanced chemiluminescence (ECL) detection reagents (Amersham) and quantitated using a computer-assisted image analyzer (Kodak 1D Image Analysis Software, Scientific Imaging Systems, New Haven, CT, USA).

Measurement of caspase-3/-7 activity

Caspase-3/-7 activity was measured by Apo-ONE homogenous caspase-3/-7 assay (Promega) according to the manufacturer's instructions. In brief, 100 μ l Apo-ONE caspase-3/-7 reagent was added to each well of a black 96-well plate containing 100 μ l blank, control cells (medium only) or treated cells (TNF- α or estradiol/TNF- α) in culture. The contents of wells were gently mixed using a plate shaker at 300 r.p.m. from 30 s. The fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Data analysis

Data were analyzed by ANOVA followed by Fisher's protected least-squares difference test.

Results

Effect of 17 β -estradiol on TNF- α -induced cytotoxicity in T cells

TNF- α treatment (10 nM) revealed 10–30% cytotoxicity in T cells. 17 β -estradiol treatment (1–100 nM) showed dose-dependent decreases of TNF- α -induced cytotoxicity in T cells (Fig. 1). In contrast, 17 β -estradiol treatment (1000 nM) did not affect TNF- α -induced cytotoxicity in T cells (Fig. 1).

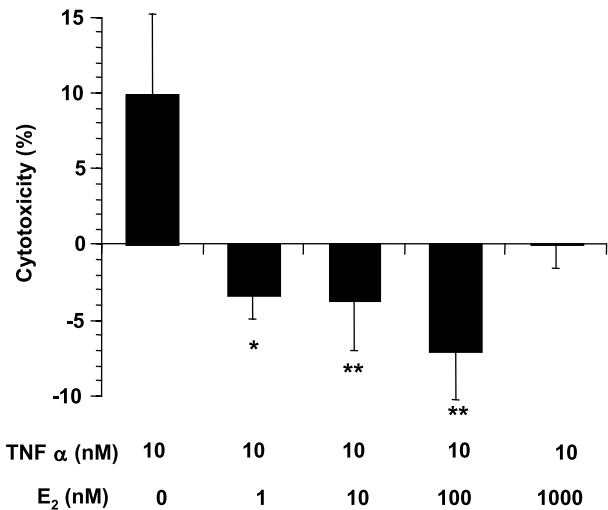


Figure 1 Effect of 17 β -estradiol (E₂) on TNF- α -induced cytotoxicity in T cells using LDH assay. The percentage of cytotoxicity was determined using the formula given in the Materials and Methods section and a low percentage means a low cytotoxicity. Data represent means \pm S.E.M. (n=6). The same experiment was performed three times and showed identical results. * and ** denote significant alterations at $P < 0.05$ and $P < 0.01$ respectively when compared with TNF- α without the 17 β -estradiol group as determined by one-way ANOVA followed by Fisher's protected least-squares difference test. The data shown are from a representative experiment.

Identification of ER α and ER β in T lymphocytes using immunocytochemistry

ER α and ER β immunoreactivity were clearly observed in T cells by immunocytochemistry. ER α and/or ER β immunoreactivity were colocalized with some CD3-positive cells (Fig. 2A). ER β immunoreactivity was co-localized with some ER α -positive cells (Fig. 2B). The percentages of ER α , ER β , ER α /ER β and no ER α /no ER β -positive cells are shown in Table 1. In addition, type 1 TNF- α receptor immunoreactivity was observed in T cells using immunocytochemistry (data not shown).

Effect of the ER antagonist ICI 172,780 on 17 β -estradiol with TNF- α in T cells

ER antagonist, ICI 172,780 (100 nM) alone did not affect cytotoxicity in T cells (data not shown). 17 β -estradiol treatment (100 nM) significantly decreased TNF- α -induced cytotoxicity in T cells. In contrast, ICI 172,780 (100 nM) completely abolished 17 β -estradiol cytoprotective effects in T cells (Fig. 2C).

Effect of 17 β -estradiol with TNF- α on NF- κ B protein levels from the nuclear extracts in T cells

In order to evaluate the modulation of NF- κ B protein followed by TNF- α /17 β -estradiol treatment, we examined

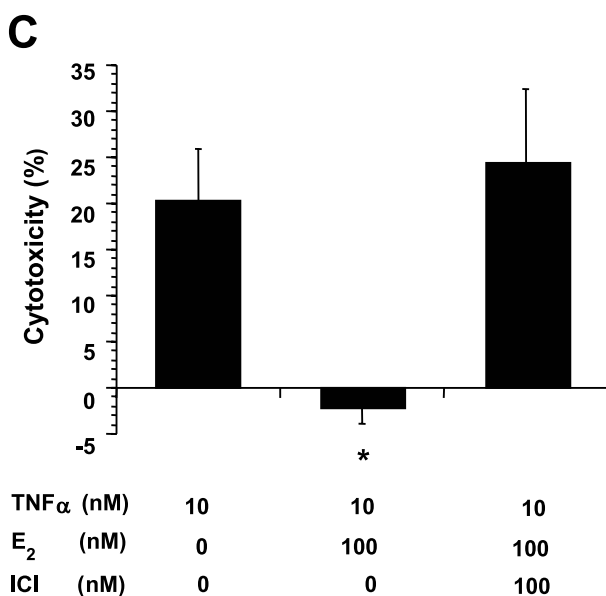
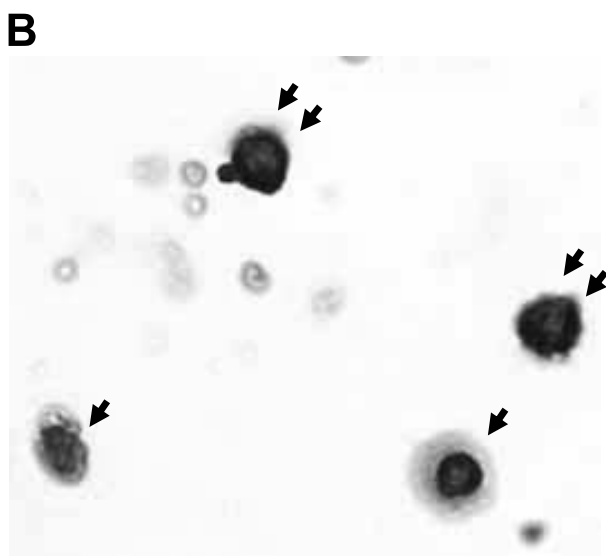


Table 1 Percentages of each cell type. The separate counts were performed three times on 300 cells and data represent means \pm s.d.

Immunoreactivity	% of immunopositive cells
ER α only	76.3 \pm 9.6
ER β only	1.0 \pm 0.58
ER α and ER β	8.7 \pm 3.2
No ER α and no ER β	14.0 \pm 6.1

NF- κ B protein levels in the nuclear extracts using Western blotting. TNF- α tended to increase NF- κ B protein levels in the nuclear extracts but it did not reach statistical significance. In contrast, NF- κ B protein levels followed by TNF- α with 17 β -estradiol treatment were significantly increased compared with NF- κ B protein levels in untreated group (Fig. 3).

Effect of NF- κ B blocker PDTC on 17 β -estradiol with TNF- α in T cells

NF- κ B blocker PDTC (10^{-4} M) alone did not affect cytotoxicity in T cells (data not shown). 17 β -estradiol treatment (100 nM) again clearly decreased TNF- α -induced cytotoxicity in T cells. In contrast, PDTC (10^{-4} M) completely abolished 17 β -estradiol cytoprotective effects in T cells (Fig. 4).

Effect of 17 β -estradiol with TNF- α on caspase-3/-7 activity in T cells

We examined the regulation of caspase-3/-7 activity after TNF- α /17 β -estradiol treatment in T cells. TNF- α significantly increased caspase-3/-7 activity. 17 β -estradiol treatment significantly reduced TNF- α -induced caspase-3/-7 activity (Fig. 5).

Discussion

Regulation of apoptosis is considered an important mechanism for controlling the number of monocytes

Figure 2 Identification of ER α and ER β in T cells using immunocytochemistry. (A) CD3- and ER α -positive cells are indicated by double arrows. In contrast, cells stained only for CD3 are shown by a single arrow. (B) Both ER α - and ER β -positive cells are indicated by double arrows. T cells stained only for ER α are shown by single arrows. Note, the number of ER α -positive cells was greater than that of ER β -positive cells. (C) Effect of ER antagonist, ICI 172,780 (ICI) on 17 β -estradiol (E₂)/TNF- α treatment in T cells. Data represent means \pm s.e.m. ($n=6$). * denotes significant alterations at $P<0.05$ when compared with the group treated with TNF- α without 17 β -estradiol, as determined by one-way ANOVA followed by Fisher's protected least-squares difference test.

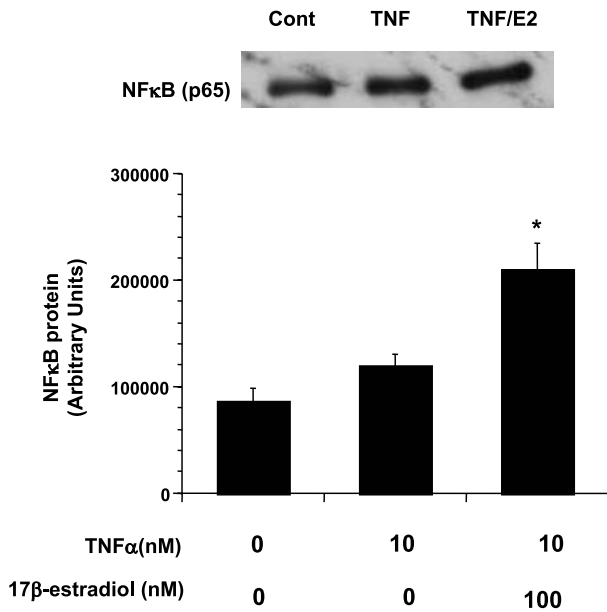


Figure 3 Effect of TNF-α with or without 17β-estradiol (E2) on NF-κB protein levels in T cells using Western blotting. A representative example of Western blotting and statistical analysis of NF-κB protein levels followed by TNF-α/17β-estradiol is shown. Data represent means ± S.E.M. (n=3). * denotes significant alterations at P<0.01 when compared with control group (Cont) as determined by one-way ANOVA followed by Fisher's protected least-squares difference test. Separate experiments were performed three times and showed identical results.

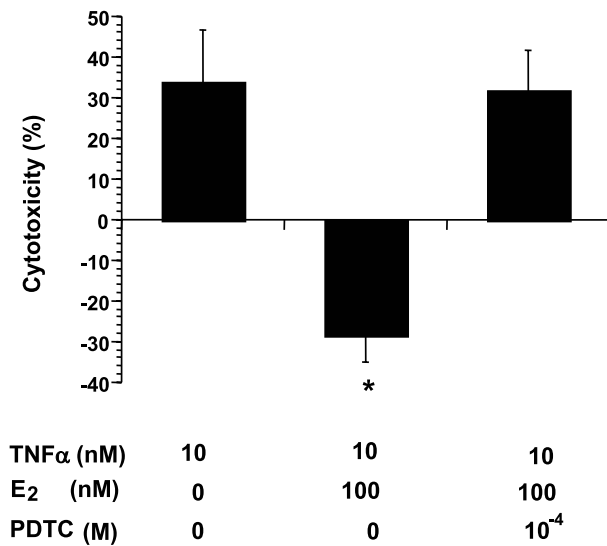


Figure 4 Effect of NF-κB blocker PDTC on 17β-estradiol (E₂)/TNF-α treatment in T cells. Data represent means ± S.E.M. (n=6). * denotes significant alterations at P<0.01 when compared with the group treated with TNF-α but without 17β-estradiol as determined by one-way ANOVA followed by Fisher's protected least-squares difference test.

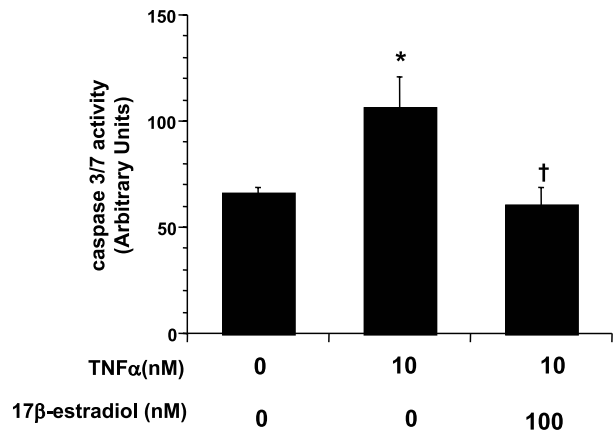


Figure 5 Effect of TNF-α with or without 17β-estradiol on caspase-3/-7 activity in T cells. Data represent means ± S.E.M. (n=3). * and † denote significant alterations at P<0.05 when compared with the control group and the TNF-α without 17β-estradiol group, respectively, as determined by one-way ANOVA followed by Fisher's protected least-squares difference test.

available and therefore the intensity of the physiological response to infection or wound healing. Failure in the control of programmed cell death may become particularly relevant in pathological conditions such as tumor growth, and autoimmune and chronic inflammatory disorders, where apoptosis has been identified as a key factor in disease progression or remission (van der Loosdrecht *et al.* 1993, Mountz *et al.* 1994, Deshpande *et al.* 1997, Thatte & Dahanukar 1997). It has recently been reported that estradiol treatment resulted in a dose-dependent, receptor-mediated inhibition of TNF-α-induced endothelial cell apoptosis, providing another mechanism that may account for the atheroprotective effect of estrogens (Spyridopoulos *et al.* 1997).

In the present study, TNF-α treatment (10 nM) showed 10–30% cytotoxicity in T cells. 17β-estradiol treatment (1–100 nM) dose dependently decreased TNF-α-induced cytotoxicity in T cells. These observations are in agreement with the report that treatment with estradiol or progesterone increased survival and prevented apoptosis induced by TNF-α in both undifferentiated and macrophage-like phorbol 12-myristate 13-acetate-differentiated U937 cells, as assessed by Trypan Blue-exclusion cell counting, thymidine incorporation and annexin V labeling, followed by flow cytometry and DNA fragmentation studies (Vegeto *et al.* 1999). Estrogens modulate target-cell activity by binding to specific intracellular receptors, which are hormone-regulated transcription factors (Katzenellenbogen *et al.* 1996, Kuiper *et al.* 1996). ER effects have been focused on the ERα identified and cloned several years ago (Green *et al.* 1986, Greene *et al.* 1986).

Recently, a second ER, designated ERβ, has been described and shown to share common structural and

functional characteristics with ER α (Kuiper *et al.* 1996, Mosselman *et al.* 1996). In the present study, ER α and ER β protein were detected in T cells using immunocytochemistry. These observations are in agreement with the report that PBMCs and lymphocytes contain ERs (Carbone *et al.* 1986, Tubiana *et al.* 1986, Weusten *et al.* 1986). During menopause there is a significant decrease in the percentage of ER-positive monocytes, and an increase in blood monocyte number, which declines following estrogen-replacement therapy to values of the young (Ben-Hur *et al.* 1995). The pure ER antagonist ICI 172,780 completely abolished 17 β -estradiol cytoprotective effects in T cells, further supporting the importance of ER for 17 β -estradiol activity on TNF- α -induced cytotoxicity. However, additional studies including cell-surface ER are required to investigate the functional nature of ER for cell protection.

TNF- α -induced signaling is believed to diverge; TRAF2/RIP recruitment leads to activation of downstream kinases in the NF- κ B and c-Jun N-terminal kinase (JNK) pathways, leading to survival, while FADD recruitment leads to apoptosis (Hsu *et al.* 1996a). It is possible that the presence of RIP within the TNFR1 signaling complex could have either prevented or decreased the recruitment of FADD and caspase-8 by competing for binding to TRADD (Harper *et al.* 2003, Hsu H. *et al.* 1996b). In unstimulated cells, NF- κ B is found in cytoplasm and is bound to the inhibitory I κ B- α and I κ B- β , which prevent it from entering the nucleus (Baldwin 1996). The entry of NF- κ B from the cytoplasm to the nucleus is regulated by I κ B, whose induction and binding to NF- κ B prevents the translocation of NF- κ B into the nucleus, where it binds to specific sequences in the promoter regions of target genes (Scheinman *et al.* 1995, Barnes & Karin 1997). A recent study showed that PDTC appears to initiate apoptosis by blocking cytoplasmic NF- κ B translocation to the nucleus and both TNF- α and PDTC alone cause apoptosis and reduce cell growth, but their combined effects are additive in reducing cell growth of DU-145 and ALVA-101 human prostate cancer cells (Gunawardena *et al.* 2002). In the present study, TNF- α with 17 β -estradiol increased NF- κ B protein levels in nuclear extracts and PDTC completely abolished 17 β -estradiol cytoprotective effects in T cells. These results are keeping with the observation by Cutolo *et al.* (2003) that the enhancing role of estrogens on the immune/inflammatory response was exerted by activating the NF- κ B complex. In addition, 17 β -estradiol significantly reduced TNF- α -induced caspase-3/-7 activity in T cells. These observations are in agreement with the report that estrone antagonized not only the N-methyl-D-aspartate (NMDA)-induced caspase-3-like activity, but also NMDA-mediated LDH release in hippocampal neurons (Kajta *et al.* 2004). Furthermore, a recent report demonstrated that preincubation with 17 β -estradiol completely prevented both TNF-related apoptosis-inducing ligand

(TRAIL)-induced DR4 and DR5 upregulation and apoptosis (Cantarella *et al.* 2004), further supporting that 17 β -estradiol suppressed TNF- α -induced apoptosis signaling pathway. Taken together, the reduced cytotoxicity could be a functional consequence of increased NF- κ B and decreased caspase activity by the estrogen.

Although further studies including functional assays of NF- κ B are needed to assess cellular responses to estrogens in T cells, the present studies provide an experimental basis for understanding the protective effect of 17 β -estradiol during inflammation and apoptosis.

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