Published in final edited form as:

Cytokine. 2008 July ; 43(1): 88–92. doi:10.1016/j.cyto.2008.04.001.

The Role of Estrogen Receptor Subtypes on Hepatic Neutrophil Accumulation following Trauma-Hemorrhage: Direct Modulation of CINC-1 Production by Kupffer Cells

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Abstract

Although 17 β -estradiol (E2) administration following trauma-hemorrhage (T-H) reduces liver injury by decreasing neutrophil accumulation via estrogen receptor (ER)- α , it remains unclear whether cytokine-induced neutrophil chemoattractant (CINC)-1 production by Kupffer cells (KC) is directly modulated by ER- α under such condition. Male rats underwent laparotomy and hemorrhagic shock (40 mmHg for 90 min), followed by resuscitation with four times the shed blood volume in the form of Ringer's lactate. 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 (10% DMSO) was administered subcutaneously during resuscitation; rats were sacrificed 24 hrs thereafter. KC were isolated and cultured with ER agonists to examine if they directly affect CINC-1 production. T-H increased plasma alanine aminotransferase (ALT; hepatic injury) and hepatic myeloperoxidase (MPO) activity. E2, PPT and DPN administration reduced increased ALT; however, PPT was more effective than DPN. PPT and E2, but not DPN significantly attenuated increased hepatic MPO activity and CINC-1 levels. PPT addition in vitro (10^{-7} and 10^{-6} M) significantly reduced KC CINC-1 production. In summary, the salutary effects of E2 against hepatic injury are mediated predominantly via ER- α which directly modulates KC CINC-1 production and hepatic neutrophil accumulation following T-H.

Keywords

Hemorrhagic shock; PMN; gender; liver; 17 β -estradiol

Introduction

Studies have shown that neutrophils are activated in the early phase of hemorrhagic shock [1,2], and the activated neutrophils produce organ injury under those conditions [3]. Previous studies have also shown that hepatic injury following hemorrhagic shock is associated with an increase in hepatic neutrophil accumulation [4,5]. The activated neutrophils appeared to

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infiltrate various tissues in parallel with increased expression of local cytokine-induced neutrophil chemoattractant (CINC) levels after hemorrhagic shock [6].

Gender-dimorphic immune and organ responses occur following hemorrhagic shock and low flow conditions [7–15]. Androgens have been shown to be responsible for producing the immunosuppression following trauma-hemorrhage in males. In contrast, female sex steroids exhibit immunoprotective properties after hemorrhagic shock and sepsis [7,16]. Estrogen administration in male animals reduces proinflammatory cytokine production and hepatic injury following trauma-hemorrhage [17,18]. Gender dimorphism in neutrophil priming and activation following trauma-hemorrhage has also been reported previously. The increased accumulation of neutrophils in lung is associated with the elevation in pulmonary CINC-1 levels [19]. Deitch et al. reported that neutrophil activation appeared to be modulated by sex hormones after trauma-hemorrhagic shock [20]. Thus, female sex steroids appeared to be closely related to decreasing hepatic injury by modulating neutrophil function following hemorrhagic shock.

There are two estrogen receptor (ER) subtypes, ER- α and ER- β , which are differentially expressed in different tissues [21]. Although the effects of 17 β -estradiol (E2) on decreasing hepatic injury [22] and neutrophil accumulation [6] appear to be mediated via ER- α , it remains unclear whether CINC-1 production by Kupffer cells is directly modulated by ER- α under such condition. The aim of our study, therefore, was to determine whether the salutary effects of E2 on Kupffer cell CINC-1 production are mediated via ER- α or ER- β . To test this, we first examined the effects of E2, ER- α agonist propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) administration on hepatic injury, measuring hepatic myeloperoxidase (MPO) activity, and CINC-1 production following trauma-hemorrhage. We then examined whether E2, PPT and DPN directly modulate Kupffer cell CINC-1 production. To study this, Kupffer cells harvested from sham and trauma-hemorrhage animals were cultured in vitro with E2, PPT, DPN and their ability to produce CINC-1 was assessed.

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 with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Experimental procedure

A non-heparinized rat model of trauma-hemorrhage was used, as previously described [23]. Briefly, male Sprague-Dawley rats were fasted overnight before the experiment, but allowed water *ad libitum*. The rats were anesthetized using isoflurane (Attane, Minrad Inc., Bethlehem, PA) inhalation. A 5-cm midline laparotomy was performed to induce soft tissue trauma. The abdominal wound was then closed in layers and polyethylene (PE-50, Becton Dickinson & Co., Sparks, MD) catheters were placed in both femoral arteries and the right femoral vein. The catheters were subsequently tunneled through to the dorsal side and the incision sites were closed. The rats were then placed into a Plexiglas box (21×9×5 cm) in a prone position and allowed to awaken, following which they were bled rapidly to a mean arterial pressure (MAP) of 35–40 mmHg within 10 min. The time at which the animals could no longer maintain MAP of 35–40 mmHg without infusing some fluid was defined as maximum bleed-out volume. The rats were maintained at this MAP until 40% of the shed blood volume was returned in the form of Ringer's lactate. The animals were then resuscitated with 4 times the volume of shed blood with Ringer's lactate over 60 min. Thirty minutes before the end of the resuscitation, the rats

received ER- α agonist PPT (5 μ g/kg BW), ER- β agonist DPN (5 μ g/kg BW), E2 (50 μ g/kg BW), or an equal volume of vehicle (0.2 mL, 10% DMSO, Sigma, St. Louis, MO) subcutaneously [24]. Following resuscitation, the catheters were removed, the vessels were ligated and skin incisions were closed with sutures. Sham-operated animals underwent the same groin dissection, which included ligation of the femoral arteries and vein, but the animals were neither subjected to trauma-hemorrhage nor resuscitated. The animals were returned to their cages and were allowed food and water *ad libitum*. At 24 hrs after trauma-hemorrhage or sham operation, the rats were anesthetized with isoflurane and exsanguinated to collect samples.

Measurement of hepatic injury

Blood samples were obtained at 24 hr after the end of resuscitation or sham operation. After centrifugation, plasma samples were separated, immediately frozen and stored at -80°C until assayed. Hepatic injury was determined by measuring alanine aminotransferase (ALT) using a commercially available colorimetric reaction kit according to the manufacturer's instructions (Sigma) [1]. Plasma CINC-1 levels were also determined using ELISA kits (R&D, Minneapolis, MN) [6].

Measurement of hepatic MPO activity

MPO activity in homogenates of whole liver was determined as previously described [25]. All reagents were purchased from Sigma Chemical Co. Briefly, equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 mL buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifuging at 300 g at 4°C , and the supernatants were stored at -80°C . Protein content in the samples was determined using the Bio-Rad (Hercules, CA) assay kit. The samples were incubated with substrate o-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μ L 50 mM phosphate buffer, 3 μ L substrate solution (containing 20 mg/mL o-dianisidine hydrochloride), and 3 μ L H_2O_2 (20 mM). Sample (10 μ L) was added to each well to start the reaction. Standard MPO (Sigma Chemical Co.) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 μ L sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by using the curve obtained from the standard MPO.

Determination of hepatic CINC-1 levels

Hepatic CINC-1 levels were determined using ELISA kit (R&D) according to the manufacturer's instructions, as previously described [19]. Briefly, the samples were homogenized in 10 times volume of PBS (pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). The homogenates were centrifuged at 2000 g for 20 min at 4°C and the supernatant was assayed for CINC-1 levels.

The direct effect of E2, PPT and DPN on CINC-1 production by Kupffer cells

Another group of trauma-hemorrhage or sham-operated rats was used to evaluate the in vitro effects of E2, PPT and DPN on CINC-1 production in the isolated Kupffer cells. Kupffer cells were obtained by an in situ collagenase digestion method at 24 hrs after the completion of resuscitation or sham operation, as previously described [26]. Briefly, regional vessels in the left lobe were ligated and the tissue samples were removed from the ligated left lobe. The remaining lobes of the liver were then perfused by oxygenized Hank's balanced salt solution (HBSS; GIBCO-BRL, Gaithersburg, MD) for 10 min to wash out the blood and were again perfused for 5 min by 0.03 % collagenase (Sigma). After the digestion, cell suspension was filtered through a sieve and centrifuged at 50 g for 3 min to separate parenchymal cells and

non-parenchymal cells. Non-parenchymal cells were collected and centrifuged over 16% Histodenz (Sigma) for 45 min (2000 g; 4°C). The cells at the interface were collected and washed twice by centrifugation with HBSS (450 g, 10 min, 4°C). The pellet was then resuspended in William's E medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL). After counting the cell numbers and assessing the cell viability by Trypan blue exclusion (average viability >95%), the concentration was adjusted to 1×10^6 cells/mL in William's E medium and 1.0 mL of cell suspension was added per well in a 24-well culture dish. After incubating for 2 hrs, the plates were washed with warm HBSS to remove non-adherent cells and 1.0 mL of William's E medium was added. The adherent Kupffer cells were incubated with vehicle or various concentrations of E2, PPT and DPN with or without LPS (1 µg/mL) for 24 hrs. Cell free supernatants were collected. Production of CINC-1 in the supernatant was evaluated using ELISA kit (R&D).

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical differences among groups were determined by one way analysis of variance (ANOVA) followed by Fisher's LSD as a post hoc test. Differences were considered significant if $p < 0.05$.

Results

Alteration in plasma levels of ALT

Trauma-hemorrhage significantly increased plasma ALT levels (Fig. 1). PPT treatment attenuated the trauma-hemorrhage-induced increase in plasma ALT but the levels remained higher than shams. Although DPN treatment also decreased plasma ALT levels significantly, the levels were higher than those in the PPT-treated rats. E2 also significantly decreased the elevated ALT levels following trauma-hemorrhage.

Alteration in hepatic MPO activity

Hepatic MPO activity significantly increased following trauma-hemorrhage (Fig. 2). PPT and E2 administration following trauma-hemorrhage normalized the hepatic MPO activity. DPN treatment had a tendency to reduce the elevated MPO activity; however, the values were not significantly different than the levels observed in the vehicle-treated rats (Fig. 2).

Alteration in plasma and hepatic CINC-1 levels

Trauma-hemorrhage induced a significant elevation in plasma and hepatic CINC-1 levels. Treatment of rats with PPT, DPN and E2 reduced the increased plasma CINC-1; however, these did not reach statistical significance and these levels remained significantly higher than shams (Fig. 3). The hepatic CINC-1 levels were significantly decreased in trauma-hemorrhage animals treated with PPT and E2, but not DPN (Fig. 3).

The effect of PPT, DPN and E2 on CINC-1 production in the isolated Kupffer cells

Trauma-hemorrhage significantly increased the levels of CINC-1 in the supernatants of Kupffer cells stimulated with LPS compared to the supernatants of Kupffer cells from sham-operated rats. PPT treatment (10^{-7} and 10^{-6} M) in vitro significantly decreased the elevated CINC-1 production in the isolated Kupffer cells in the presence as well as in the absence of LPS stimulation. The high dose DPN treatment (10^{-6} M) significantly reduced the increase in CINC-1 production from Kupffer cells with LPS stimulation. The addition of E2 (10^{-6} M) also reduced the elevated CINC-1 levels significantly (Fig. 4).

Discussion

The present study shows that both PPT and DPN significantly decreased hepatic injury following trauma-hemorrhage; however, the salutary effects of ER- α agonist, PPT, are more significant than those observed with the ER- β agonist, DPN. Our findings suggest that ER- α agonist may predominantly downregulate hepatic injury by reducing hepatic MPO activity and CINC-1 production compared to ER- β following trauma-hemorrhage. Furthermore, the elevated CINC-1 production in the isolated Kupffer cells was reduced in vitro by both PPT and DPN addition; however, the reduction was more effective in the PPT-treated group compared to the DPN-treated group. The salutary effects of E2 in decreasing hepatic injury are therefore mediated predominantly via ER- α which modulates CINC-1 production in Kupffer cells and decreased hepatic neutrophil accumulation following trauma-hemorrhage.

Previous studies have suggested that Kupffer cells play an important role in producing hepatic dysfunction and that occurs via neutrophil accumulation and activation under various stress conditions [27,28]. Horie et al. reported that pretreatment with gadolinium chloride (GdCl₃), which reduces Kupffer cell function, attenuated the liver leukostasis and impaired sinusoidal perfusion following gut ischemia/reperfusion [29]. Liu et al. demonstrated that Kupffer cells and neutrophils have important roles in the development of hepatic injury in a model of endotoxin-enhanced liver injury after hepatic ischemia [30]. Mosher et al. showed that Kupffer cell depression with GdCl₃ significantly decreased the extent of liver injury by reducing hepatic neutrophil infiltration in a mouse hepatic ischemia-reperfusion model [31]. O'Neill et al. reported that prior Kupffer cell blockade with GdCl₃ markedly decreased circulating IL-6 levels following trauma-hemorrhage [32]. Although the suppression of Kupffer cells with GdCl₃ most likely decreases hepatic injury following hemorrhagic shock, Paxian et al. demonstrated that Kupffer cell blockade with GdCl₃ 24 hrs prior to hemorrhage aggravated hepatocellular injury since it abolished heme oxygenase-1 (HO-1) mRNA and protein induction after hemorrhage [33]. They concluded that oxygen-free radicals released by Kupffer cells may serve as paracrine regulators of a hepatocellular stress gene which is necessary to maintain liver blood flow and liver integrity after hemorrhagic shock. Our previous studies have shown that E2 administration in male rats following trauma-hemorrhage decreased hepatic injury by enhancing the induction of hepatic HO-1 mRNA and protein [18] and reducing Kupffer cell IL-6 production [26]. Therefore, Kupffer cells may play both beneficial and detrimental roles in hepatic injury following hemorrhagic shock. Nonetheless, E2 administration in male rats following trauma-hemorrhage appears to modulate Kupffer cell function by enhancing anti-inflammatory cascade and reducing pro-inflammatory cascade.

Although the present findings indicate that PPT directly modulates Kupffer cell CINC-1 production after trauma-hemorrhage, the precise mechanism by which this occurs remains unknown. CINC has been reported to play a potential role in neutrophil infiltration in response to various forms of inflammation [34–37]. This CINC is an 8-kDa polypeptide originally identified in conditioned media from IL-1 β -stimulated rat glomerular epithelial cells and subsequently purified from NRK52E rat epithelioid cells [38]. CINC consists of 72 amino acids with homology to human peptides possessing gro/melanoma growth stimulatory activities, indicating that rat CINC belongs to the IL-8 superfamily [39]. Kupffer cells generate various inflammatory mediators, including cytokines, chemokines, eicosanoids, reactive oxygen intermediates, and other factors. Hsieh et al. demonstrated that the protective effects of E2 on Kupffer cell function are mediated via downregulation of TLR4-dependent p38 mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF- κ B) signaling following trauma-hemorrhage, which prevents the systemic release of cytokines or chemokines [40]. Our recent study reported that the salutary effects of E2 on Kupffer cell functions are mediated predominantly via ER- α and normalization of MAPK following trauma-hemorrhage [24]. Therefore, the E2-mediated direct downregulation of CINC-1 production by Kupffer cells may

also be related to MAPK and NF- κ B signaling following trauma-hemorrhage. Further studies are, however, needed to pinpoint the precise mechanism by which Kupffer cell CINC-1 production is directly modulated by E2.

Studies indicate that there are two ER subtypes, ER- α and ER- β [6,41]. Since the two subtypes of ER have different tissue distribution, it appears important to determine which subtype of ER contributes to the salutary effects of E2 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 . Conversely, DPN, which is a selective ER- β agonist, has only 70-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ER- β than ER- α [41]. Although the present study indicates that DPN administration after trauma-hemorrhage significantly attenuated hepatic injury and CINC-1 production in the isolated Kupffer cells, DPN administration did not produce the same effects as E2, while administration of PPT reduced hepatic injury to the same extent as E2. These results therefore suggest that ER- α agonist is as effective as E2 in decreasing hepatic injury and Kupffer cell CINC-1 production following trauma-hemorrhage. Since DPN has a relatively low selectivity, it is possible that the effects of DPN on hepatic injury and CINC-1 production are mediated by ER- α . Moreover, although we have not determined the expression of ER on Kupffer cells in this study, findings from our 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 [6]. Other investigators have also shown tissue-specific ER expression [42]. Thus, it is also likely that the predominant expression of ER- α in the liver may contribute to the selective action of ER- α agonist and thereby to the different regulation of the mechanism observed in response to PPT and DPN following trauma-hemorrhage.

It could be argued that the present study was performed at a single time point, i.e., at 24 hrs after treatment, and thus it remains unclear whether the salutary effects of E2 or PPT are evident at time periods earlier than 24 hrs or whether the salutary effects are sustained for periods of time longer than 24 hrs after treatment. Our previous studies have, however, shown that if the improvement in organ function by any pharmacological agent was evident at 2, 5 or 24 hrs after treatment that those effects were sustained for prolonged intervals and they also improved the survival of animals [7]. Thus, although a time point other than 24 hrs was not examined in this study, it would appear that the salutary effects of E2 or PPT on the measured parameters in different organs would be evident even if one measured those effects at another time point following trauma-hemorrhage.

In conclusion, since PPT and DPN decreased hepatic injury following trauma-hemorrhage, both ER- α and ER- β may be responsible for the E2-mediated reduction in hepatic injury following trauma-hemorrhage. However, PPT appears to be more effective in reducing hepatic injury as well as in reducing other parameters such as hepatic MPO activity and CINC-1 production compared to DPN. Kupffer cell CINC-1 production in vitro was also directly and predominantly moderated by PPT compared to DPN. Therefore, the salutary effects of E2 in decreasing hepatic injury are mediated predominantly via ER- α which modulated Kupffer cell CINC-1 production and hepatic neutrophil accumulation following trauma-hemorrhage.

ACKNOWLEDGEMENTS

The authors thank Bobbi Smith for her skill and assistance in preparing this manuscript. This work was supported by NIH grant R37 GM39519.

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Fig. 1. Effects of PPT, DPN and E2 on plasma alanine aminotransferase (ALT) at 24 hrs after sham operation or trauma-hemorrhage (T-H). Data are presented as mean $\pm$ SEM (n=6 animals/group). * p <0.05 vs. sham, † p <0.05 vs. T-H.

Fig. 2. Effects of PPT, DPN and E2 on hepatic myeloperoxidase (MPO) activity at 24 hrs after sham operation or trauma-hemorrhage (T-H). Data are presented as mean $\pm$ SEM (n = 6 animals/group). * p <0.05 vs. sham, † p <0.05 vs. T-H.

Fig. 3. Effects of PPT, DPN and E2 on plasma [A] and hepatic cytokine-induced neutrophil chemoattractant (CINC)-1 [B] at 24 hrs after sham operation or trauma-hemorrhage (T-H). Data are presented as mean $\pm$ SEM (n = 6 animals/group). * p <0.05 vs. sham, † p <0.05 vs. T-H.

Fig. 4. Effects of PPT, DPN and E2 on cytokine-induced neutrophil chemoattractant (CINC)-1 production in the isolated Kupffer cells. CINC-1 production without LPS [A] and with LPS [B] in Kupffer cells (KC). Data are presented as mean $\pm$ SEM (n = 6 animals/group). * p < 0.05 vs. corresponding control group. White bar: sham; black bar: trauma-hemorrhage.