Salutary effects of estrogen receptor-β agonist on lung injury after trauma-hemorrhage

Huang-Ping Yu,1,2 Ya-Ching Hsieh,1 Takao Suzuki,1 Tomoharu Shimizu,1 Mashkoor A. Choudhry, Martin G. Schwacha, and Irshad H. Chaudry1

1Center for Surgical Research and Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama; and 2Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan, Taiwan

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Am J Physiol Lung Cell Mol Physiol 290: L1004–L1009, 2006. First published December 16, 2005; doi:10.1152/ajplung.00504.2005.—Although 17β-estradiol (E2) administration after trauma-hemorrhage attenuates lung injury in male rodents, it is not known whether the salutary effects are mediated via estrogen receptor (ER)-α or ER-β. We hypothesized that the salutary effects of E2 lung are mediated via ER-β. Male Sprague-Dawley rats underwent trauma-hemorrhage (mean blood pressure 40 mmHg for 90 min, then resuscitation). E2 (50 μg/kg), ER-α agonist propyl pyrazole triol (PPT; 5 μg/kg), ER-β agonist diarylpropionitrile (DPN; 5 μg/kg), or vehicle (10% DMSO) was injected subcutaneously during resuscitation. At 24 h after trauma-hemorrhage or sham operation, bronchoalveolar fluid (BALF) was collected for protein concentration, LDH activity, and nitrate/nitrite and IL-6 levels. Moreover, lung tissue was used for inducible nitric oxide synthase (iNOS) mRNA/protein expression, nitrate/nitrite and IL-6 levels, and wet/dry weight ratio (n = 6 rats/group). One-way ANOVA and Tukey’s test were used for statistical analysis. The results indicate that E2 downregulated lung iNOS expression after trauma-hemorrhage. Protein concentration, LDH activity, and nitrate/nitrite and IL-6 levels in BALF and nitrate/nitrite and IL-6 levels in the lung increased significantly after trauma-hemorrhage; however, administration of DPN but not PPT significantly improved all parameters. Moreover, DPN treatment attenuated trauma-hemorrhage-mediated increase in iNOS mRNA/protein expression in the lung. In contrast, no significant change in the above parameters was observed with PPT. Thus the salutary effects of E2 on attenuation of lung injury are mediated via ER-β, and ER-β-induced downregulation of iNOS likely plays an important role in the DPN-mediated lung protection after trauma-hemorrhage.

shock; propyl pyrazole triol; diarylpropionitrile

HEMORRAGIC SHOCK RESULTS in the development of acute respiratory distress syndrome and multiple organ dysfunction syndrome in patients sustaining major mechanical trauma (8, 20). Patients who survive the initial traumatic insult remain susceptible to sepsis, multiple organ failure, septic shock, and death (4, 26, 36). Cellular dysfunction occurs in many organs, including heart, lung, liver, and gut after hemorrhagic shock, and these alterations persist for a prolonged period of time despite fluid resuscitation (12, 28, 32, 33).

Sex hormones are known to modulate immune functions in animals and in humans under normal and stress conditions (11). Studies have shown that proestrus female mice show normal immune responses; however, male mice have markedly altered immune responses after trauma-hemorrhage (35). Studies have also demonstrated that male sex steroids appear to be responsible for producing depression in cell and organ functions after trauma-hemorrhage (34). Additional support for this notion comes from studies that showed castration of male animals 14 days before trauma-hemorrhage attenuated the lung injury observed in noncastrated animals under those conditions (2). Furthermore, administration of flutamide, a testosterone receptor antagonist, after trauma-hemorrhage improved the depressed organ function in male animals (24). These studies suggest that male and female sex steroids, such as 5α-dihy-drotestosterone and 17β-estradiol (E2), have an opposite effect on cell and organ function after injury.

E2 is the predominant circulating sex hormone in females and has been shown to protect lung after adverse circulatory conditions such as trauma-hemorrhage (2). Studies have also shown that these salutary effects of E2 are mediated via estrogen receptors (ER), which are expressed in various organs (15).

Nitric oxide (NO) is produced as part of the inflammatory response (27). NO is generated by inducible nitric oxide synthase (iNOS), which is expressed in many cell types including alveolar macrophages (1, 18). A variety of inflammatory cytokines, including IL-6, induce the expression of iNOS (10). Furthermore, iNOS expression has been reported in the lung (31) after several inflammatory conditions such as sepsis (18) and hemorrhagic shock (30). Thus the above-mentioned pathophysiological conditions are known to be associated with an elevated NO production (18, 27, 30). Previous studies have shown that E2 can decrease iNOS expression and NO production in the lung and protect against lung injury (6, 7). In addition, it appears that the salutary effects of E2 on attenuation of iNOS expression and NO production in the lung are receptor dependent. Support for this suggestion comes from a study that showed administration of E2 with the ER antagonist ICI 182,780 abolished the salutary effects of E2 in the lung (7).

Although two primary subtypes of ERs (ER-α and ER-β) are known to exist, it remains unknown which of the two subtypes is responsible for producing the salutary effects of E2 after trauma-hemorrhage. Because our recent studies have shown that the salutary effects of E2 on the myocardium after trauma-hemorrhage are mediated via the ER-β (37), we hypothesized that ER-β is also responsible for mediating the salutary effects of E2 in the lung after trauma-hemorrhage. The aim of our study was to determine whether ER-β mediates the salutary effects of E2 on the lung following hemorrhagic shock.

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study, therefore, was to determine which ER is predominantly responsible for the salutary effects of E2 in attenuation of lung injury after trauma-hemorrhage and whether these effects are mediated via modulation of iNOS under those conditions.

MATERIALS AND METHODS

Trauma-hemorrhage procedure. Our previously described non-heparinized rat model of trauma-hemorrhage was used in this study (38). Briefly, male Sprague-Dawley rats (275–325 g; Charles River, Wilmington, MA) were fasted overnight before the experiment but were allowed water ad libitum. The rats were anesthetized by isoflurane (Attane; Minrad, Bethlehem, PA) inhalation before the induction of soft tissue trauma via a 5-cm midline laparotomy. The abdomen was closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (PE-50 tubing; Becton Dickinson, Sparks, MD). The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. Rats were then allowed to awaken and bled to a mean arterial pressure (MAP) of 40 mmHg. This level of hypotension was continued until the animals were no longer able to maintain MAP of 40 mmHg unless additional fluid in the form of Ringer lactate (RL) was administered. This interval was defined as maximum bleed-out time, and the amount of withdrawn blood was noted. After this, the rats were maintained at a MAP of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of RL. The animals were then resuscitated with four times the volume of the shed blood over 60 min with RL. Thirty minutes before the end of the resuscitation period, the rats received E2 (50 μg/kg subcutaneously), ER-α agonist propyl pyrazole triol (PPT; 5 μg/kg subcutaneously), ER-β agonist diarylpropionitrile (DPN; 5 μg/kg subcutaneously), or an equal volume of the vehicle (0.2 ml, 10% DMSO, Sigma). The doses of E2, PPT, and DPN used in this study were the same as those used in our recent study that examined the effect of these agents on cardioprotection after trauma-hemorrhage and resuscitation (37). The catheters were then removed, the vessels ligated, and the skin incisions closed with sutures. Sham-operated animals underwent the same groin dissection, which included the ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. The animals were then returned to their cages and were allowed food and water ad libitum. All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Preparation of lung tissue and collection of bronchoalveolar fluid. At 24 h after the completion of fluid resuscitation or sham operation, the animals were anesthetized with isoflurane and then killed. The chest was opened, and the left side of the lung was obtained after clamping the hilum. Excess blood was blotted, and the left upper lobe of the lung was obtained after the animals were anesthetized with isoflurane and then killed. The lung tissue was measured using a commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

IL-6 assay. Concentration of IL-6 in cell-free BALF and lung tissue was determined using ELISA kits (Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

Protein assay in lung lavage. Cell-free BALF was evaluated for total protein content (DC Protein Assay, Bio-Rad).

Lactate dehydrogenase activity assay. To evaluate lactate dehydrogenase (LDH) activity in cell-free BALF, a commercially available kit was used (LDH Optimiert; Roche Diagnostics, Mannheim, Germany).

Statistical analysis. Results are presented as means ± SE. The data were analyzed using one-way analysis of variance and Tukey’s test, and differences were considered significant at a P value of ≤0.05.

RESULTS

Effect of ER-α agonist PPT, ER-β agonist DPN, and E2 on lung iNOS mRNA and protein expression. Trauma-hemorrhage induced a significant increase in lung iNOS mRNA expression compared with sham (Fig. 1A). E2 treatment prevented trauma-hemorrhage-mediated increase in iNOS mRNA expression in the lung; however, it remained higher than shams. Furthermore, DPN administration also attenuated the increase in iNOS mRNA in the lung after trauma-hemorrhage. In contrast, PPT did not prevent the increase in iNOS mRNA expression after trauma-hemorrhage. In addition to mRNA expression, we also examined the effect of PPT, DPN, and E2 on the iNOS protein level. Consistent with mRNA expression, the results as shown in Fig. 1B indicate that the trauma-hemorrhage-induced increase in iNOS expression was attenuated in rats treated with E2 and DPN. Administration of PPT, on the other hand, did not influence iNOS protein expression in the lung after trauma-hemorrhage.

Effect of ER-α agonist PPT, ER-β agonist DPN, and E2 on lung tissue edema. There was a significant increase in wet/dry weight ratio in the lung tissue obtained from trauma-hemorrhage rats compared with sham-operated rats (Fig. 2), suggesting that trauma-hemorrhage increases water content in the lung tissue. Administration of E2 and ER-β agonist DPN significantly prevented the increase in wet/dry weight ratio after trauma-hemorrhage. In contrast, treatment of animals with PPT did not influence this ratio after trauma-hemorrhage.
Effect of ER-α agonist PPT, ER-β agonist DPN, and E2 on lung and BALF nitrate/nitrite levels. Trauma-hemorrhage significantly increased nitrate/nitrite levels in the lung and BALF (Fig. 3, A and B). However, treatment with DPN or E2, but not PPT, prevented the increase in lung and BALF nitrate/nitrite levels.

Effect of ER-α agonist PPT, ER-β agonist DPN, and E2 on lung and BALF IL-6 levels. There was a significant increase in IL-6 levels in the lung and BALF (Fig. 4, A and B) after trauma-hemorrhage. Administration of DPN or E2 after trauma-hemorrhage prevented the trauma-hemorrhage-induced increase in IL-6 levels.

Fig. 1. Inducible nitric oxide synthase (iNOS) mRNA (A) and protein (B) expressions in the lung from sham rats with vehicle (sham), trauma-hemorrhage with vehicle (T-H+Veh), trauma-hemorrhage with propyl pyrazole triol (T-H+PPT), trauma-hemorrhage with diarylpropionitrile (T-H+DPN), and trauma-hemorrhage with 17β-estradiol (T-H+E2). For equal protein loading, membranes were reprobed for β-actin using mouse monoclonal antibody. The intensity of the bands was analyzed using densitometry and plotted as histograms in B. In each experiment, the densitometric values obtained from sham operation with vehicle are normalized to 1, and then the fold increases in other groups over sham values are calculated. Data are shown as means ± SE of 5 animals in each group. *P < 0.05 compared with sham; #P > 0.05 compared with T-H+Veh. RQ, relative quantification.

Fig. 2. Lung tissue wet/dry weight ratio from sham rats receiving vehicle (sham), trauma-hemorrhage with vehicle (T-H+Veh), trauma-hemorrhage with propyl pyrazole triol (T-H+PPT), trauma-hemorrhage with diarylpropionitrile (T-H+DPN), and trauma-hemorrhage with 17β-estradiol (T-H+E2). Data are shown as means ± SE of 6 rats in each group. *P < 0.05 compared with sham, T-H+DPN, and T-H+E2; #P > 0.05 compared with T-H+Veh.

Fig. 3. Nitrate/nitrite levels in the lung (A) and bronchoalveolar fluid (BALF) (B) from sham rats with vehicle (sham), trauma-hemorrhage with vehicle (T-H+Veh), trauma-hemorrhage with propyl pyrazole triol (T-H+PPT), trauma-hemorrhage with diarylpropionitrile (T-H+DPN), and trauma-hemorrhage with 17β-estradiol (T-H+E2). Data are shown as means ± SE of 6 rats in each group. *P < 0.05 compared with sham, T-H+DPN, and T-H+E2; #P > 0.05 compared with T-H+Veh.
increase in lung and BALF IL-6 levels. However, treatment of animals with PPT did not influence lung and BALF IL-6 expressions.

**Effect of ER-α agonist PPT, ER-β agonist DPN, and E2 on BALF total protein content and LDH activity.** Trauma-hemorrhage significantly increased total protein content and LDH activity in BALF (Figs. 5 and 6). However, treatment with DPN or E2 prevented the trauma-hemorrhage-induced increase in BALF total protein content and LDH activity. In contrast, no significant change in the above parameters was observed in trauma-hemorrhage rats treated with PPT.

**DISCUSSION**

Previous studies have shown that lung injury is significantly increased in male animals after trauma-hemorrhage (2). In contrast, female rats in the proestrus state, a state in which plasma levels of estradiol were found to be the highest, showed attenuation of lung injury after trauma-hemorrhage (2). However, ovariectomized females displayed depression in lung function after trauma-hemorrhage, similar to those observed in males (2). Thus it appears that female sex steroids have protective effects on attenuation of lung injury after trauma-hemorrhage.

There are two major subtypes of ERs, ER-α and ER-β (15, 25). In this study, we attempted to determine which of the estrogen receptors plays a predominant role in lung protection after trauma-hemorrhage. Our results indicate that iNOS expression and water content in lung tissue, nitrate/nitrite and IL-6 levels, total protein content, and LDH activity in the BALF were significantly increased after trauma-hemorrhage. However, treatment of rats with ER-β agonist DPN after trauma-hemorrhage demonstrated significant improvement in the above parameters at 24 h after trauma-hemorrhage. In contrast to DPN, treatment of rats with ER-α agonist PPT did not attenuate the above parameters of lung injury after trauma-hemorrhage.

DPN acts as an agonist on both ER subtypes but has 70-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ER-β than with...
ER-α (19). PPT, on the other hand, is a selective agonist for the ER-α subtype and binds to ER-α with high affinity, displaying 410-fold binding selectivity over ER-β (29). Previous studies have shown that there are differences in the tissue distribution of the ER subtypes (15). Consistent with these findings, we also found differences in ER-α and -β distribution in various organs. Our findings suggest that ER-β mRNA expression is higher than ER-α mRNA expression in the lung (data not shown). Therefore, the described differences between the ER subtypes in tissue distribution might contribute to the selective action of ER agonists in different tissues (15). Thus our results provide evidence that after trauma-hemorrhage, E2-induced attenuation of lung injury is mediated via ER-β activation.

iNOS has been previously shown to be overexpressed in rodent lung after hemorrhagic shock (13, 23). Hierholzer and colleagues (9) have also reported that iNOS inhibition resulted in a marked reduction of lung injury produced by hemorrhagic shock. These findings support the view that an enhanced formation of NO from iNOS plays an important role in lung injury after hemorrhagic shock (9). In agreement with those studies, our present study also shows that there is a positive correlation between lung injury and iNOS expression. In addition, under stressful conditions, iNOS can directly produce superoxide radicals (14). It is therefore possible that iNOS in the lung produces both NO and superoxide concurrently.

It is known that iNOS can be transcriptionally regulated in rodent cells (7). The complexity of iNOS promoter suggests that a substantial number of factors may participate in its regulation (16, 22). By interacting with AP-1 or NF-κB components, estrogen could directly influence iNOS mRNA synthesis (17). Furthermore, a few studies have examined the effects of E2 and gender on iNOS expression in the lung (6, 7). Ovariectomy increased the level of iNOS in female lung, and this could be attenuated by E2 replacement therapy (6). Additional studies have shown that E2 treatment reduced the level of iNOS in male rats (21). Consistent with these findings, we observed downregulation of lung iNOS after trauma-hemorrhage with DPN or E2 administration. These findings corroborate previous studies by Baker and colleagues (3), which showed that ER-β knockout mice display increased iNOS expression after endotoxemia compared with wild-type mice. Together, these findings suggest that DPN or E2 might attenuate lung injury after trauma-hemorrhage through downregulation of iNOS.

It could be argued that the present study utilized measurement at a single time point, i.e., at 24 h after treatment, and thus it remains unclear whether the salutary effects of E2 or DPN are sustained for periods of time longer than 24 h after treatment. In this regard, our previous studies have shown that if the improvement in organ functions 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 (5). Thus although a time point other than 24 h was not examined in this study, based on our previous studies, it would appear that the salutary effects of E2 or DPN on the measured parameters in the lung would be evident even if one measured those effects at another time point after trauma-hemorrhage.

It can also be argued that we should have administered PPT or DPN alone in sham groups in these studies to determine whether that per se has any adverse effects. In this regard, our recent study has shown that administration of PPT or DPN alone in sham groups did not produce any deleterious or salutary effects (37). Because PPT or DPN administration by themselves did not influence organ function in sham animals, administration of PPT or DPN alone was therefore not carried out in this study.

In conclusion, our results suggest that similar to E2, DPN, which is an ER-β agonist, also provides protection in the lung after trauma-hemorrhage. Furthermore, we found that down-regulation of iNOS likely plays a significant role in the DPN-mediated attenuation of lung injury after trauma-hemorrhage. Additional studies using ER-β knockout mice under these conditions will provide further support to this notion.

GRANTS

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