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17 β -Estradiol's Salutary Effects on Splenic Dendritic Cell Functions Following Trauma-Hemorrhage are Mediated via Estrogen Receptor- α

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Abstract

Although 17 β -estradiol administration following trauma-hemorrhage attenuates Kupffer cell, splenic and peritoneal macrophage functions, it remains unknown whether 17 β -estradiol has any salutary effects on splenic dendritic cell (DC) functions and if so, whether such effects are mediated via the estrogen receptors (ER). We hypothesized that 17 β -estradiol administration following trauma-hemorrhage has salutary effects on splenic DC functions. Male C3H/HeN (6-8 weeks) mice were randomly assigned to sham operation or trauma-hemorrhage. Trauma-hemorrhage was induced by midline laparotomy and ~90 min of hemorrhagic shock (blood pressure [BP] 35 mmHg), followed by fluid resuscitation (4 $\times$ the shed blood volume in the form of Ringer's lactate). Estrogen receptor (ER)- α agonist propyl pyrazole triol (PPT; 5 μ g/kg), ER- β agonist diarylpropionitrile (DPN; 5 μ g/kg), 17 β -estradiol (50 μ g/kg), or vehicle (10% DMSO) was injected subcutaneously during resuscitation. Two hrs later, the mice were sacrificed, splenic DCs were isolated and the changes in their apoptosis, co-stimulating factors and MHC class II expression, ability to produce cytokines, and antigen presentation capacity were measured. Apoptosis of splenic DC increased following trauma-hemorrhage; however, 17 β -estradiol administration after trauma-hemorrhage normalized the rate of apoptosis. Moreover, splenic DC cytokines production, co-stimulating factors and MHC class II expression, and antigen presentation capacity were significantly decreased following trauma-hemorrhage; however, 17 β -estradiol as well as PPT also prevented these depressions. In contrast, DPN did not attenuate splenic DC functions following trauma-hemorrhage. Since PPT administration following trauma-hemorrhage was more effective in normalizing splenic DC functions than DPN, the salutary effects of 17 β -estradiol on splenic DC functions are mediated predominantly via ER- α .

Keywords

Shock; trauma; hemorrhage; splenic dendritic cell; estradiol; estrogen receptors

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Introduction

Many studies have demonstrated that trauma-hemorrhage induces marked alterations in various immune functions (Meldrum et al., 1997; Murphy et al., 2005; Murphy et al., 2004; Noel et al., 2005; Shukla et al., 2004; Zuckerbraun et al., 2005). Severe hemorrhage followed by adequate resuscitation with blood and crystalloid solutions is known to produce depression of immune system, such as lymphocyte functions and macrophage functions (Chaudry and Ayala, 1993). Traumatic injury due to burn or soft-tissue damage is also known to induce a profound depression of cell-mediated and humoral immunity (Frink et al., 2007; Purcell et al., 2006). The trauma/injury-induced immunosuppression is also associated with an increased susceptibility to subsequent sepsis, organ failure and mortality (Baue, 2005; Baue, 2006; Menges et al., 1999; Ulloa and Tracey, 2005).

Previous studies have demonstrated gender dimorphism in both immunological and physiological responses following trauma-hemorrhage (Choudhry et al., 2005; Coyle et al., 2006; Frink et al., 2007). In general, these studies have concluded that males are susceptible to the deleterious effects of hemorrhage shock, whereas proestrus females, with elevated systemic estrogen levels, are protected. With regard to the immune system, proestrus female mice showed normal immune response; however, male mice have markedly altered immune responses following trauma-hemorrhage (Angele et al., 2000). In addition, male animals treated with 17 β -estradiol following trauma-hemorrhage display an immunological response that closely mimics that of proestrus females (Suzuki et al., 2006; Yu et al., 2006). Studies have also demonstrated that male sex steroid appear to be responsible for producing the depression in cell and organ functions following trauma-hemorrhage (Angele et al., 1999; Shimizu et al., 2007). These studies therefore suggest that male and female sex steroids have opposite effects on immune functions following trauma-hemorrhage.

Dendritic cells (DCs) are the most potent antigen presenting cells (APC) that are intimately involved in the initiation of innate and adaptive immunity (Liu, 2001; Palucka and Banchereau, 1999; Steinman and Hemmi, 2006). Immature DCs are strategically located in tissues that represent pathogen entry routes. After microbial invasion and tissue destruction, immature DCs become activated and undergo maturation. Mature DCs reduce endocytic and phagocytic capacity, but they acquire an antigen-presenting capacity and migratory functions that allow antigen-loaded DCs to move to the T-cell areas or lymphoid tissues. Mature DCs also express high levels of MHC class II and co-stimulatory molecules, including CD40, CD80, CD83 and CD86, on their surface. They also have the ability to activate both T helper 1 (Th1) and Th2 cell responses. Our previous studies have shown that trauma-hemorrhage causes a decrease in splenic DCs maturation (Kawasaki et al., 2006). Although treatment with 17 β -estradiol following trauma-hemorrhage alters the circulating plasma cytokine levels and improves lymphocyte and macrophage immune functions, it remains unknown whether 17 β -estradiol also modulates splenic DC function under those conditions. The aim of this study, therefore, was to investigate the effect of 17 β -estradiol on splenic DC functions following trauma-hemorrhage. In addition, using estrogen receptor (ER) specific agonist, we investigated which of the two estrogen receptors is more critical in mediating the effects of 17 β -estradiol on splenic DC.

Materials and Methods

Mice

Male C3H/HeN mice (Charles River Laboratories, Wilmington, MA) 6–8 weeks old and weighing 20–25 g, were used in the experiments. These mice were allowed to acclimatize in the animal facility for one week before the experiments. All animal experiments were conducted in accordance with guidelines set forth in the Animal Welfare Act and the *Guide*

for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH) and approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Trauma-hemorrhag

Animals were fasted overnight but allowed water *ad lib*. They were anesthetized with isoflurane (Attane; Minrad, Buffalo, NY) and restrained in supine position. A 2.0-cm midline laparotomy (i.e., induction of soft tissue trauma) was performed and then closed aseptically in two layers using 6-0 Ethilon™ sutures (Ethicon, Somerville, NJ). Subsequently, both femoral arteries were aseptically catheterized with polyethylene-10 tubing (Clay-Adams, Parsippany, NJ) and the animals were allowed to awaken. Blood pressure was monitored continuously through one of the femoral artery catheters using a blood pressure analyzer (Digi-Med BPA-190; Micro-Med, Louisville, KY). Upon awakening, the animals were bled through the other catheter to a mean arterial pressure (MAP) of 35 ± 5 mmHg that was maintained for 90 min. At the end of that period, animals were resuscitated with four times the shed blood volume in the form of lactated Ringer's solution over 30 min. Fifteen minutes before the end of the resuscitation, the mice received estrogen receptor (ER)- α agonist propyl pyrazole triol (PPT; 5 μ g/kg BW), ER- β agonist diarylpropionitrile (DPN; 5 μ g/kg BW), 17 β -estradiol (50 μ g/kg BW), or an equal volume of the vehicle (~0.02 ml, 10% dimethyl sulfoxide; DMSO) subcutaneously (Suzuki et al., 2006; Yu et al., 2006). Lidocaine was applied to the groin incision sites, the catheters were removed, the vessels were ligated, and the incisions were then closed. Sham-treated animals underwent the same anesthetic and surgical procedures, but neither hemorrhage nor fluid resuscitation was performed. The animals were anesthetized by isoflurane inhalation 2 h following trauma-hemorrhage and resuscitation, and blood and spleen were collected for analysis.

Plasma collection and storage

Blood was obtained by cardiac puncture and centrifuged at $400 \times g$ for 10 min at 4°C. Plasma was immediately frozen and stored at -80°C until analyzed.

Quantitation of plasma estrogen levels

Plasma estradiol levels were determined by EIA kit (Cayman Chemical, Ann Arbor, MI).

Isolation of splenic dendritic cells and flow cytometric analysis

Spleens were digested by Liberase CI (Roche, Indianapolis, IN) and teased apart by repeated pipetting in PBS containing 5% FCS and 5 mM EDTA. The red blood cells were osmotically lysed and splenocytes were blocked with 1 μ g/ml Fc block (clone: 93) antibody for 15 min on ice. Cell suspensions were enriched with anti-CD11c magnetic beads and positive selection columns MS+ according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Flow cytometric analysis demonstrated that cells contained >90% CD11c-positive cells. Cell suspensions were stained with antibody against surface markers MHC II (clone: M5/114.15.2), CD40 (clone: 1C10), CD80 (clone: 16-10A1), CD83 (clone: Michel-17), or CD86 (clone: GL1) for DCs (eBioscience, San Diego, CA). Cells were acquired using a BD LSRII™ (BD Biosciences, San Diego, CA), and 10,000 events were collected for analysis.

Determination of cytokines level in plasma and CD11c-positive cell culture supernatants

Purified CD11c-positive cells (1×10^5 cells/well) from spleen were cultured in 96-well tissue culture plates after resuspension in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated FBS (Gibco) and 50 ng/ml gentamycin with or without LPS (10 μ g/ml). After 24 h incubation at 37°C, 5% CO₂, the plate was centrifuged at $400 \times g$ for 10 min. Supernatants were collected and frozen at -80°C until use. Plasma samples were collected and

clarified by centrifugation as described above. The levels of IL-6, IL-10, IL-12p70, IFN- γ , MCP-1, and TNF- α in the plasma and DC supernatant were measured using Cytometric Bead Array (CBA) mouse inflammation kit (BD Biosciences) according to the manufacturer's instructions. IL-12p40 levels in cell supernatants were measured by ELISA (BD Pharmingen, San Diego, CA).

Apoptosis assay

Apoptotic cells were detected using the AnnexinV-FITC kit (BD Biosciences). Briefly, a total of 1×10^5 purified CD11c-positive cells were incubated with 5 μ l of AnnexinV-FITC and 5 μ l of propidium iodide (PI) in binding buffer for 15 min. Apoptotic DCs were identified by staining with AnnexinV positive and PI negative (early apoptotic cells) or AnnexinV positive and PI positive (late apoptotic cells). Quantitative analysis was performed by BD LSR.™ (BD Biosciences) with 10,000 events acquired.

Antigen presentation

The capacity of purified splenic CD11c-positive cells to present antigen to D10.G4.1 cloned helper T-cells (ATCC, Manassas, VA) was carried out according to the method as previously described (Kawasaki et al., 2006). In brief, the CD11c-positive cells were incubated for 30 min (37°C, 5% CO₂, in the dark) with 50 μ g/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) following which the cells were washed (4 times) in PBS, and a series of dilutions was made. The DCs were then cocultured with 2×10^4 cells of D10.G4.1 in the presence or absence of 300 μ g/ml conalbumin (Sigma Chemical) for 48 hrs (37°C, 5% CO₂). 5-bromo-2'-deoxyuridine (BrdU) was added to the cells and cells were reincubated for 24 hrs. After incubation, T-cell proliferation was determined using Cell Proliferation Biotrak ELISA System™ (Amersham Biosciences, Piscataway, NJ).

ER- α and ER- β mRNA expression in splenic DC

The expression levels of ER - α , ER - β , and β -2 microglobulin mRNAs were determined by real-time, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using cells from experimental and sham animals that were harvested but not stimulated with LPS *in vitro*. Total RNA was extracted from isolated splenic DCs using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Aliquots of 0.5 μ g of total RNA were reversed to cDNA using a commercially available reverse-transcriptional kit (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA) in a 50 μ l reaction mix. PCR was carried out in a 20 ml reaction mix containing 9 ml of cDNA, 10 ml of 2 X TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 ml of primers and TaqMan probe mix (Applied Biosystems). The following validated primers and TaqMan MGB probes (6-FAM labeled) were used: ER- α (assay ID: Mm00433149_m1), ER- β (Mm00599816_m1), and β -2 microglobulin (Mm00437762_m1). All real-time PCR assay were performed in duplicate using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with the following program: 50°C for 2 min to activate uracil N-glycosylase enzyme, 95°C for 10 min to denature uracil N-glycosylase and activate DNA polymerase, 45 cycles at 95°C for 20 s and at 60°C for 1 min. β -2 microglobulin was used as an internal control. The relative expression levels of individual target genes were normalized to the internal control.

Statistical analysis

The data were presented as mean $\pm$ standard error (SE). One-way analysis of variance (ANOVA) and Tukey's test were used for the comparison between groups, and the differences were considered to be significant at $p < 0.05$.

Results

Plasma estrogen concentration

Plasma estrogen levels were significantly higher in 17 β -estradiol treatment groups than vehicle groups both in sham and trauma-hemorrhage animals (Fig. 1).

Plasma cytokines concentration

Plasma IL-6, IL-10, TNF- α and MCP-1 concentrations were significantly increased at 2 hrs following trauma-hemorrhage (Fig. 2). In sham-operated mice, 17 β -estradiol administration did not alter cytokines concentration. However, administration of 17 β -estradiol or PPT following trauma-hemorrhage attenuated the increase in cytokine concentrations under those conditions. Administration of DPN also reduced the elevated cytokines levels, but the plasma cytokines remained significantly higher than those in trauma-hemorrhage mice treated with E2 or PPT. Plasma IL-12p70 and IFN- γ concentrations were not detectable in sham and trauma-hemorrhage groups.

Effect of E2, PPT, and DPN on splenic DC apoptosis

As shown in Fig. 3, the percentage of both AnnexinV positive and PI negative cells (early phase of apoptosis) and AnnexinV positive and PI positive cells (late phase of apoptosis) were significantly increased following trauma-hemorrhage. Administration of 17 β -estradiol following trauma-hemorrhage normalized the percentage of both early phase and late phase of apoptotic cells under those conditions. Although PPT administration following trauma-hemorrhage also normalized the percentage of apoptotic cells, DPN administration did not affect the apoptotic rate under those conditions.

Effects of E2, PPT, and DPN on MHC-II and co-stimulating factors expression on splenic DCs

The maturation of DCs is closely involved in its activation of immune functions. The mature DCs highly express MHC-II and co-stimulatory factors, such as CD40, CD80, CD83, and CD86 on their surface (Steinman and Hemmi, 2006). We have previously demonstrated that trauma-hemorrhage impairs the maturation of splenic DC (Kawasaki et al., 2006). To examine whether administration of 17 β -estradiol following trauma-hemorrhage affects DC maturation; we analyzed the expression of CD40, CD80, CD83, CD86, and MHC-II on splenic DC surface following trauma-hemorrhage. Figure 4 shows the effect of 17 β -estradiol treatment on the expression of MHC-II and co-stimulating factors on the surface of splenic DCs. Following trauma-hemorrhage, CD40 (Fig. 4A), CD83 (Fig. 4C), and MHC-II (Fig. 4E) expression were significantly decreased. However, CD80 (Fig. 4B) and CD86 (Fig. 4D) expression was not significantly different between sham and trauma-hemorrhage mice. Administration of 17 β -estradiol following trauma-hemorrhage attenuated the suppression of CD40, CD83, and MHC-II expression under those conditions. In addition to 17 β -estradiol, we also examined the effect of PPT and DPN on DCs maturation. The results demonstrate that PPT administration also normalized CD40, CD83, and MHC-II expression following trauma-hemorrhage; however, DPN treatment did not affect these expressions. Similar to 17 β -estradiol, CD80 and CD86 expression was not affected by PPT administration.

Effects of E2, PPT, and DPN on cytokines production by splenic DCs

There were no significant differences between sham and trauma-hemorrhage DCs production of TNF- α , IL-6, IL-10, and IL-12p40 without stimulation (data not shown). After stimulation with LPS, the concentrations of these cytokines increased significantly in both sham and trauma-hemorrhage mice. There was no significant difference between sham and trauma-hemorrhage mice in LPS-induced IL-10 production; however, LPS-induced production of TNF- α , IL-6, and IL-12p40 were significantly suppressed in splenic DCs following trauma-

hemorrhage (Fig. 5). Administration of 17 β -estradiol normalized the production of these cytokines following trauma-hemorrhage. Furthermore, PPT treatment also restored the depressed cytokines production. In contrast, DPN treatment did not attenuate cytokines production following trauma-hemorrhage. The levels of IL-12p70, MCP-1, and IFN- γ were not detectable in both sham and trauma-hemorrhage groups with or without LPS stimulation.

Effects of E2, PPT, and DPN on antigen-presenting capacity of DCs after trauma-hemorrhage

The CD11c-positive cells isolated from trauma-hemorrhage mice showed a lower capacity to stimulate D10.G4.1 T-cell line proliferation than those from sham controls in the presence of conalbumin (the antigen to which the D10.G4.1 cell responds following appropriate antigen processing and presentation by the DC) (Fig. 6). Administration of 17 β -estradiol or PPT following trauma-hemorrhage attenuated the suppressed antigen-presenting capacity of DCs under those conditions; however, ER- β agonist DPN did not improve DCs antigen-presenting function.

Effect of trauma-hemorrhage on splenic DC ER- α and ER- β expression

To determine if trauma-hemorrhage influenced ER- α and ER- β gene expression in splenic DC, we used quantitative RT-PCR to assess changes in expression of ER- α and ER- β mRNA in purified splenic DCs from sham or trauma-hemorrhage mice treated with vehicle. Although trauma-hemorrhage did not significantly influence the expression of ER- α and ER- β mRNA in the splenic DC, the results suggest that the splenic DC expresses ER- α mRNA predominantly (Fig. 7).

Discussion

The present study demonstrates that E2 treatment following trauma-hemorrhage reduces splenic DCs apoptosis. The depressed cytokines production ability of splenic DCs following trauma-hemorrhage was also normalized by E2 treatment. In addition, the surface antigen expression studies demonstrate that the trauma-hemorrhage-induced downregulation of DCs were attenuated by E2 administration under those conditions. Furthermore, the suppressed splenic DCs antigen presentation function following trauma-hemorrhage is also attenuated by E2 treatment. Mice treated with ER- α agonist PPT also show the same effects as E2 treated mice, but apparent changes were observed in the ER- β agonist DPN treated mice. These results therefore suggest that E2 produces its salutary effects on splenic DCs immune functions via ER- α .

Estrogen is well known as the key regulator of cell growth, differentiation, and function (Crandall et al., 1980; Hewitt et al., 2005; Medina et al., 2000; Robinson et al., 1997). Previous studies have shown that sepsis induces splenic DCs apoptosis (Hotchkiss et al., 2002; Wesche et al., 2005). Our results also demonstrate that trauma-hemorrhage induces splenic DCs apoptosis (Kawasaki et al., 2006). Nonetheless, administration of E2 following trauma-hemorrhage reduces splenic DCs apoptosis. This finding is in accordance with the recent report which showed that E2 increases the viability of splenic DCs *in vitro* (Yang et al., 2006). Previous studies have also demonstrated that E2 suppresses lymphocytes (Jimenez Del Rio and Velez-Pardo, 2000) and B cells apoptosis (Grimaldi et al., 2002; Peeva et al., 2005). Nonetheless, there are some reports which showed E2 induces apoptosis of thymocytes (Hoffman-Goetz et al., 2001; Zoller and Kersh, 2006). The reason for E2-induced thymocytes apoptosis as well as the precise mechanism by which E2 decreases splenic DCs apoptosis remains unknown. However, previous study demonstrated that estrogen activates protein kinase C (PKC) pathways in lymphocytes. For instance, PKC activation has been associated with decreased apoptosis in the spleen, but in the thymus with increased apoptosis (Perandones et al., 1993). Therefore, one potential mechanism of E2 in decreasing splenic DCs apoptosis

following trauma-hemorrhage could be due to increased level of PKC in splenic DCs. PKC is one of the pathways to LPS-induced TNF- α and IL-12 production in DCs (Aksoy et al., 2002). Although we did not measure PKC level in splenic DCs, LPS-induced TNF- α and IL-12 production of splenic DCs were markedly suppressed following trauma-hemorrhage. Furthermore, the production of these cytokines was significantly increased by E2 treatment. These results thus indicate that trauma-hemorrhage down-regulates PKC in splenic DC and E2 treatment up-regulates PKC activation. These results collectively suggest that E2 prevents splenic DCs apoptosis via an up-regulating of PKC level.

As the antigen-presenting cells, it is important for the DCs to mature and migrate to lymphoid tissues in order to initiate the activation of antigen-specific T-cells (Janeway and Medzhitov, 2002; Liu, 2001; Steinman and Hemmi, 2006). Mature DCs have potent antigen-presenting function, high expression of co-stimulating factors and MHC-II antigen, and increased cytokine production capacity. One of the major consequences of trauma-hemorrhage is severe depression of immune functions. Our recent studies have indicated that the defective splenic DC function such as suppressed functional maturation is a major underlying cause of defective immunity following trauma-hemorrhage (Kawasaki et al., 2006). There was no significant difference in the expression of DC surface CD80 and CD86 between cells from sham and trauma-hemorrhage animals; however, CD40, CD83, and MHC-II were significantly suppressed following trauma-hemorrhage. Furthermore, cytokines (TNF- α , IL-6, and IL-12) production by DCs was also decreased following trauma-hemorrhage. Administration of E2 following trauma-hemorrhage increased the expression of CD40, CD83, and MHC-II in trauma-hemorrhage mice. In addition, E2 also restored cytokines production capacity of splenic DCs following trauma-hemorrhage. These results thus indicate that E2 treatment improved the depressed functional maturation that is observed in splenic DCs from trauma-hemorrhage mice. Indeed, splenic DCs from E2 treated trauma-hemorrhage mice have highly antigen-presenting capability compared to splenic DCs from trauma-hemorrhage mice. Immature DCs are known for their ability to capture antigen by endocytosis or macropinocytosis; however, mature DCs lose the capacity to capture antigen. In accordance with our results, Yang et al (Yang et al., 2006) have shown that E2 decreases the endocytosis of splenic DCs. Our results, therefore, strongly suggest that DCs maturation is impaired following trauma-hemorrhage; however, E2 administration following trauma-hemorrhage restores the maturation of splenic DCs to shams levels.

The predominant biological effects of E2 are mediated through two estrogen receptors, ER- α and ER- β ? (Giguere, 2002). These two receptors are differentially expressed in different tissues (Harris et al., 2002). Accordingly, we examined which subtype of receptor was predominantly responsible for producing the salutary effects of E2 on splenic DC functions following trauma-hemorrhage. Our results demonstrate that mice treated with ER- α agonist PPT displayed improvement in splenic DCs cytokines production following trauma-hemorrhage. In contrast to PPT, treatment with ER- β agonist DPN did not produce any salutary effects on immune functions following trauma-hemorrhage. In addition, our results of ER- α and ER- β mRNA expression in splenic DC showed that splenic DC expresses ER- α mRNA predominantly. These results are in agreement with previous studies that showed murine splenic DC expressed ER- α mRNA but not ER- β mRNA (Lambert et al., 2004). Furthermore, other studies have reported that ER- α plays a crucial role in the full development of spleen (Staples et al., 1999) and also for antibody production by B lymphocytes in the spleen (Thurmond et al., 2000). Our previous studies have shown tissue specific site of ER in various organs following trauma-hemorrhage. For example, the beneficial effects of E2 on cardiac function, neutrophil accumulation in liver, small intestine, and lung following trauma-hemorrhage are mediated via ER- β , ER- α , both ER- α and ER- β , and ER- β , respectively (Yu et al., 2006). In addition, we recently reported that the salutary effects of E2 on Kupffer cell functions following trauma-hemorrhage are mediated predominantly via ER- α (Suzuki et al., 2006). Although the precise mechanism of the salutary

effects of E2 in reducing immunosuppression following trauma-hemorrhage via two different subtypes of ER in various tissues remains unclear, our studies indicating the existence of two ER subtypes provides, at least in part, an explanation for the selective action of estrogen in different target tissues or cells.

The results of CD83 expression and splenic DCs IL-6 productive capacity showed that ER- β agonist DPN treatment also has some effects in reversing the suppressive splenic DCs functions following trauma-hemorrhage. It should be noted, however, that ER- β agonist DPN has 72-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ER- β than ER- α (Meyers et al., 2001). On the other hand, ER- α agonist PPT has 410-fold higher binding affinity with ER- α than ER- β (Harris et al., 2002). It is therefore possible that the some of immunoprotective effects of DPN observed in this study are mediated by ER- α .

It can be argued that since plasma cytokine levels were decreased in E2 treated animals; however, cytokine release by DC's in culture was increased. Thus, it may appear that there is a discrepancy between these two results. However, previous studies from our laboratory have shown that Kupffer cells are the major source of IL-6 and IL-10 and they contribute to the increased circulatory levels of these cytokines following trauma-hemorrhage (O'Neill et al., 1994; Schneider et al., 2004). PPT and E2 administration also decreased the elevated plasma cytokine levels following trauma-hemorrhage and they also reduced Kupffer cell cytokines production (Suzuki et al., 2006). Therefore, the effects of E2 or PPT on plasma cytokine levels appear to be via their effects on Kupffer cell cytokine production as shown in our previous studies (O'Neill et al., 1994; Schneider et al., 2004).

The present study utilized measurement of various functional aspects of splenic DCs at a single time point, i.e., at 2 hrs after trauma-hemorrhage. In view of this, it remains unclear whether similar effects of E2 and PPT are maintained for periods longer than 2 hrs after trauma-hemorrhage. Our previous studies, however, have shown that if improvement in cell/organ functions by any pharmacological agent is evident early after treatment that those salutary effects are sustained for prolonged intervals and they also improved the survival of animals (Angele et al., 1999; Angele et al., 2000; Choudhry et al., 2005; Suzuki et al., 2006; Yu et al., 2006). Thus, although a time point other than 2 hrs was not examined in this study; based on our previous studies, it would appear that the salutary effects of E2 and PPT on splenic DCs would be evident even if one measured the effects at another time point following trauma-hemorrhage and resuscitation. It should also be noted that we examined only splenic DCs and thus it remains to be determined whether bone marrow DCs maturation and function are also adversely affected by trauma-hemorrhage. Furthermore, it remains unclear whether E2 and its agonists also produces salutary effects on bone marrow DCs following trauma-hemorrhage.

In conclusion, our results suggest that E2 produces immunoprotective effects on splenic DC following trauma-hemorrhage. Similar to E2, administration of ER- α agonist PPT after trauma-hemorrhage was effective in normalizing the cytokines production and antigen-presenting capacity of splenic DC. It therefore appears that the salutary effects of E2 on splenic DC functions following trauma-hemorrhage are mediated predominantly via ER- α , and that these beneficial effects are likely mediated via an attenuation of splenic DCs maturation under those conditions.

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Fig. 1. Plasma estrogen concentration in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, and trauma-hemorrhage treated with E2. At 2 hrs following resuscitation, plasma was collected for analysis. Data are shown as mean $\pm$ SEM of 6 animals in each group. * $p < 0.05$ compared to sham treated with vehicle.

Fig. 2.

Plasma tumor necrosis factor (TNF)- α (A), interleukin (IL)-6 (B), MCP-1 (C), and IL-10 (D) levels in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, trauma-hemorrhage treated with propyl pyrazole triol (PPT), trauma-hemorrhage treated with diarylpropionitrile (DPN), and trauma-hemorrhage treated with E2. At 2 hrs following resuscitation, plasma was collected for analysis. Data are shown as mean $\pm$ SEM of 6 animals in each group. * p <0.05 compared to sham treated with Veh. # p <0.05 compared to trauma-hemorrhage treated with Veh.

Fig. 3.

Changes of apoptotic rate of splenic DC in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, trauma-hemorrhage treated with propyl pyrazole triol (PPT), trauma-hemorrhage treated with diarylpropionitrile (DPN), and trauma-hemorrhage treated with E2. At 2 hrs following resuscitation, splenic DC were purified from splenocytes. Purified CD11c-positive cells were incubated with AnnexinV-FITC and propidium iodide (PI). A representative example of six independent analyses is shown (A). Percentage of early apoptotic DCs (B). Early apoptotic DCs were identified by staining with AnnexinV-positive and PI-negative. Percentage of late apoptotic DCs (C). Late apoptotic DCs were identified AnnexinV-positive and PI-positive. Data are shown as mean $\pm$ SEM of 6 animals in each group. * p <0.05 compared to other groups.

Fig. 4.

Surface phenotype of CD11c-positive splenic DCs in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, trauma-hemorrhage treated with propyl pyrazole triol (PPT), trauma-hemorrhage treated with diarylpropionitrile (DPN), and trauma-hemorrhage treated with E2. At 2 hrs following resuscitation, splenic DC were purified from splenocytes by MACS sorting, stained with antibodies against CD40 (A), CD80 (B), CD83 (C), CD86 (D), and MHC-II (E), and analyzed by flow cytometry. Data are shown as mean $\pm$ SEM of 6 animals in each group. MFI: mean fluorescence intensity. *p<0.05 compared to sham treated with Veh. #p<0.05 compared to trauma-hemorrhage treated with Veh.

Fig. 5.

IL-6, IL-10, IL-12, and TNF- α production by CD11c-positive cells in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, trauma-hemorrhage treated with propyl pyrazole triol (PPT), trauma-hemorrhage treated with diarylpropionitrile (DPN), and trauma-hemorrhage treated with E2. At 2 hrs following resuscitation, splenic DC were purified from splenocytes. Purified CD11c-positive cells were cultured in 96-well tissue culture plates with LPS (10 μ g/ml) for 24 hrs. The levels of TNF- α (A), IL-6 (B), and IL-10 (C) in DC supernatant were measured using Cytometric Bead Array (CBA). IL-12p40 levels (D) in cell supernatants were measured by ELISA. Data are shown as mean $\pm$ SEM of 6 animals in each group. *p<0.05 compared to sham treated with Veh. #p<0.05 compared to trauma-hemorrhage treated with Veh.

Fig. 6.

Changes of antigen-presenting capacity of CD11c-positive cells in sham animals treated with vehicle (Veh), sham animals treated with 17 β -estradiol (E2), trauma-hemorrhage treated with Veh, trauma-hemorrhage treated with propyl pyrazole triol (PPT), trauma-hemorrhage treated with diarylpropionitrile (DPN), and trauma-hemorrhage treated with E2. Antigen-presenting capacity was determined using antigen conalbumin specifically to stimulate D10.G4.1 cloned helper T-cells proliferation. At 2 hrs following resuscitation, splenic DCs were purified from splenocytes. DCs were then cocultured with 2×10^4 cells of D10.G4.1 in the presence or absence of 300 μ g/ml conalbumin for 48 h. 5-bromo-2'-deoxyuridine (BrdU) was added to the cells and cells were reincubated for 24 h. After incubation, T-cell proliferation was measured using ELISA. Data are shown as mean $\pm$ SEM of 6 animals in each group. * $p < 0.05$ compared to sham treated with Veh. # $p < 0.05$ compared to trauma-hemorrhage treated with Veh.

Fig. 7. ER- α and ER- β mRNA expression in splenic DC from sham or trauma-hemorrhage animals treated with vehicle. Using quantitative RT-PCR, we measured the ER- α and ER- β mRNA at 2 h following resuscitation. β -2 microglobulin was used as an internal control. The relative expression levels of individual target genes were normalized to the internal control. Data are shown as mean $\pm$ SE of 6 animals in each group.