

Estradiol's Salutary Effects on Keratinocytes Following Trauma-Hemorrhage Are Mediated by Estrogen Receptor (ER)- α and ER- β

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Although administration of 17 β -estradiol (estrogen) following trauma-hemorrhage attenuates the elevation of cytokine production and mitogen-activated protein kinase (MAPK) activation in epidermal keratinocytes, whether the salutary effects of estrogen are mediated by estrogen receptor (ER)- α or ER- β is not known. To determine which estrogen receptor is the mediator, we subjected C3H/HeN male mice to trauma-hemorrhage (2-cm midline laparotomy and bleeding of the animals to a mean blood pressure of 35 mmHg and maintaining that pressure for 90 min) followed by resuscitation with Ringer's lactate (four times the shed blood volume). At the middle of resuscitation we subcutaneously injected ER- α agonist propyl pyrazole triol (PPT; 5 μ g/kg), ER- β agonist diarylpropionitrile (DPN; 5 μ g/kg), estrogen (50 μ g/kg), or ER antagonist ICI 182,780 (150 μ g/kg). Two hours after resuscitation, we isolated keratinocytes, stimulated them with lipopolysaccharide for 24 h (5 μ g/mL for maximum cytokine production), and measured the production of interleukin (IL)-6, IL-10, IL-12, and TNF- α and the activation of MAPK. Keratinocyte cytokine production markedly increased and MAPK activation occurred following trauma-hemorrhage but were normalized by administration of estrogen, PPT, and DPN. PPT and DPN administration were equally effective in normalizing the inflammatory response of keratinocytes, indicating that both ER- α and ER- β mediate the salutary effects of estrogen on keratinocytes after trauma-hemorrhage.

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INTRODUCTION

Sex hormones are known to modulate immune function in animals and humans under normal conditions as well as under stress (1–6). Studies have indicated that proestrus female mice show normal immune response following trauma-hemorrhage whereas male mice have markedly altered immune response under those conditions (7). This difference between males and proestrus females has been shown to be due to the presence of high circulating levels of sex hormone in females. Additional support

for the protective role of estrogen comes from studies demonstrating that administration of 17 β -estradiol (estrogen) in males following trauma-hemorrhage restored the altered immune functions (8,9). Additionally, administration of estrogen in ovariectomized females following trauma-hemorrhage normalized immune functions (10).

Studies dealing with cardiovascular, neurovascular, and neurotrauma stress responses have delineated various mechanisms by which estrogen exerts its beneficial effects, including interacting with

the immune system (11–13). Studies have also shown that the salutary effects of estrogen are mediated via the estrogen receptors (ERs). Estrogen binds to intracellular receptors, ER- α and ER- β , that regulate gene transcription factors. Previous studies from our laboratory have shown that the salutary effects of estrogen are mediated via ERs (14–17). Yu *et al.* have shown that treatment of animals with the ER- β agonist diarylpropionitrile (DPN) following trauma-hemorrhage had protective effects on the lungs (14). Another study by Suzuki *et al.* showed that administration of ER- α agonist propyl pyrazole triol (PPT) following trauma-hemorrhage normalized Kupffer cell cytokine production and activation of mitogen-activated protein kinase (MAPK) (18). Thus, although different tissues have ERs, the subtype of ERs varies, with some tissues having predominantly ER- α , others having predom-

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inantly ER- β , and some tissues having both (19–21).

Skin, the largest immune organ of the body, is also influenced by estrogen. Studies have shown the delay in wound healing observed in elderly patients of both sexes can be significantly improved by the application of estrogen at the wound site (22,23). The presence of ERs in human skin has also been demonstrated (24). These studies, however, used whole skin homogenate and did not distinguish between the different cellular components of the skin or between the different ERs. Studies using fetal human skin have shown that both ER- α and ER- β are expressed in human skin at mid-gestation (25). Verdier-Sevrain *et al.* have also shown that human keratinocytes express both ER- α and ER- β (26).

We previously showed that trauma-hemorrhage induces a keratinocyte inflammatory response and that administration of estrogen following trauma-hemorrhage normalizes the inflammatory response of keratinocytes (27). Although the ER- α and ER- β subtypes of ER are known to exist, it remains unknown which of the two receptors is responsible for producing the salutary effects of estrogen on epidermal keratinocytes following trauma-hemorrhage. Accordingly, we investigated the roles of ER- α and ER- β in estrogen-mediated salutary effects on the epidermal keratinocytes following trauma-hemorrhage.

MATERIALS AND METHODS

Animals

Inbred male C3H/HeN mice (Charles River Laboratories, Wilmington, MA, USA), 6 wks of age [body weight (BW) 24–27 g] were used in this study. The mice were allowed to acclimatize in the animal facility for at least 1 wk prior to experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and were performed in accordance with the 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.

Trauma-Hemorrhage Procedure

Mice were fasted overnight but allowed free access to water. The animals were anesthetized with isoflurane (Minrad, Bethlehem, PA, USA) and restrained in a supine position. A midline laparotomy (2 cm) was performed, which was closed in two layers with sutures (Ethilon 6/0; Ethicon, Somerville, NJ, USA). Both femoral arteries and the right femoral vein were then cannulated with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ, USA). The areas of the incisions were bathed with lidocaine to prevent discomfort to the animals. Blood pressure was measured via one of the arterial lines using a blood pressure analyzer (Digi-Med, Louisville, KY, USA). Within 10 min after animals had awakened, they were bled through the arterial catheter until their mean arterial blood pressure reached 35 ± 5 mmHg, which was maintained for 90 min. Mice then received estrogen (50 $\mu\text{g}/\text{kg}$ BW), or an equal volume and concentration of vehicle (cyclodextrin), PPT (5 $\mu\text{g}/\text{kg}$ BW), DPN (5 $\mu\text{g}/\text{kg}$ BW), and/or ICI 182,780 (ICI; 150 $\mu\text{g}/\text{kg}$ BW) subcutaneously at the middle of resuscitation. We have used these doses in our previous studies and found them effective in protecting both immune and other organ function following trauma-hemorrhage (5,15,18,19,21,27).

The animals were resuscitated by administration via the venous line of Ringer's lactate at four times the shed blood volume. After the catheters were removed, the incisions were closed, and the animals were returned to their cages. Sham-operated animals underwent the same surgical procedures but were neither hemorrhaged nor resuscitated (28).

Preparation of Keratinocytes

Epidermal sheet isolation. The mice were anesthetized at 2 h after resuscitation and the skin was shaved. Longitudinal strips (about 5 strips, each 0.5 cm

wide) of skin were removed and the excess fat removed. The skin strips were then digested in Dispase II-RPMI solution, 0.5% (Roche Cat #10165859; Indianapolis, IN, USA) for 30 min at 37°C in a shaker at 20 side-to-side movements/min. Following this, the strips were transferred to RPMI solution containing 10% fetal bovine serum (FBS) and kept on ice. The wet strips were then transferred to a Petri dish, dermis side down; the end of the dermis was secured with tweezers and the epidermis was separated from the dermis by use of a set of tweezers.

Keratinocyte isolation. Keratinocytes were isolated by use of the method described previously (29) with some modification. Epidermis sheets were floated, dermis-side down, in 20 mL of 0.15% trypsin solution incubated at 37°C and in a shaker at 20 side-to-side movements/min with agitation every 5 min for 45–50 min. In the last 30 min, 2 $\mu\text{L}/\text{mL}$ of DNAase was added to the trypsin solution and vigorously pipetted for a few minutes to help break up the cells. The cells were filtered through a 250- μm sieve into a tube containing equal volume of RPMI supplemented with FBS and antibiotics. Following this, the cells were centrifuged for 5 min at 290g (1200 rpm) at 4°C, the supernatant was discarded, and the pellet was resuspended in 20 mL of 0.002% DNAase and culture medium. The suspension was allowed to sit for a few minutes, then the cells were filtered through a 250- μm sieve into a Petri dish. The cells were then passed through a 26-gauge needle and centrifuged for 5 min at 290g (1200 rpm) at 4°C. The supernatant was discarded and cells were resuspended in 10 mL of keratinocyte serum-free growth medium (Gibco Cat #10725-018; Carlsbad, CA, USA). Trypan blue staining of the isolated cells from different groups (that is, sham, trauma-hemorrhage, and estrogen-treated) showed the typical keratinocyte morphology with viability greater than 95%. Furthermore, there were no significant changes in keratinocyte numbers in any group. The attached cells were allowed to grow for 24 h in

keratinocyte growth medium. The keratinocytes thus obtained were cultured in the presence or absence of 5 µg/mL lipopolysaccharide (LPS) (*Escherichia coli*, 055:B5; Sigma Chemical Co. St. Louis, MO, USA) for the measurement of various parameters as listed below. It should be noted that the process of epidermal isolation and keratinocyte processing may serve as an additional cellular stressor. Although the keratinocytes may be stressed following isolation, the same stress was also applied to the sham animals. We also understand that LPS acts as a cellular stressor; however, we selected the dose of LPS used in this study (5 µg/mL) after performing a dose-response study in which LPS was used at 100 ng/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL (data not shown). The results showed that keratinocytes responded poorly when they were stimulated with LPS at concentrations of 100 ng/mL and 1 µg/mL, and that optimal cytokine production by keratinocytes was achieved when they were stimulated with 5 µg/mL for 24 h.

Cell extract preparation. Keratinocyte cell extracts were prepared for the measurement of MAPK. In brief, the adherent keratinocyte cells were stimulated with 5 µg/mL LPS for 30 min and lysed with 60 µL/3 × 10⁵ cells of ice-cold lysis buffer (150 mM NaCl, 1 mM MgCl₂, 50 mM HEPES, 1 mM EDTA, 0.5% Triton × 100, 10% glycerol, 200 µM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 µM phenylmethylsulfonyl fluoride, glycerol, Triton X-100, and deionized water) for 60 min at 4°C. Cell lysates were centrifuged at 9,600g for 10 min at 4°C; supernatants were stored at -80°C until used for Western blot analysis.

Western Blot Analysis

Cell extracts (15 µg protein) were electrophoretically separated onto 10% sodium dodecyl sulfate-polyacrylamide gels as described previously (27). Proteins were electroblotted to Immobilon-P membranes (Healthcare, Waukesha, WI, USA) by use of a Semidry Transblot

System (Bio-Rad, Hercules, CA, USA) at 11 V for 45 min. Membranes were then blocked for 2 h at room temperature with 5% nonfat dry milk and incubated overnight at 4°C with antibodies against phospho-p38, phospho-p44/42 (phospho-ERK1/2), phospho-JNK, non-phospho-p38, non-phospho-ERK, and non-phospho-JNK (Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in Tris-buffered saline with Tween 20 containing 0.5% nonfat dry milk. The membranes were then incubated for 1 h with goat anti-rabbit or goat anti-mouse secondary antibody (diluted 1:2000), and the protein bands were developed with an enhanced chemiluminescence reagent followed by exposure to Biomax Light Film. After the films were developed, the density of the bands was determined using Ambis Optic Imaging System (GE Health Care, Chalfont St Giles, UK).

Cytokine Analysis

Keratinocytes were cultured with or without 5 µg/mL LPS for 24 h, and the supernatants were collected for cytokine measurement [interleukin (IL)-6, IL-10, IL-12 and TNF-α] using a cytometric bead array according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA) and as described previously (30). We incubated 50 µL of mixed capture beads with 50 µL of supernatant and 50 µL phycoerythrin detection reagents for 2 h at room temperature. The immunocomplexes were then washed and analyzed by use of an LSRII flow cytometer (BD Biosciences). Data processing was carried with a FACS Diva and BD cytometric bead array software.

Statistics

Statistical analysis was performed with Sigma-Stat computer software (SPSS, Chicago, IL, USA). We performed comparison between groups by using one-way ANOVA followed by Tukey's test. A *P* value of less than 0.05 was considered to be statistically significant for all analyses.

RESULTS

Effects of ER Antagonist ICI on Estrogen-Mediated Cytokine Production by Epidermal Keratinocytes

Mice subjected to trauma-hemorrhage had significant increase in keratinocyte cytokine (IL-6, IL-10, IL-12, and TNF-α) production compared with sham-treated mice. Administration of estrogen following trauma-hemorrhage normalized cytokine production under those conditions; however, coadministration of ICI along with estrogen blocked the salutary effect of estrogen on keratinocytes following trauma-hemorrhage (Figure 1).

Effect of ER Antagonist ICI on the Effects of Estrogen on MAPK Activation in Keratinocytes following Trauma-Hemorrhage

Significant increases in activation of p38, ERK, and JNK (MAPK) protein were evident in mice after trauma-hemorrhage compared with sham-treated mice (Figure 2). Estrogen significantly decreased the activation of MAPK proteins following trauma-hemorrhage; however, ICI blocked the beneficial effects of estrogen on MAPK activation (see Figure 2).

Expression of ER-α and ER-β on Epidermal Keratinocytes

Using mouse monoclonal ER-α and ER-β antibodies, we examined the expression of ER-α and ER-β on keratinocytes following trauma-hemorrhage. Trauma-hemorrhage significantly decreased ER-α and ER-β expression; however, administration of estrogen, PPT, and DPN normalized the expression of ER-α and ER-β in keratinocytes (Figure 3).

Effect of PPT and DPN on Keratinocyte Cytokine Production

Production of keratinocyte cytokines (IL-6, IL-10, IL-12, and TNF-α) was significantly increased following trauma-hemorrhage. However, administration of PPT, DPN, or estrogen following trauma-hemorrhage normalized the cytokine production by keratinocytes (Figure 4).

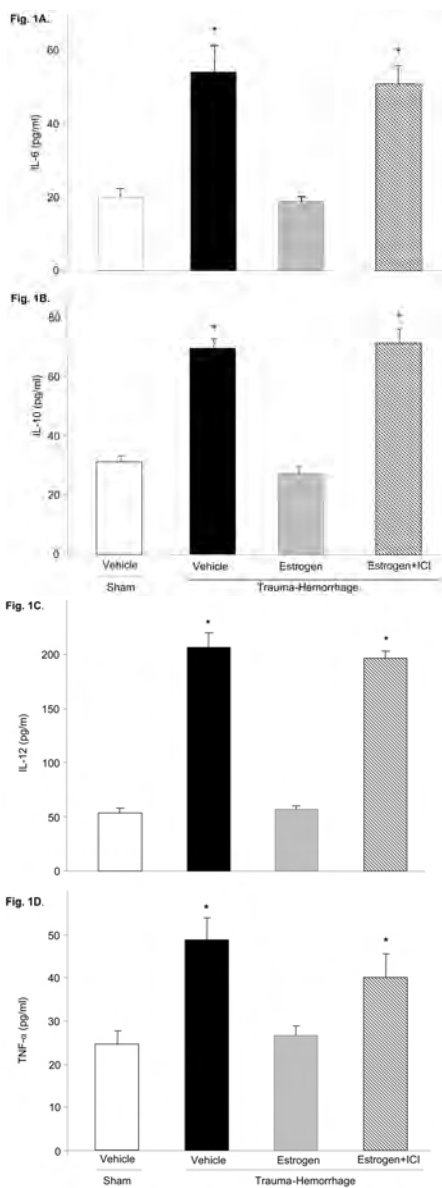


Figure 1. Effect of ICI 182,780 (ICI) and estrogen on production of cytokines IL-6 (A), IL-10 (B), IL-12 (C), and TNF- α (D) by epidermal keratinocytes. For details, see Materials and Methods. Data are mean \pm SEM from six animals in each group. * $P < 0.05$ versus sham and trauma-hemorrhage estrogen-treated groups.

Effects of PPT and DPN on p38, JNK, and ERK Phosphorylation in Keratinocytes

Trauma-hemorrhage significantly increased the phosphorylation of p38, ERK, and JNK in keratinocytes; however, ad-

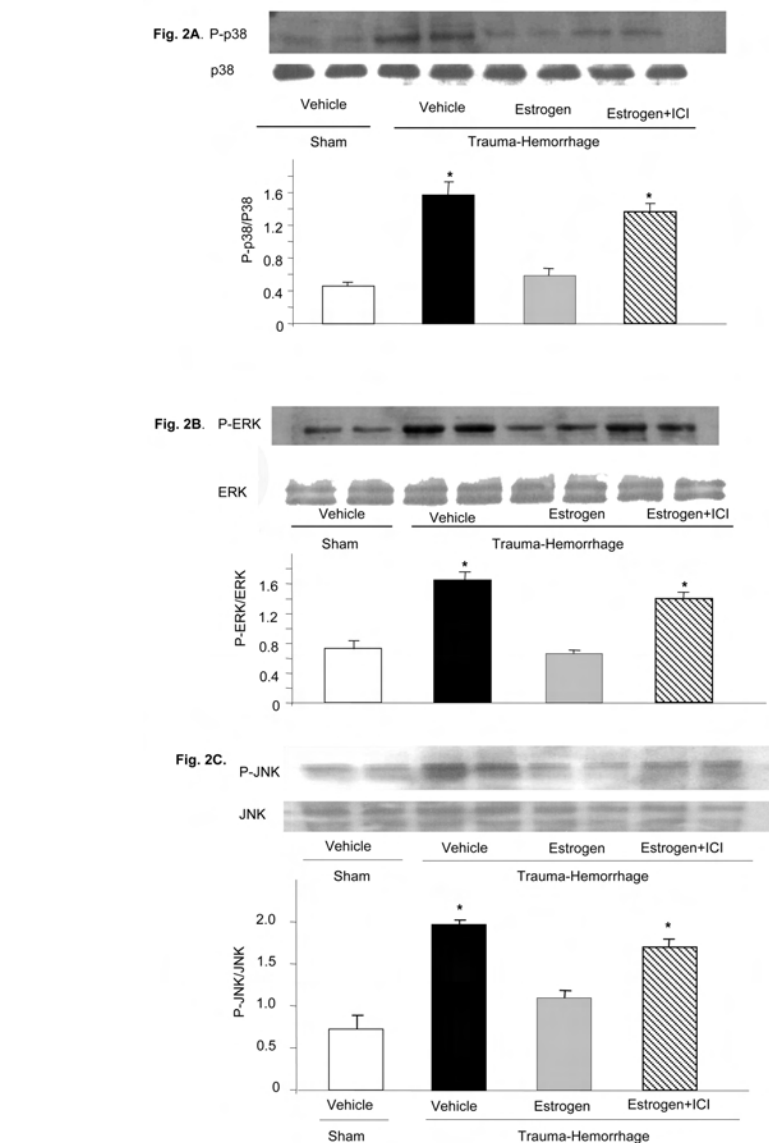


Figure 2. Effects of ICI 182,780 (ICI) on p38 (A), ERK (B), and JNK (C) activation in keratinocytes. Blots from six animals in each group were analyzed using densitometry. Densitometric values for phosphorylation were normalized to p38, ERK, and JNK protein and are shown in bar graph as mean \pm SEM. * $P < 0.05$ versus sham and trauma-hemorrhage estrogen-treated groups.

ministration of estrogen, PPT, or DPN following trauma-hemorrhage normalized p38, JNK, and ERK phosphorylation in epidermal keratinocytes (Figure 5).

DISCUSSION

The epidermis is an important site of host interactions and keratinocytes, the main cell type in the epidermis (> 90%) (31,32). Epidermal keratinocytes differ from other immune cells because they

not only form a physical barrier, they also initiate and regulate the cutaneous inflammation and immune response by producing a number of cytokines.

The present results show that administration of ER- α agonist PPT and ER- β agonist DPN or estrogen following trauma-hemorrhage prevented the increased production by epidermal keratinocytes of proinflammatory cytokines IL-6 and TNF and antiinflammatory cy-

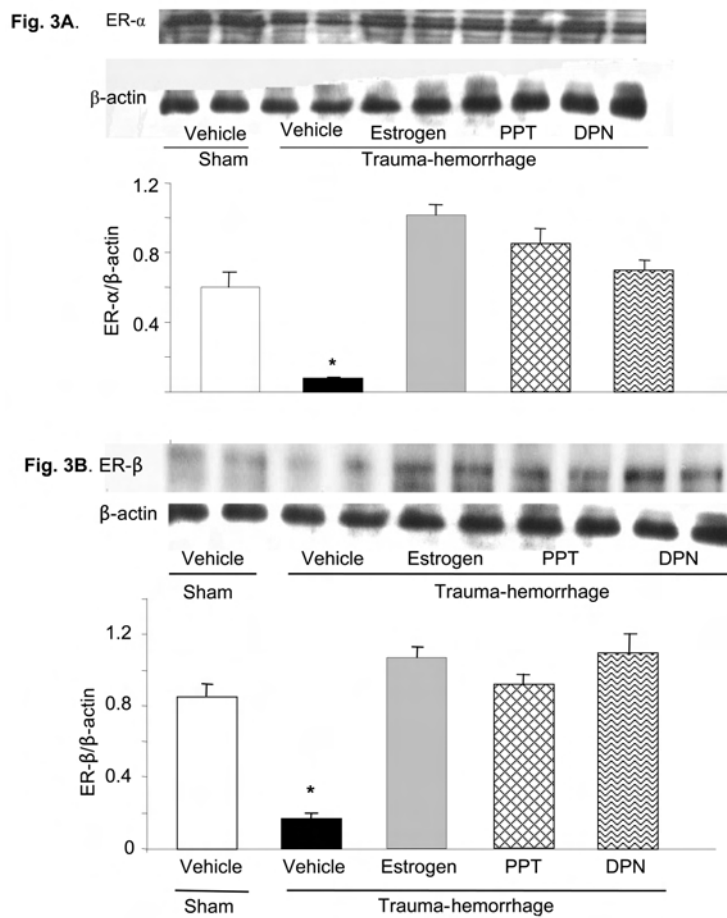


Figure 3. Expression of ER- α (A) and ER- β (B) on epidermal keratinocytes following trauma-hemorrhage. The blots were stripped and reprobbed for β -actin for equal protein loading in various lanes. Blots from six animals in each group were analyzed using densitometry. Densitometric values were normalized to β -actin and are shown in bar graphs as mean \pm SEM. * $P < 0.05$ versus all other groups.

tokine IL-10. Moreover, PPT, DPN, or estrogen administration after trauma-hemorrhage prevented the activation of p38, ERK1/2, and JNK in keratinocytes. Our previous studies have shown that trauma-hemorrhage induces an inflammatory response in epidermal keratinocytes, as evidenced by an increase in the production of IL-6, IL-10, and TNF- α (27). However, administration of estrogen following trauma-hemorrhage normalized keratinocyte cytokine production under those conditions. It is thus tempting to suggest that the decrease in proinflammatory IL-6 and antiinflammatory IL-10 might balance each other off with essentially no subsequent response.

Whether or not this is indeed true remains to be determined.

A number of studies from our and other laboratories have shown that the female sex hormone estrogen has salutary effects on immune response following trauma-hemorrhage (7,8,12,19,28,30, 33,34). It is well known that estrogen mediates its effect via receptors ER- α and ER- β . The aim of the present study was to test the hypothesis that the immunoprotective effect of estrogen on epidermal keratinocytes following trauma-hemorrhage was ER-dependent. Accordingly, male mice were subjected to trauma-hemorrhage or sham operation and treated with the specific ER antago-

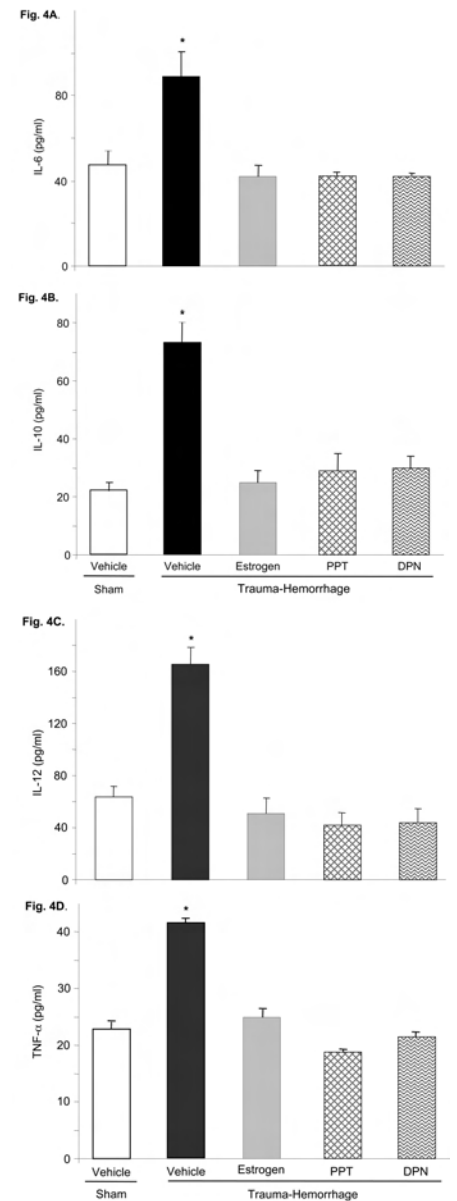


Figure 4. Effects of propyl pyrazole (PPT) and diarylpropionitrile (DPN) on production of cytokines IL-6 (A), IL-10 (B), IL-12 (C), and TNF- α (D) by keratinocytes after trauma-hemorrhage. Keratinocytes were isolated from sham animals and trauma-hemorrhage animals treated with vehicle, DPN, PPT or estrogen. The isolated keratinocytes were stimulated with LPS for 24 hr, supernatants were harvested, and IL-6, IL-10, IL-12 and TNF- α levels in the supernatants were analyzed by use of a cytokine bead array. Data are mean \pm SEM from six animals in each group. * $P < 0.05$ compared with all other groups.

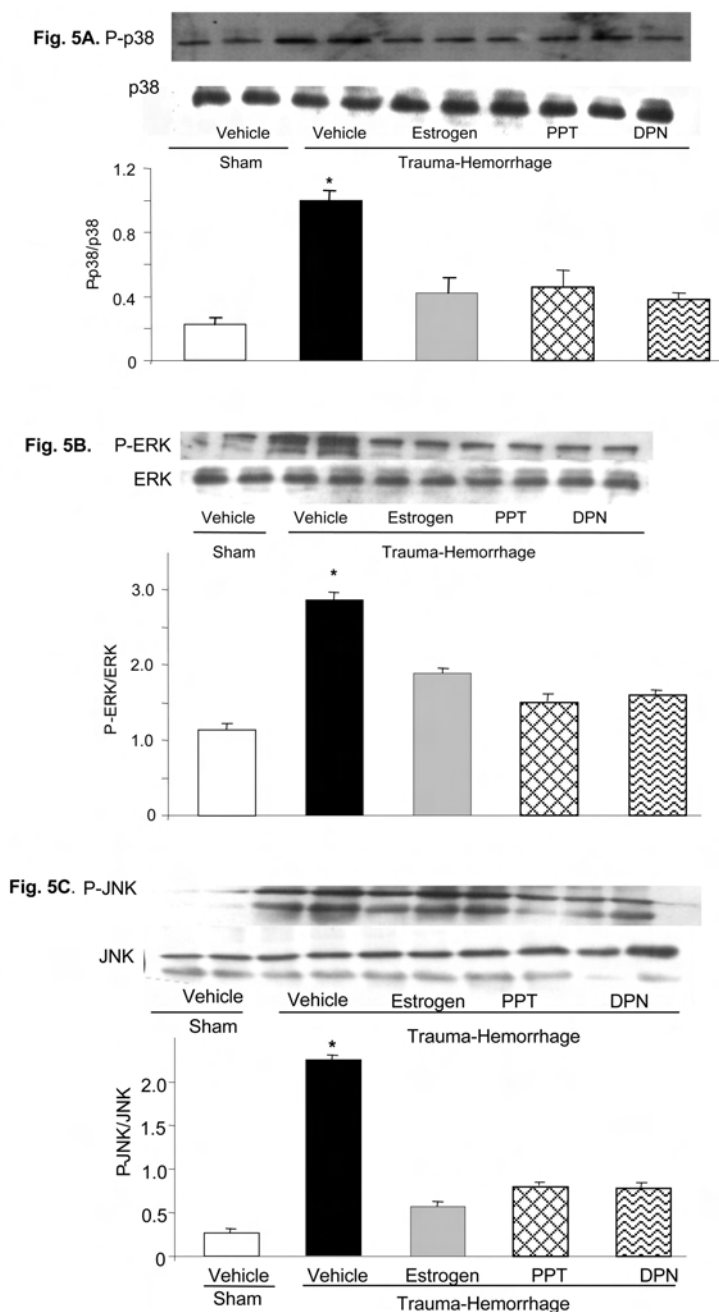


Figure 5. Effects of propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) on keratinocytes p38 (A), ERK (B), and JNK (C) phosphorylation following trauma-hemorrhage. The blots were stripped and reprobbed for p38, ERK, and JNK total protein contents in various lanes. Blots from six animals in each group were analyzed using densitometry. Densitometric values for phosphorylation were normalized to p38, ERK, and JNK protein and are shown in bar graph as mean ± SEM. **P* < 0.05 compared with all other groups.

nist ICI. Our results showed that the salutary effects of estrogen on keratinocytes were blocked if ICI was administered along with estrogen. ICI, via its ac-

tive metabolites, acts as a pure antagonist of both ER- α and - β (35). ICI binds to ERs competitively, and its binding leads to degradation and downregulation of

ERs. Furthermore ICI administration is followed by multiple changes in the ERs, which contribute to the blockade of estrogen action. These changes include impaired dimerization, increased turnover, and disrupted nuclear localization of the ERs (14).

Our present results indicate that at 2 h following trauma-hemorrhage, cytokine production and MAPK activation in keratinocytes from male mice were markedly increased. Administration of a single dose of estrogen during resuscitation attenuated the increase in these inflammatory markers. However, keratinocytes from animals treated with ICI and estrogen following trauma-hemorrhage displayed depressed immune function and thus the salutary effects of estrogen were blocked in those animals. These findings are in line with previous studies from our laboratory, which showed that administration of ICI blocked the salutary effects of estrogen in heart tissue (17). Because the ER antagonist depressed the estrogen-mediated keratinocyte immune responses, the results indicate that the protective effects of estrogen on keratinocyte immune response following trauma-hemorrhage are mediated via ERs.

There are two known ER subtypes, ER- α and ER- β . Because the two subtypes of ERs have different tissue distribution, it appears important to determine which subtype of ER contributes to the effect of estrogen in keratinocytes. In this study, we used the selective agonists for ER subtypes to determine which subtype has a predominant role in the effects of estrogen on immune cell cytokine production following trauma-hemorrhage. PPT, a selective ER- α agonist, binds to ER- α with 410-fold higher affinity than ER- β (36). In contrast, DPN, a selective ER- β agonist, has 70-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ER- β than with ER- α (2). Nonetheless, despite the specificity of the ER- α and ER- β agonist, the possibility that there may be some overlap in these stimuli cannot be ruled out. Our present

results showed that administration of ER- α agonist PPT and ER- β agonist DPN following trauma-hemorrhage significantly attenuated cytokine production by keratinocytes. These results therefore indicate that the ER- α agonist and ER- β agonist were both as effective as estrogen in preventing the increase in keratinocyte cytokine production following trauma-hemorrhage.

Although the present findings showed the salutary effects of PPT and DPN in keratinocyte cytokine production after trauma-hemorrhage, the precise mechanism by which this occurs remains unknown. MAPKs play an important role in the regulation of macrophage activation as well as other functions (4,37–42). Moreover, the production of various cytokines by macrophages involves MAPK signal transduction pathways (38,43–46). The present results demonstrate that p38, ERK1/2, and JNK were activated in keratinocytes after trauma-hemorrhage, a finding that suggests that the stress of trauma-hemorrhage *per se* caused the activation of some signaling pathways leading to MAPKs. Administration of PPT, DPN, or estrogen following trauma-hemorrhage, however, prevented MAPK activation in keratinocytes. This result suggests that the salutary effect of estrogen on MAPKs in keratinocytes is mediated via ER- α and ER- β . Studies have indicated that p38 activation is required for IL-6 and IL-10 expression. Although the ERK pathway has been shown to control TNF- α expression (4,47–51), neither IL-6 nor IL-10 appears to be ERK independent. JNK is also involved in IL-6 and TNF- α expression. MAPKs regulate cytokine production both at the transcriptional level via various transcriptional factors and at the posttranscriptional level via stabilizing mRNA and promoting their translations (8,37). Although the present study indicates that PPT, DPN, or estrogen administration following trauma-hemorrhage attenuated the activation of p38, ERK, and JNK, further studies are required to determine whether all three or any one of them has a predominant role in producing the

salutary effects under those conditions. Furthermore, although our results indicate that ER activation influences MAPKs, the precise mechanism by which ER activation influences the MAPKs remains unknown and should be examined in the future.

It can be argued that the present study used measurements at a single time point, that is, at 2 h after treatment, and thus it remains unclear whether the salutary effects of estrogen, PPT, and DPN on cytokine and MAPKs are sustained for a longer period of time after treatment. Although a later time point was not examined in this study, our previous studies have shown that if the improvement in cell and organ function by any pharmacological agent is evident at 2, 5, or 24 h after treatment, those salutary effects are sustained for prolonged intervals and they also improve the survival of animals (8,28,33). Thus, it would appear that the salutary effects of estrogen, PPT, and DPN on keratinocyte cytokine release and MAPK activation would be evident even if one measured those effects at another time point after trauma-hemorrhage and resuscitation.

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