Tissue compartment-specific role of estrogen receptor subtypes in immune cell cytokine production following trauma-hemorrhage

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PREVIOUS STUDIES HAVE SHOWN that circulatory proinflammatory cytokine levels are markedly increased (5, 8) and there is increased susceptibility to subsequent sepsis (6, 36) in male animals following trauma-hemorrhage. Additional findings indicate that the capacity of Kupffer cells to produce various proinflammatory cytokines increases following trauma-hemorrhage; however, the capacity to produce those cytokines by splenic and peritoneal macrophages decreases at the same time (4, 38, 42). In contrast to males, proestrus female mice, which have high circulating levels of estrogen and progesterone, maintain immune function in multiple compartments to determine overall immune status. In view of this, the aim of our study was to determine which subtype is predominantly responsible for producing the salutary effects of 17β-estradiol on immune function. Since the two subtypes of ER have different tissue distribution (17), it appears important to determine which subtype of ER contributes to the effects of 17β-estradiol on immune function following trauma-hemorrhage and whether tissue specificity exists.

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 were 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 (13, 37). Briefly, male Sprague-Dawley rats (275–325 g) were fasted overnight before the experiment but allowed water ad libitum. The 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 cm × 9 x 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 volume. MAP was maintained at 40 mmHg until 40% of the shed blood volume was returned in the form of Ringer solution.

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lactate. The animals were resuscitated with 4× the shed blood volume with Ringer 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 (BW)], ER-β agonist diarylpropionitrile (DPN) (5 μg/kg BW), 17β-estradiol (50 μg/kg BW), 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 the 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.

**Plasma collection.** Blood samples were obtained and placed in microcentrifuge tubes, and plasma was separated by centrifugation, immediately frozen, and stored at −80°C until assayed.

**Kupffer cell isolation.** Kupffer cells were isolated by an in situ collagenase digestion method (40). Briefly, the liver was perfused with oxygenated HBSS (Invitrogen, Grant Island, NY) for 10 min to wash the adhered and was perfused with 0.04% collagenase (Sigma, St. Louis, MO) for 5 min. After the digestion and mechanical disruption of the liver, the cell suspension was filtered through a sieve and centrifuged at 50 g for 3 min to separate parenchymal from nonparenchymal cells. Nonparenchymal cells were collected and centrifuged at 16% HistoDenz (Sigma) for 45 min (2,000 g, 4°C). The cells at the interface were collected and washed twice by centrifugation with HBSS (450 g, 10 min, 4°C). The cell fractions were then washed, counted, and suspended (1 × 10⁶ cells/ml) in William’s E medium (Invitrogen) in 24-well plates. After 2 h of incubation (37°C at 5% CO₂), nonadherent cells were removed, and 1 ml of fresh William’s E medium containing 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen) was added to the adhered Kupffer cells. After an incubation period of 24 h with 1 μg/ml LPS (from E. coli, O55:B5, Sigma), the supernatants were harvested and frozen at −80°C until assayed.

**Isolation of splenic macrophages.** Spleens were removed aseptically and placed into 50-ml conical tube with cold PBS (3). 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, counted, and suspended (1 × 10⁶ cells/ml) in RPMI-1640 and incubated in 24-well plates. After 2 h of incubation (37°C at 5% CO₂), nonadherent cells were removed, and 1 ml of fresh RPMI-1640 containing 10% FBS and antibiotics was added to the adhered splenic macrophages. Following an incubation period of 24 h with 1 μg/ml LPS, the supernatants were harvested and frozen at −80°C until assayed.

**Isolation of alveolar macrophages.** Alveolar macrophages were collected by performing the bronchoalveolar lavage, as described by Leeper-Woodford and Detmer (20). The lungs were lavaged with a total of 50 ml of HBSS (Invitrogen). The cell fractions were then washed, counted, and suspended (5 × 10⁷ cells/ml) in RPMI-1640 (Invitrogen) in 24-well plates. After 2 h of incubation (37°C at 5% CO₂), nonadherent cells were removed, and 1 ml of fresh RPMI-1640 containing 10% FBS and antibiotics was added to the adhered alveolar macrophages. Following an incubation period of 24 h with 1 μg/ml LPS, the supernatants were harvested and frozen at −80°C until assayed.

**Isolation of peripheral blood mononuclear cells.** Peripheral blood mononuclear cells (PBMC) were puriﬁed by density gradient method, as previously described for mice (29). Brieﬂy, heparinized whole blood was obtained via the aorta, and, after centrifugation, plasma was removed. Cell fractions were mixed with 10 ml of cold HBSS, then layered on top of 10 ml Ficoll-Paque (Plus solution; Amersham Biosciences, Uppsala, Sweden) in two 50-ml tubes. The tubes were centrifuged at 400 g at 18°C for 30 min. PBMC were collected from the interface and then washed, counted, and suspended (1 × 10⁸ cells/ml) in RPMI-1640 containing 10% heat-inactivated FBS and antibiotics in 24-well plates. After an incubation period of 24 h (37°C at 5% CO₂) with 1 μg/ml LPS, the supernatants were harvested and frozen at −80°C until assayed.

**Plasma cytokine level.** Plasma concentrations of IL-6 and IL-10 were signiﬁcantly elevated following trauma-hemorrhage in vehicle-treated rats (Fig. 1). Administration of PPT or 17β-estradiol following trauma-hemorrhage markedly attenuated the increase in plasma IL-6 and IL-10 levels. Administration of DPN also reduced the elevation in plasma IL-6 and IL-10 levels, but the levels of these cytokines still remained signiﬁcantly higher than that in sham animals. TNF-α was not detected in plasma samples in any groups.

**IL-6 production.** As shown in Fig. 2, IL-6 production by Kupffer cells increased following trauma-hemorrhage. On the other hand, the capacity to produce IL-6 was decreased in splenic macrophages, alveolar macrophages, and PBMC. PPT or 17β-estradiol administration following trauma-hemorrhage normalized the production of IL-6 by Kupffer cells and splenic macrophages, whereas DPN or 17β-estradiol treatment prevented the suppression of IL-6 production by alveolar macrophages and PBMC. Although the alteration in IL-6 production was also attenuated by DPN treatment in Kupffer cells and splenic macrophages, and by PPT administration in alveolar macrophages, the increase was not completely normalized.
macrophages and PBMC following trauma-hemorrhage, the capacity to produce IL-6 still remained significantly higher in Kupffer cells and lower in splenic macrophages, alveolar macrophages, and PBMC compared with shams.

**TNF-α production.** Similar to IL-6, the capacity of TNF-α production was increased in Kupffer cells and decreased in splenic macrophages, alveolar macrophages, and PBMC following trauma-hemorrhage (Fig. 3). Administration of PPT or 17β-estradiol administration following trauma-hemorrhage prevented the suppression in production of TNF-α by alveolar macrophages and PBMC. Although the decrease in TNF-α production was also attenuated by DPN administration in splenic macrophages and by PPT in alveolar macrophages and PBMC, TNF-α production by those cells was not normalized to the same level as that in sham animals. Moreover, administration of DPN following trauma-hemorrhage had no significant effects on TNF-α production by Kupffer cells.

**IL-10 production.** In contrast to IL-6 and TNF-α, IL-10 production capacity was increased following trauma-hemorrhage in Kupffer cells, splenic macrophages, alveolar macrophages, and PBMC (Fig. 4). PPT or 17β-estradiol administration following trauma-hemorrhage prevented the elevation of IL-10 production by Kupffer cells, whereas DPN or 17β-estradiol treatment normalized PBMC IL-10 production. The increases in IL-10 production by splenic and alveolar macrophages were normalized by administration of either of the agonists as well as 17β-estradiol.

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**Fig. 2.** IL-6 production by Kupffer cells, splenic macrophages, alveolar macrophages, and peripheral blood mononuclear cells (PBMC) from sham, T-H, Veh, T-H+PPT, T-H+DPN, and T-H+E2 animals. Kupffer cells, splenic macrophages, alveolar macrophages, and PBMC were isolated 24 h after T-H and cultured with 1 μg/ml LPS for 24 h. IL-6 levels in culture supernatants were determined by ELISA. Values are means ± SE of 5–6 animals in each group; ANOVA. *P < 0.05 compared with all other groups; #P < 0.05 compared with sham.

**Fig. 3.** TNF-α production by Kupffer cells, splenic macrophages, alveolar macrophages, and peripheral blood mononuclear cells (PBMC) from sham, T-H, Veh, T-H+PPT, T-H+DPN, and T-H+E2 animals. Kupffer cells, splenic macrophages, alveolar macrophages, and PBMC were isolated 24 h after T-H and cultured with 1 μg/ml LPS for 24 h. TNF-α levels in culture supernatants were determined by ELISA. Values are means ± SE of 5–6 animals in each group; ANOVA. *P < 0.05 compared with all other groups, except the panel showing Kupffer cell response where #P < 0.05 compared with sham.
proinflammatory cytokines, IL-6 and TNF-α.

Kupffer cells, but their production by splenic macrophages, alveolar macrophages, and PBMC was decreased following trauma-hemorrhage, consistent with the previous findings (4, 14, 23, 38). Moreover, the production capacity of the anti-inflammatory cytokine IL-10 by Kupffer cells, splenic macrophages, alveolar macrophages, and PBMC was increased. Administration of 17β-estrogen following trauma-hemorrhage prevented all of these alterations.

Previous studies have demonstrated that proestrus female animals, with high circulating levels of estrogen, have normal immune functions compared with male mice following trauma-hemorrhage (2). Furthermore, proestrus females have a significantly lower mortality following trauma-hemorrhage and induction of subsequent sepsis than male mice (2, 10, 21). Our laboratory’s previous studies have shown that administration of a single dose of 17β-estrogen following trauma-hemorrhage improved macrophage and lymphocyte functions (10), which is consistent with the present results. However, blockade of ERs by administration of EM-800 abolished the salutary effects of 17β-estrogen (15), suggesting that the salutary effects of 17β-estrogen are mediated via ER.

There are two known ER subtypes, termed ER-α and ER-β. In this study, we used the selective agonists for ER subtypes to determine which subtype has a predominant role in the effects of 17β-estrogen on immune cell cytokine production following trauma-hemorrhage. PPT is a selective ER-α agonist, which binds to ER-α with 410-fold higher affinity than ER-β (32). In contrast, 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 (22). Our present results showed that administration of ER-α agonist PPT following trauma-hemorrhage was more effective than administration of ER-β agonist DPN, and its effects were the same as 17β-estrogen on IL-6 and TNF-α production capacity of Kupffer cells and splenic macrophages. In contrast, in alveolar macrophages and PBMC, DPN administration was more potent than PPT and was as effective as 17β-estrogen on such cytokine productive capacity. These results suggested that effects of 17β-estrogen on proinflammatory cytokine production were mediated predominantly via ER-α in Kupffer cells and splenic macrophages and via ER-β in alveolar macrophages and PBMC.

In contrast to proinflammatory cytokine, anti-inflammatory cytokine IL-10 productive capacity was increased following trauma-hemorrhage in immune cells in all of the four different compartments analyzed in this study. PPT treatment following trauma-hemorrhage prevented elevation of IL-10 production by Kupffer cells to the same extent as 17β-estrogen, whereas DPN normalized IL-10 production by PBMC. In addition, both PPT and DPN administration normalized splenic and alveolar macrophage IL-10 production capacity. The reason for different responses in IL-10 production from proinflammatory cytokines might be because IL-10 production is affected by circulatory proinflammatory mediator or controlled by a different mechanism from proinflammatory responses (7, 19, 28). However, further studies need to be performed to elucidate the details.

The present findings indicated that PPT administration had the same effects as 17β-estrogen in preventing the elevation of plasma IL-6 and IL-10 levels following trauma-hemorrhage. Since previous studies from our laboratory have shown that
Kupffer cells are the major source of IL-6 and IL-10 and contribute to the increased circulatory levels of these cytokines following trauma-hemorrhage (24, 28), the effects of 17β-estrogen or PPT on plasma cytokine levels might be via their effects on Kupffer cell cytokine production.

Macrophages are a heterogeneous population. Myeloid precursor cells or monocytes leave the bone marrow, circulate in peripheral blood, and move into peripheral tissues. Then they differentiate differently into tissue-specific macrophages, which have different morphology and function, due to the effects of different combinations of colony-stimulating factors produced locally (33). Previous studies have indicated that injury (i.e., burn, trauma, and sepsis) differentially influences immune functions in various tissue compartments (9, 15, 29), suggesting heterogeneity of macrophages or other immune cells. The present results also indicate that immune cells in different compartments showed different responses to the trauma-hemorrhage or the treatment of selective ER agonists.

Although we did not determine the expression of ER subtypes in this study, our laboratory’s previous studies have shown organ-specific expression of ER-α and ER-β and that liver has relatively more ER-α compared with ER-β, while ER-β is expressed relatively more than ER-α in lung (41). Moreover, PPT treatment prevented increased myeloperoxidase (MPO) activity in the liver following trauma-hemorrhage, whereas DPN normalized MPO activity in lung. These findings are compatible with the results of this study and showed that the effects of 17β-estradiol are mediated via ER-α in Kupffer cells and via ER-β in alveolar macrophages, and such effects on Kupffer cells and alveolar macrophages might contribute to the effects on MPO activity in liver and lung, respectively. It has also been reported that both ER-α and ER-β are expressed in spleen, but that ER-α is important for the function of splenic macrophages (18, 30, 31), which is consistent with our present finding of predominance of ER-α response in splenic macrophages.

PBMC include various cell populations. Previous studies have shown that ERs are differentially expressed in PBMC subsets. CD4+ T cells express relatively high levels of ER-α, whereas B cells express high levels of ER-β. CD8+ T cells and monocytes express low but comparable levels of both ERs (25). Monocytes are a major source of IL-6 and TNF-α from LPS-stimulated PBMC (1). However, B cells are also activated by LPS and produce IL-6 (11). These findings appear to support our data that the effects of 17β-estradiol on PBMC proinflammatory cytokine production are mediated predominantly via ER-β.

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 17β-estradiol or selective agonists on immune cell 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 also improve the survival of animals (2, 10, 27). 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 selective agonists on immune cell cytokine release would be evident, even if one measured those effects at another time point following trauma-hemorrhage and resuscitation.

It could also be suggested that we should have administered PPT or DPN alone in sham groups in these studies to determine whether each of those per se has any adverse or salutary effects. However, our recent study has shown that administration of PPT or DPN alone in sham groups did not produce any deleterious or salutary effects (12). Since PPT or DPN administration in itself did not influence organ function in sham animals, administration of PPT or DPN alone was, therefore, not carried out in this study.

In summary, our results indicate that the beneficial effects of 17β-estradiol on proinflammatory cytokine production following trauma-hemorrhage are mediated predominantly via ER-α in Kupffer cells and splenic macrophages and via ER-β in alveolar macrophages and PBMC. On the other hand, the effects of 17β-estradiol on anti-inflammatory cytokine production are mediated predominantly via ER-α in Kupffer cells, via ER-β in PBMC, and via both subtypes equally in splenic macrophages and alveolar macrophages. Although the present study provides evidence that immune cells in different compartments respond to 17β-estradiol via different ER subtypes, it remains to be determined which agonist of ER has relatively more beneficial effects on the total immune system. Studies to examine the effect of selective ER agonist on the survival of animals following trauma-hemorrhage and subsequent sepsis will provide important information to elucidate which agonist is more effective in improving the entire immune function and has a larger potential for clinical application.

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