Published in final edited form as:

Cytokine. 2010 August ; 51(2): 166–172. doi:10.1016/j.cyto.2010.03.016.

SALUTARY EFFECTS OF 17 β -ESTRADIOL ON PEYER'S PATCH T CELL FUNCTIONS FOLLOWING TRAUMA-HEMORRHAGE

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Abstract

Although 17 β -estradiol (E2) administration following trauma-hemorrhage (T-H) improves immune functions in male rodents, it remains unclear whether E2 has salutary effects on Peyer's patch (PP) T cell functions. We hypothesized that T-H induces PP T cell dysfunction and E2 administration following T-H will improve PP T cell function. T-H was induced in male C3H/HeN mice (6–8 weeks) by midline laparotomy and ~90 min of hemorrhagic shock (blood pressure 35 mmHg), followed by fluid resuscitation (4x 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), E2 (50 μ g/kg), or vehicle was injected subcutaneously at resuscitation onset. Two hrs later, mice were sacrificed and PP T cells isolated. PP T cell capacity to produce cytokines in response to in vitro stimulation, PP T cell proliferation and MAPK (p38, ERK-1/2, JNK) activation were measured. Results indicate PP T cell proliferation, cytokine production and MAPK activation decreased significantly following T-H. E2, PPT or DPN administration normalized these parameters. Since PPT or DPN administration following T-H was effective in normalizing PP T cell functions, the salutary effects of E2 are mediated via ER- α and ER- β .

Keywords

shock; ER- α ; ER- β ; cell signaling; estrogen

1. Introduction

Many studies demonstrated that trauma-hemorrhage induces a marked alteration in immune cell functions, including T cell activation, proliferation and cytokine release [1–8]. Immune cell dysfunctions are pivotal in the development of multiple organ failure and infectious complications in trauma patients [9–11]. Studies have shown that monocytes or macrophages

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from the injured host exhibit depressed HLA-DR expression and antigen-presenting function and this appears to be correlated with the development of T cell suppression [12].

Gut is the major reservoir of bacteria within the body. The gut-associated lymphoid tissues (GALT) consist of intraepithelial (IE), lamina propria (LP) compartments, Peyer's patches (PP) and mesenteric lymph nodes (MLN). The immune cells of these compartments contribute to the active immune response against infection [13,14]. Following bacterial entry, cells of gastrointestinal mucosa rapidly initiate the innate and acquired immune response. Within the first few hours after bacterial invasion, the intestinal mucosa produces mediators that orchestrate the onset of an early inflammatory response. Characteristics of this program include the increased production and release of chemokines, cytokines and nitric oxide [15,16]. These molecules can act as early signals to activate an acute mucosal inflammatory response and enhance the ability of epithelial cells to produce cytokines that regulate mucosal immune responses [15,16].

Previous studies have demonstrated gender dimorphism in both immunological and cardiovascular responses following trauma-hemorrhage [17–19]. 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 [20]. In addition, male animals treated with 17 β -estradiol (E2) following trauma-hemorrhage display an immunological response that closely mimics that of proestrus females [21,22]. Studies have also demonstrated that male sex steroids appear to be responsible for producing the depression in cell and organ functions following trauma-hemorrhage [20,23]. These studies therefore suggest that male and female sex steroids have opposite effects on immune functions following trauma-hemorrhage. Although studies have shown that E2 administration following trauma-hemorrhage improves both immune and cardiac functions in male rodents, it remains unclear whether trauma-hemorrhage and E2 also affect PP T cell functions. We hypothesized that trauma-hemorrhage induces PP dysfunctions and administration of E2 following trauma-hemorrhage improves PP functions. The aim of this study, therefore, was to investigate the effect of E2 on PP T cell 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 E2 on PP T cells.

2. Materials and Methods

2.1. Mice

Male C3H/HeN mice (6–8 weeks old and 20–25 g) obtained from Charles River Laboratories, Wilmington, MA, were used in the experiments. Mice were allowed to acclimatize to the animal facility for one week prior to the experiments. 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.

2.2. Trauma-hemorrhage

Animals were fasted overnight but allowed water ad lib. They were anesthetized with isoflurane (Attane; Minrad, Buffalo, NY) and restrained in a 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 EthilonTM 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. At the onset of the resuscitation, the mice received estrogen receptor (ER)- α agonist propyl pyrazole triol (PPT; 5 μ g/kg body weight [BW]), ER- β agonist diarylpropionitrile (DPN; 5 μ g/kg BW), E2 (50 μ g/kg BW), or an equal volume of the vehicle (~0.02 ml, 10% dimethyl sulfoxide; DMSO) subcutaneously [21,22]. Lidocaine was applied to the groin incision sites, the catheters were removed, the vessels were ligated, and the incisions were then closed. Sham-operated animals underwent the same anesthetic and surgical procedures, but neither hemorrhage nor fluid resuscitation was performed.

2.3. Isolation and culturing of T cells from PP

Animals were anesthetized by isoflurane inhalation 2 hrs following trauma-hemorrhage and resuscitation, and intestine was exposed via midline incision. PP were carefully excised from the intestine wall, then dissociated using the neutral protease enzyme collagenase type V (Sigma, St Louis, Mo) (40 U/mL) in Hanks' balanced salt solution (HBSS; Fisher Scientific, Atlanta, GA) containing 10 mM HEPES, 50 μ g/ml gentamicin, 100 U/ml penicillin, and 100 μ g/ml streptomycin for 60 min at 37° in a water shaker (150 rpm). After collagenase digestion, the cell suspension was incubated with nylon wool-packed columns. These columns were pre-equilibrated with HBSS containing 10 mM HEPES, 50 μ g/ml gentamicin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5% fetal calf serum (FCS). The columns containing cells were incubated at 37°C for 50–60 min. T cells were obtained by eluting the columns with 15 ml of HBSS at a flow rate of 1 drop/sec [24,25]. The cells were resuspended in RPMI-1640 (Fisher Scientific) supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μ g/ml gentamicin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS, then counted. The number of PP T cells was counted using a microscope. Between 10 and 15 fields were counted per slide depending upon the number of cells present (i.e., at least 200 cells were counted). Flow cytometric analysis demonstrated that cells contained >96% CD3-positive cells.

2.4. Apoptosis assay

Apoptotic cells were detected using the AnnexinV-FITC kit (BD Biosciences). Briefly, a total of 1×10^5 purified PP T 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 PP T cells were identified by staining with AnnexinV positive and PI negative. Quantitative analysis was performed by BD LSRII™ (BD Biosciences, San Diego, CA) with 10,000 events acquired.

2.5. Cell proliferation assay

ELISA (Amersham Biosciences, Piscataway, NJ) kit was used to determine the proliferation of PP T cells. Briefly, PP T cells (1×10^6 cells/ml) were plated into a 96-well plate with or without ConA (5 μ g/ml) stimulation and cultured at 37°C, 5% CO₂ and 95% humidity for 48 hrs. 5-bromo-2'-deoxyuridine (BrdU) was added to the cell suspension and incubated for an additional 8 hrs for PP T cells. The plates were then centrifuged at $400 \times g$, 4°C for 10 min, supernatants carefully removed, and the cells dried using a hairdryer for 15 min. The cells were incubated with blocking buffer for 30 min at room temperature after which the blocking buffer was removed by centrifugation. Peroxidase-labeled anti-BrdU reagent was then added, and the plates were incubated for an additional 90 min at room temperature. The plates were washed 3 times with PBS, and after the addition of 3,3',5,5'-tetramethylbenzidine (TMB) and 1 M sulphuric acid, the intensity of the color was measured with a Bio-tek (PowerWave, Winooski, VT) plate reader.

2.6. Determination of cytokines production

The nylon wool enriched PP T cells were cultured in 96-well tissue culture plates with ConA (5 µg/ml) for 48 hrs. The levels of IL-2, IL-4, IL-5 and IFN-γ in culture supernatants were determined by cytometric bead array (BD Bioscience) according to the manufacturer's instruction.

2.7. Detection of phosphorylated (activated) mitogen-activated protein kinase (MAPK) signaling molecules by FACS

We measured intracellular signaling molecules by fluorescein-activated cell sorter analysis, as described previously [26]. Briefly, PP T cells were incubated with 5 µg/ml of ConA for 15 min. Following incubation, the cells were rapidly fixed with 2% paraformaldehyde for 10 min at 37°C, permeabilized with ice-cold methanol (100%) for an additional 10 min, and washed with phosphate-buffered saline, supplemented with 1% bovine serum albumin and 0.1% sodium azide. Prior to antibody staining, samples were incubated with Fc block antibody (eBiosciences, San Diego, CA) to prevent nonspecific binding, and then stained with unlabeled, primary mouse monoclonal antibodies specific for phosphorylated (active) forms of p38, ERK1/2, or SAPK/JNK (Cell Signaling, Beverly, MA). An isotype-matched mouse IgG was used as a nonspecific staining control. Cells were then washed and stained with a goat anti-mouse FITC-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells were subsequently washed twice and resuspended in 0.5% paraformaldehyde. Flow cytometry was performed using the LSR II instrument (BD Biosciences), and the results were analyzed using the accompanying FACSDiva software (BD Biosciences).

2.8. Statistical analysis

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

3. Results

3.1. Effect of E2, PPT, and DPN on Peyer's patch T cell number and apoptotic rate

The percentage of apoptotic cells was significantly increased following trauma-hemorrhage (Fig. 1A). The number of PP T cells was found to be significantly decreased following trauma-hemorrhage compared to sham mice (Fig. 1B). These results demonstrate that apoptosis is a possible cause of PP T cell loss following trauma-hemorrhage. However, administration of E2, PPT, or DPN following trauma-hemorrhage normalized the number of PP T cells and apoptotic rate of PP T cells under those conditions. Administration of E2 did not influence PPT cell number and apoptosis in sham animals.

3.2. Effects of E2, PPT, and DPN on cytokines production by PP T cells

There were no significant differences between sham and trauma-hemorrhage PP T cell production of IFN-γ, IL-2, IL-4, and IL-5 without stimulation (data not shown). Following stimulation with ConA, the concentrations of these cytokines increased significantly in both sham and trauma-hemorrhage mice. However, ConA-induced production of IFN-γ, IL-2, and IL-4 were significantly depressed in PP T cells following trauma-hemorrhage (Fig. 2). There was no significant difference in ConA-induced IL-5 production between sham and trauma-hemorrhage mice. Administration of E2 normalized the production of these cytokines following trauma-hemorrhage. Furthermore, PPT or DPN treatment also prevented the decrease in PPT cell cytokine production.

3.3. Effects of E2, PPT, and DPN on PP T cell proliferation after trauma-hemorrhage

The effect of trauma-hemorrhage on the ability of PP T cells to proliferate in response to stimulation with ConA is shown in Fig. 3. The proliferation of PP T cells in response to stimulation with ConA was increased compared to non-stimulation controls. The PP T cells isolated from trauma-hemorrhage mice showed a lower capacity of proliferation than those from sham controls in the presence of ConA. Administration of E2, PPT, or DPN following trauma-hemorrhage attenuated the suppressed proliferation capacity of PP T cells under those conditions.

3.4. Effect of E2, PPT, and DPN on activation of p38, ERK1/2, and SAPK/JNK after trauma-hemorrhage

Since ConA was used to stimulate cytokine production by PP T cells, we examined whether trauma-hemorrhage-induced inhibition of cytokine production resulted from the impaired phosphorylation of MAPK. To evaluate activation of p38, ERK1/2, and SAPK/JNK, we used a flow cytometric approach as previously described [26] and the results from these analyses are shown in Fig. 4. Phosphorylation of p38, ERK1/2, and SAPK/JNK was detected in isolated PP T cells in the absence of ConA stimulation. Unstimulated cells did not exhibit any significant differences in the levels of phosphorylated p38, ERK1/2, and SAPK/JNK between sham and trauma-hemorrhage. The activation of p38 MAPK, ERK1/2, and SAPK/JNK was significantly increased in response to ConA stimulation. Trauma-hemorrhage induced a suppression of p38 MAPK and ERK 1/2 activation in PP T cells; however, trauma-hemorrhage had no effect on SAPK/JNK activation of PP T cells. Administration of E2, PPT, or DPN following trauma-hemorrhage attenuated the suppressed p38 MAPK and ERK 1/2 activation of PP T cells under those conditions.

4. Discussion

The present study demonstrates that trauma-hemorrhage causes an increase in PPT cell apoptosis and a decrease in T cell proliferation and cytokine production. E2 treatment following trauma-hemorrhage reduces PP T cell apoptosis. The depressed PP T cell proliferation and cytokine production following trauma-hemorrhage was also normalized by E2 treatment. Furthermore, trauma-hemorrhage induced a downregulation of MAPK activation which was also attenuated by E2 administration. Mice treated with ER- α agonist PPT or ER- β agonist DPN also show the same effects as with E2 administration. These results therefore suggest that E2 produces its salutary effects on PP T cell immune functions via both ER- α and ER- β .

Owens and Berg [27] have previously shown an increase in spontaneous bacterial translocation from intestine to extra intestinal organs in athymic homozygous (nu/nu) nude mice compared with heterozygous strains (nu/+). Furthermore, Choudhry et al. [28] have shown that depletion of T cells in healthy rats resulted in increased bacterial accumulation in MLN. These studies suggested that T cells play an important role in defense against gut bacteria. In this study, we observed PP T cell dysfunction following trauma-hemorrhage. It is well known that trauma-hemorrhage induces alteration in immune cell functions [1–3]. Since T cell-mediated immunity is critical to defense against bacterial infection, including the bacteria derived from intestine, impaired PP T cell functions as observed after trauma-hemorrhage would be expected to contribute to an increase in bacterial translocation. This in turn may contribute to sepsis and organ failure which is the major cause of death in the injured host [1–3].

We examined the role of MAPK pathway in altered PP T cell functions following trauma-hemorrhage. Several lines of evidence suggest that MAPK pathways play significant roles in mediating signals triggered by cytokines and growth factors, and are involved in cell proliferation, cell differentiation, and cell death [29–31]. We found a decrease in p38 and ERK

1/2 in PP T cells following trauma-hemorrhage. However, there was no change in SAPK/JNK activity in PP T cells following trauma-hemorrhage. Although a role of SAPK/JNK along with p38 and ERK1/2 has been suggested in T cell proliferation and IL-2 production [32], the present study demonstrated that SAPK/JNK may not be critical to PP T cell suppression following trauma-hemorrhage. Thus, the suppression in p38 and ERK1/2 likely plays a major role in trauma-hemorrhage-induced suppression of T cell proliferation and IL-2 production. Studies have shown that p38 plays a role in the differentiation of T cells into Th1 subtypes and the production of Th1 cytokines including IL-2 and IFN- γ [32]. Thus alterations in p38 as observed in PP T cells following trauma-hemorrhage may cause a decrease in IL-2 and IFN- γ production under those conditions. Previous studies have shown that IFN- γ produced by the intestinal T cell helps in resolution *Yersinia enterocolitica* [33] and *S. typhimurium* [34] infection. Furthermore, IFN- γ -deficient mice showed impaired ability to kill bacteria [35]. It is interesting to note that along with Th1 cytokines (IL-2 and IFN- γ) the production Th2 cytokine (IL-4) is also depressed following trauma-hemorrhage. Previous study have demonstrated that intestinal IgA levels are correlated with intestinal Th2 cytokine concentration [36]. Those authors [36] found that intravenous total parenteral nutrition decreases production of Th2 cytokine and induces severely impaired mucosal immunity. Recent study also showed that parenteral nutrition blunts GALT lymphocyte ERK1/2 phosphorylation and this may be an important mechanism underlying the impaired immunologic and physiologic barrier of the gut in parenterally fed animals [37]. Moreover, phosphorylation of ERK1/2 is essential for proper expression of the tight junction proteins to normal gut barrier function [38]. The Ras-ERK MAPK cascade regulates GATA3 protein which is critical for the differentiation of Th2 cells [39]. Thus, depressed Th2 cytokine production following trauma-hemorrhage in PP T cells shown in this study may be mediated via suppression of ERK1/2 activation.

Estrogen (E2) is well known as the key regulator of cell growth, differentiation, and function [40–42]. Previous studies have demonstrated that E2 suppresses lymphocyte apoptosis [43]. In addition, previous study demonstrated that E2 activates protein kinase C (PKC) pathways in lymphocytes [44]. Therefore, one potential mechanism of E2 in preventing PP T cell apoptosis following trauma-hemorrhage could be due to increased level of PKC in PP T cells. Furthermore, Fazal et al. [45] indicated downregulation of Ca²⁺ signaling in T cells as an important factor in T cell suppression following burn injury. A similar role of Ca²⁺ signaling is implicated in the regulation of IL-2 gene in T cells [46]. Recent studies have indicated the MAPK pathway may also be modulated by activation of Ca²⁺ signaling [47]. Our results demonstrated that the intracellular pathway by which trauma-hemorrhage suppresses PP T cell function is MAPK. Although we did not examine the effect of trauma-hemorrhage on PKC and Ca²⁺ signaling, it seems reasonable to surmise that trauma-hemorrhage-induced signaling derangements in T cells may be linked to crosstalk between MAPK and Ca²⁺ signaling mechanisms. Therefore, we postulate that inhibition of PKC and Ca²⁺ signaling is another possible mechanism by which trauma-hemorrhage suppresses PP T cell function. However, further studies are required to confirm this assumption.

The predominant biological effects of E2 are mediated through two estrogen receptors, ER- α and ER- β [48]. These receptors are differentially expressed in different tissues [49]. Accordingly, we examined which of the two subtypes was predominantly responsible for producing the salutary effects of E2 on PP T cell functions following trauma-hemorrhage. Our results demonstrate that mice treated with ER- α agonist PPT or ER- β agonist DPN displayed improvement in PP T cell functions following trauma-hemorrhage. Our previous studies have shown tissue-specific sites 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 [50]. In addition, we also reported that the salutary effects of E2 on Kupffer cell functions following trauma-hemorrhage are mediated predominantly via

ER- α [21]. Furthermore, our previous results of ER- α and ER- β mRNA expression in splenic DC showed that splenic DC predominantly express ER- α mRNA [51]. 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.

In summary, our results suggest that E2 produces immunoprotective effects on PP T cells following trauma-hemorrhage. Similar to E2, administration of ER- α agonist PPT or ER- β agonist DPN after trauma-hemorrhage was equally effective in normalizing the PP T cell immune functions. It therefore appears that the salutary effects of E2 on PP T cell functions following trauma-hemorrhage are mediated via both ER- α and ER- β . These beneficial effects are likely mediated via attenuation of MAPK activation under those conditions.

Acknowledgments

This work was supported by NIH grant RO1 GM37127. The authors wish to thank Bobbi Smith for her skill and assistance in preparing this manuscript.

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Fig. 1.

Changes in apoptotic rate and cell numbers of Peyer's patch T cells. At 2 hrs following resuscitation, Peyer's patch T cells were purified and incubated with AnnexinV-FITC and propidium iodide (PI). **(A)** Percentage of apoptotic Peyer's patch T cells. Apoptotic cells were identified by staining with AnnexinV-positive and PI-negative. **(B)** Number of Peyer's patch T cells. Data are shown as mean $\pm$ SEM of 6 mice in each group. * $p < 0.05$ compared to other groups. E2: 17 β -estradiol; PPT: propyl pyrazole triol (estrogen receptor- α agonist); DPN: diarylpropionitrile (ER- β agonist); VEH: vehicle.

Fig. 2. IL-2, IL-4, IL-5, and IFN- γ production by Peyer's patch T cells. At 2 hrs following resuscitation, Peyer's patch T cells were purified and cultured in 96-well tissue culture plates with ConA (5 μ g/ml) for 48 hrs. The levels of IFN- γ (A), IL-2 (B), IL-4 (C), and IL-5 levels (D) in cell supernatant were measured using Cytometric Bead Array. Data are shown as mean $\pm$ SEM of 6 mice in each group. *p<0.05 compared to other groups.

Fig. 3. Proliferation of Peyer's patch T cells. At 2 hrs following resuscitation, Peyer's patch T cells (1×10^6 cells/ml) were cultured with or without ConA ($5 \mu\text{g/ml}$) for 48 hrs. Payer's patch T cell proliferation was determined using BrdU labeling. Data are mean $\pm$ SE of 6 mice in each group. ANOVA, * $p < 0.05$ compared to other groups

Figure 4A

A.

Figure 4B

Fig. 4.

MAPK activation in Peyer's patch T cells. At 2 hrs following resuscitation, Peyer's patch T cells were stimulated with or without ConA (5 μ g/ml) for 15 min. Levels of phosphorylated p38 (p-p38), ERK $\frac{1}{2}$ (p-ERK), and SAPK/JNK (p-SAPK) were measured after permeabilization with methanol. (A) A representative example of six independent analyses. (B) Levels of phosphorylated p38, ERK $\frac{1}{2}$, and SAPK/JNK. Data are mean $\pm$ SE of 6 mice in each group. ANOVA; * p <0.05 compared to other groups.