Salutary effects of 17β-estradiol on T-cell signaling and cytokine production after trauma-hemorrhage are mediated primarily via estrogen receptor-α

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Suzuki T, Shimizu T, Yu HP, Hsieh YC, Choudhry MA, Chaudry IH. Salutary effects of 17β-estradiol on T-cell signaling and cytokine production after trauma-hemorrhage are mediated primarily via estrogen receptor-α. Am J Physiol Cell Physiol 292: C2103–C2111, 2007. First published February 7, 2007; doi:10.1152/ajpcell.00488.2006.—Although 17β-estradiol (E2) administration following trauma-hemorrhage prevents the suppression in splenocyte cytokine production, it remains unknown whether the salutary effects of 17β-estradiol are mediated via estrogen receptor (ER)-α or ER-β. Moreover, it is unknown which signaling pathways are involved in 17β-estradiol’s salutary effects. Utilizing an ER-α- or ER-β-specific agonist, we examined the role of ER-α and ER-β in E2-mediated restoration of T-cell cytokine production following trauma-hemorrhage. Moreover, since MAPK, NF-κB, and activator protein (AP)-1 are known to regulate T-cell cytokine production, we also examined the activation of MAPK, NF-κB, and AP-1. Male rats underwent trauma-hemorrhage (mean arterial pressure 40 mmHg for 90 min) and fluid resuscitation. ER-α agonist propyl pyrazole triol (PPT; 5 μg/kg), ER-β agonist diarylpropionitrile (DPN; 5 μg/kg), 17β-estradiol (50 μg/kg), or vehicle (10% DMSO) was injected subcutaneously during resuscitation. Twenty-four hours thereafter, splenic T cells were isolated, and their IL-2 and IFN-γ production and MAPK, NF-κB, and AP-1 activation were measured. T-cell IL-2 and IFN-γ production was decreased following trauma-hemorrhage, and this was accompanied with a decrease in T-cell MAPK, NF-κB, and AP-1 activation. PPT or 17β-estradiol administration following trauma-hemorrhage normalized those parameters, while DPN administration had no effect. Since PPT, but not DPN, administration following trauma-hemorrhage was as effective as 17β-estradiol in preventing the T-cell suppression, it appears that ER-α plays a predominant role in mediating the salutary effects of 17β-estradiol on T cells following trauma-hemorrhage, and that such effects are likely mediated via normalization of MAPK, NF-κB, and AP-1 signaling pathways.

PREVIOUS STUDIES HAVE SHOWN that splenocyte proliferation and cytokine production are decreased in male animals following trauma-hemorrhage (1, 39, 51). However, such suppression in splenocyte function was not observed in proestrus female animals under those conditions (1). Additional studies have shown that male sex steroids produce deleterious effects on splenocyte function (37), but female sex steroids maintain/enhance the splenocyte functions following trauma-hemorrhage (1). Studies have also indicated that administration of a single dose of 17β-estradiol (E2) following trauma-hemorrhage in males normalizes splenocyte functions (21). It has also been shown that there are two major subtypes of estrogen receptors (ERs), namely ER-α and ER-β (22, 45). Furthermore, studies have indicated a specific role of ER-α and -β in cytokine production by peripheral blood mononuclear cells and other tissue macrophages following trauma-hemorrhage (52). While the effects of 17β-estradiol on splenocytes following trauma-hemorrhage appear to be mediated via ER (20), it remains unknown which subtypes of ER are responsible for producing the salutary effects of E2 on splenic T cells under those conditions. Furthermore, the precise mechanism by which 17β-estradiol mediates its salutary effect following trauma-hemorrhage remains unknown.

Mitogen-activated protein kinases (MAPKs) are signaling molecules that play an important role in the regulation of immune responses including T-cell activation and cytokine production (5, 7, 10, 28). There are three major MAPK-dependent pathways: p38, extracellular-regulated protein kinase (ERK)1/2, and c-Jun NH2-terminal kinase (JNK). All three MAPK families are activated by dual phosphorylation on adjacent threonine and tyrosine residues. In addition to MAPKs, nuclear factor (NF)-κB and activator protein (AP)-1 are also implicated in T-cell cytokine production (10, 17, 40). Furthermore, it is well known that MAPKs activate AP-1 (8, 10, 17, 50), and recent studies have revealed that NF-κB is also activated by MAPKs (3, 18). Thus NF-κB and AP-1 are likely the downstream factors of MAPK signaling pathways for T-cell cytokine production. However, it is not known whether these kinases and transcription factors are involved in mediating the salutary effects of 17β-estradiol on splenic T cells following trauma-hemorrhage. The aim of our study, therefore, was to determine whether the salutary effects of 17β-estradiol on cytokine production by splenic T cells following trauma-hemorrhage are mediated via ER-α or ER-β, and whether MAPKs, NF-κB, or AP-1 plays a role in mediating those salutary effects of 17β-estradiol under those conditions.

MATERIALS AND METHODS

Animals. Adult male (275–325 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. All experiments were performed in adherence to the National Institutes of Health Guidelines for the Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Trauma-hemorrhage procedure. A nonheparinized rat model of trauma-hemorrhage, as described previously, was used in this study (14, 49). Briefly, male Sprague-Dawley rats (275–325 g) were fasted overnight before the experiment but allowed water ad libitum. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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rats were anesthetized by isoflurane (Attane; Minrad, Bethlehem, PA) inhalation before the induction of soft tissue trauma (i.e., 5-cm midline laparotomy). The abdominal incision was then closed in two layers, and polyethylene catheters (PE-50; Becton-Dickinson, Sparks, MD) were placed in both femoral arteries and the right femoral vein. The rats were then placed into a Plexiglas box (21 × 9 × 5 cm) in a prone position and allowed to awaken, after which they were bled rapidly within 10 min to a mean arterial pressure (MAP) of 35–40 mmHg. The time at which the animals could no longer maintain a MAP of 35–40 mmHg without infusion of some fluid was defined as maximum bleed-out time. MAP was maintained at 40 mmHg until 40% of the shed blood was returned in the form of Ringer’s lactate. The animals were resuscitated with 4 × the shed blood volume with Ringer’s lactate over 60 min. Thirty minutes before the end of the resuscitation, the rats received ER-α agonist propyl pyrazole triol (PPT; 5 μg/kg body wt), ER-β agonist diarylpropionitrile (DPN; 5 μg/kg body wt), 17β-estradiol (50 μg/kg body wt), or an equal volume of the vehicle (0.2 ml, 10% DMSO) subcutaneously. Following resuscitation, the catheters were removed, the vessels ligated, and skin incisions closed with sutures. Sham-operated animals underwent laparotomy and the same groin dissection, which included ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. At 24 h after trauma-hemorrhage or sham operation, the rats were anesthetized with isoflurane and exsanguinated to collect samples.

Isolation of splenic T cells. Spleens were removed aseptically and placed into a 50-ml conical tube with cold PBS (44). The spleens were then gently ground between frosted microscope slides to produce single cell suspension and centrifuged at 400 g at 4°C for 15 min. The erythrocytes were lysed with Buffer EL (Qiagen, Valencia, CA). The remaining cells were then washed and loaded into a nylon wool column. After 1 h of incubation (37°C at 5% CO2), T cells were eluted from the column and suspended (1 × 106 cells/ml) in RPMI 1640 (Invitrogen, Grant Island, NY) containing 10% fetal bovine serum and antibiotics. T cells are the only cells that are CD3 positive, and CD3

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inhibitors (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions (41). Briefly, cell lysates of T cells stimulated with 1 μg/ml anti-CD3 for 30 min were incubated in a 96-well plate coated with an oligonucleotide containing the NF-κB or AP-1 consensus binding site. Activated transcription factors from the lysates specifically bound to the respective immobilized oligonucleotide were detected using the antibodies to NF-κB p65, c-Fos, or c-Jun followed by a secondary antibody conjugated to horseradish peroxidase in an ELISA-like assay.

Statistical analysis. Data are presented as means ± SE. Statistical differences between groups were determined by one-way ANOVA followed by Tukey’s test as a post hoc test. The differences were considered significant if P < 0.05.

RESULTS

Cytokine production. IL-2 and IFN-γ production by splenic T cells decreased following trauma-hemorrhage (Fig. 1). However, ER-α agonist PPT or 17β-estradiol administration following trauma-hemorrhage normalized the production of these cytokines. On the other hand, ER-β agonist DPN administration had no effect on IL-2 and IFN-γ production by T cells following trauma-hemorrhage.

p38, ERK1/2, and JNK phosphorylation. To determine MAPK activation, phosphorylation of p38, ERK1/2, and JNK in T cells with and without anti-CD3 stimulation was evaluated (Figs. 2–4). In unstimulated T cells, there was no significant difference in the phosphorylation of p38 (Fig. 2, A and B), ERK1/2 (Fig. 3, A and B), and JNK (Fig. 4, A and B) in T cells

Fig. 1. IL-2 and IFN-γ production by splenic T cells from sham animals treated with vehicle (Sham), trauma-hemorrhage animals (T-H) treated with vehicle (T-H+Veh), T-H treated with propyl pyrazole triol (T-H+PPT), T-H treated with diarylpropionitrile (T-H+DPN), and T-H treated with 17β-estradiol (T-H+E2). T cells were isolated 24 h after T-H and cultured in 24-well plates precoated with anti-CD3 (2 μg/ml) for 24 h. Cytokine levels in culture supernatants were determined by ELISA. Data are means ± SE of 5–6 animals in each group. *P < 0.05 compared with shams (ANOVA).
obtained from rats following trauma-hemorrhage compared with sham rat T cells. The stimulation with anti-CD3 resulted in a significant increase in the phosphorylation of p38, ERK1/2, and JNK in T cells derived from control animals; however, anti-CD3-induced phosphorylation of p38, ERK1/2, and JNK was significantly suppressed in T cells from rats subjected to trauma-hemorrhage compared with those from sham-operated rats (Figs. 2–4, A and B). Administration of PPT or 17β-estradiol following trauma-hemorrhage prevented the suppression of p38, ERK1/2, and JNK phosphorylation in T cells. On the contrary, administration of DPN following trauma-hemorrhage did not affect the phosphorylation of p38, ERK1/2, and JNK in T cells. These results did not change after normalization by calculating the ratio of phosphorylation to the total protein expression (Figs. 2–4, D). There was no significant difference in p38, ERK1/2, and JNK protein expression in T cells from rats subjected to trauma-hemorrhage compared with rats subjected to sham operation (Figs. 2–4, C).

**DISCUSSION**

Our results indicate that IL-2 and IFN-γ production by splenic T cells decreased following trauma-hemorrhage. This was accompanied by the suppression of the phosphorylation of p38, ERK1/2, and JNK and the decrease in DNA binding activity of NF-κB and AP-1. Administration of ER-α agonist

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**DNA binding activity of NF-κB and AP-1.** To determine the activation of NF-κB and AP-1, DNA binding activity of p65 subunit (NF-κB) or c-Fos and c-Jun (AP-1) was evaluated. The results showed that DNA binding activity of NF-κB (Fig. 5A) and AP-1 (Fig. 5B) in splenic T cells was decreased following trauma-hemorrhage compared with shams. However, PPT or 17β-estradiol administration following trauma-hemorrhage normalized DNA binding activity of these transcription factors. In contrast, DPN administration did not significantly influence NF-κB and AP-1 DNA binding activity in T cells.
PPT or 17β-estradiol following trauma-hemorrhage restored the production of IL-2 and IFN-γ by splenic T cells to the levels observed in sham animals. Moreover, PPT or 17β-estradiol administration following trauma-hemorrhage also prevented the suppression of the activation of MAPKs, NF-κB, and AP-1 in T cells. In contrast, administration of ER-α agonist DPN following trauma-hemorrhage had no effect on the above parameters. These findings therefore suggest that the effects of 17β-estradiol on T cells are mediated via ER-α.

T cells play a critical role in host defense against bacterial infection (6, 19, 24). Previous studies from our laboratory have shown that the depressed T-cell functions following trauma-hemorrhage were associated with an increased susceptibility to subsequent sepsis (29). It therefore appears important to prevent the suppression of T-cell functions following trauma-hemorrhage to maintain the immune functions and decrease subsequent sepsis/infections. Previous studies have demonstrated that proestrus female animals, with high circulating levels of estrogen, did not show decreased cytokine production by T cells, and they had normal immune functions compared with male mice following trauma-hemorrhage (1, 16). Furthermore, proestrus females had a significantly lower mortality following trauma-hemorrhage and induction of subsequent sepsis than male mice (1, 4). Our previous studies have also shown that administration of a single dose of 17β-estradiol following trauma-hemorrhage in male mice restored macrophage and lymphocyte functions to sham levels (4). However, blockade of ER by administration of EM-800 following trauma-hemorrhage abolished the salutary effects of 17β-estradiol (21), suggesting that the salutary effects of 17β-estradiol are mediated via ER.

Fig. 3. ERK phosphorylation and protein expression in T cells with anti-CD3 stimulation (anti-CD3+) or without stimulation (NS) from Sham, T-H+Veh, T-H+PPT, T-H+DPN, and T-H+E2. T cells were isolated 24 h after T-H, stimulated with 1 μg/ml anti-CD3 for 5 min, and lysed. Lysates were then analyzed for ERK phosphorylation (p-ERK) and protein expression (A). Blots were reprobed for β-actin for equal protein loading in various lanes. ERK blots obtained from 5 animals were analyzed using densitometry, and densitometric values for phosphorylation and total protein were normalized to β-actin and are shown as means ± SE in B and C, respectively. In addition, densitometric values for ERK phosphorylation were also normalized to total ERK protein and are shown as means ± SE in D. *P < 0.05 compared with shams.
ER-α and ER-β are the two major ER subtypes. Since the two subtypes of ER have different tissue distribution (22, 45, 52), it appears important to determine which subtype of ER contributes to the effects of 17β-estradiol in the target cells. In this study, we used the selective agonists for ER subtypes. PPT is a selective ER-α agonist, which has 410-fold binding selectivity over ER-β (42). On the other hand, DPN, which is a selective ER-β agonist, has 70-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ER-β than ER-α (30). The results of Takao et al. (46) also indicated that ER-α immunoreactivity was observed in 85% of human peripheral T cells by immunocytochemistry, whereas ER-β immunoreactivity was only detected in <10% of T cells. Another study also showed that CD4+ T cells express relatively high levels of ER-α, whereas CD8+ T cells express low but comparable levels of both ERs in human peripheral blood (31). Furthermore, a study regarding the function of ER indicated that ER-α, but not ER-β, was necessary for the enhanced E2-driven Th1 cell responsiveness (27). Additional findings suggested that treatment of ovariectomized mice with ER-α-selective agonist PPT caused thymic atrophy and significant changes in thymic CD4/CD8 phenotypic profile (25). In contrast, ER-β-selective agonist DPN alone had no effect on thymic weight, cellularity, or CD4/CD8 ratio. In addition, PPT administration induced a reduction in the percentage of mature B cells in the spleen and enhanced IFN-γ production from in vitro concanavalin A-stimulated splenocytes, whereas DPN treatment had no effects (25). These findings suggest that ER-α plays a predominant role in estrogen-induced thymic atrophy, and ER-β activation may partially downregulate ER-α-mediated effects on thymic cellularity and T-cell phenotype expression. These studies collectively suggest that ER subtype-selective agonists provide a novel approach to explore each ER subtype-mediated immunomodu-
have relatively greater ER-α mRNA expression compared with ER-β mRNA expression, which is associated with maintenance of T-lymphocyte cytokine release (36). In another study, Lambert et al. (23) showed that murine CD4+ T cells express detectable transcripts for ER-α but not ER-β. In contrast, human T cells express both ER-α and ER-β, but the density of ER-α is greater than that of ER-β (23). The findings from this study further suggest that ER-α deficiency in murine macrophages results in increased stimulation of CD4+ as determined by their ability to produce IFN-γ. However, Lambert et al. also showed that ER-α deficiency does not have a direct effect on IFN-γ secretion, and that E2 regulates IL-4 secretion by CD4+ T cells through ER-α in a classical ligand-independent manner. Thus it can be concluded from these findings that there are species-specific differences in the expression of ER-β by T cells. Nonetheless, a definitive mechanism by which E2 regulates splenic T-cell function in the absence of ER remains to be established.

Findings from several studies suggest that E2 can also mediate its effect independent of ERs, i.e., via G protein-coupled receptor (GPR)30. In this regard, GPR30 is a novel ER and has been suggested as a candidate for triggering a broad range of E2-mediated signaling (15, 34). The membrane-located GPR30 is thus an alternative to the classical ERs and is involved in the E2-mediated signaling through direct binding of E2. Studies have reported that E2 activates adenylate cyclase and induces the cAMP/PKA signaling pathway via GPR30 (15). Moreover, GPR30 acts independently of ER-α and ER-β to promote rapid E2 action (15). Additionally, overexpression of GPR30 in MDA-MB-231 breast cancer cells restores the activation of adenyl cyclase by E2, and suppression of GPR30 expression with antisense oligonucleotides or siRNA prevents E2-mediated cAMP-dependent signaling in keratinocytes and in SKBR3 breast cancer cells that lack ER-α and ER-β (34). Although we did not determine the expression of GPR30 in splenocytes in the current study, a recent study from our laboratory (15) showed that the role of GPR30 in E2-mediated increases in PKA activity and Bcl-2 levels in isolated hepatocytes. Alternatively, it is also possible that the salutary effects of the ER-α agonist on splenic T-cell functions are mediated indirectly via improved organ blood flow and attenuated gastrointestinal tract neutrophil influx (1, 52). In this regard, results from our laboratory (22) have shown that 17β-estradiol improved systemic blood flow following trauma-hemorrhage and sepsis. Moreover, the depression of splenic immune responses following trauma-hemorrhage appears to be due to gut-derived mediators. Such mediators, in turn, stimulate Kupffer cells to secrete additional agents that induce immunosuppressive exocrine effects on splenic immune functions (2). However, administration of the ER-α agonist PPT prevented hepatic injury (43), decreased expression of cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 and intercellular adhesion molecule-1, and attenuated neutrophil influx in the liver and intestine following trauma-hemorrhage (52) as well as reduced Kupffer cell inducible nitric oxide synthase levels (unpublished observation). Thus it is also possible that the ER-α agonist indirectly restores splenic immune function via improved organ perfusion and attenuated hepatic injury as well as by preventing liver and intestine neutrophil accumulation.
In addition to the classical estrogen binding receptors ER-α and ER-β, the existence of novel receptors and novel forms of ER in the plasma membrane has also been described (12, 34). Recently, it has been suggested that these receptors mediate estrogen signaling events, including the generation of second messengers as well as the activation of receptor tyrosine kinase pathways (9, 12, 33). It is therefore likely that these novel ERs play a role in the estrogen-mediated signaling pathway in splenic T cells following trauma-hemorrhage. In the present study, we demonstrated that activation of p38, ERK1/2, and JNK was suppressed in T cells following trauma-hemorrhage. However, administration of PPT or 17β-estradiol, but not DPN, following trauma-hemorrhage prevented such suppression of MAPK activation in T cells. Thus it could be postulated that a novel ER is involved in the ER-α agonist action and prevented the suppression of p38, ERK1/2, and JNK signaling in splenic T cells.

Our results indicated that, in addition to MAPK activation, NF-κB and AP-1 activity was also suppressed in T cells following trauma-hemorrhage. Administration of PPT or E2 following trauma-hemorrhage normalized the activation and activity of T cells, whereas DPN administration had no effect under those conditions. Since NF-κB and AP-1 have been shown to contribute to the expression of various genes including IL-2 and IFN-γ in T cells (10, 17, 40), the effects of 17β-estradiol or PPT on cytokine production by T cells appear to be mediated via NF-κB and AP-1 (3, 8, 10, 17, 18, 50). In addition, a number of studies have indicated that MAPKs are involved in the activation of NF-κB and AP-1. Therefore, it is also possible that these transcription factors are downstream effectors of MAPKs in T cells for the production of IL-2 and IFN-γ, and that the effects of 17β-estradiol or PPT on MAPKs lead to the normalization of NF-κB and AP-1 activity and cytokine production. However, further studies are necessary to elucidate such a relationship between MAPKs and NF-κB or AP-1 in mediating the effects of 17β-estradiol following trauma-hemorrhage.

Our previous study (38) showed that IL-2 and IFN-γ production by splenocytes from male mice increased following trauma-hemorrhage when cells were stimulated with anti-CD3; however, this appears to be in contrast to the present findings. However, it should be noted that the study cited above used a heparinized model, whereas the present study employed a nonheparinized model. In this regard, studies have shown that heparinization has protective effects against trauma-hemorrhage (32, 47, 48). However, IL-2 production by concanavalin A-stimulated purified T cells from nonheparinized mice decreased following trauma-hemorrhage (53). Moreover, studies preliminary to the present study showed that concanavalin A-stimulated T cells had responses similar to those observed in anti-CD3-stimulated T cells following trauma-hemorrhage (data not shown). Therefore, heparinization appears to be the cause of the apparent discrepancy between the present findings and those of the above-cited study.

Recently, it was suggested that the effects of 17β-estradiol on splenocytes after trauma-hemorrhage are mediated primarily by ER-β in 12-mo-old female mice ovariectomized 10 mo before the experiments (11). In contrast, the present study indicated that ER-α mediates 17β-estradiol effects on splenic T cells from young normal male rats. It therefore remains to be determined whether the difference between the present results and the previous one (11) concerning ER-β vs. ER-α is due to the use of rats vs. young mice or ovariectomized middle-aged mice. Since the animals used are quite different i.e., rats vs. mice, it is possible that the role of ER subtypes might also be different in these two rodents.

It can be argued that the present study utilized measurement at a single time point, i.e., at 24 h after treatment, and thus it remains unclear whether the salutary effects of E2 or PPT on T-cell signaling and cytokine production are sustained for periods of time longer than 24 h after treatment. Our previous studies, however, have shown that, if the improvement in cell and organ function by any pharmacological agent is evident at 2, 5, or 24 h after treatment, those salutary effects are sustained for prolonged intervals and those agents also decreased the mortality rates following the induction of trauma-hemorrhage and subsequent sepsis (1, 4, 37). Thus, although a time point other than 24 h was not examined in this study, based on our previous work, it would appear that the salutary effects of 17β-estradiol or PPT on T-cell cytokine release and MAPK, NF-κB, and AP-1 activation would be evident even if one measured those effects at another time point following trauma-hemorrhage and resuscitation.

It could be suggested that we should have administered 17β-estradiol, PPT, or DPN alone in sham groups in these studies to determine whether each per se has any adverse or salutary effects (13). However, our recent study (15) showed that administration of PPT or DPN alone in sham animals did not produce any deleterious or salutary effects. Therefore, the effects of PPT or DPN administration in sham animals were not examined in this study.

In summary, our findings suggest that the administration of ER-α agonist PPT or 17β-estradiol is equally effective in normalizing T-cell cytokine production and the activation of MAPK, NF-κB, and AP-1 following trauma-hemorrhage. Administration of ER-β agonist DPN on the other hand had no effect on those parameters under the same conditions. Thus it can be concluded that the salutary effects of 17β-estradiol on T-cell functions following trauma-hemorrhage are mediated predominantly via ER-α, and that these beneficial effects are likely mediated via restoration of T-cell MAPK, NF-κB, and AP-1. However, since our studies did not confirm the ER protein expression in rat spleen, it remains unknown whether the effects of 17β-estradiol, PPT, or DPN are mediated via the classical ER or via ER-independent mechanisms such as GPR30. Alternatively, the possibility that the salutary effects of the ER-α agonist on splenic T-cell functions are mediated indirectly via improved organ blood flow is not ruled out.

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