17β-Estradiol inhibits keratinocyte-derived chemokine production following trauma-hemorrhage

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First published November 3, 2006; doi:10.1152/ajplung.00364.2006.


TRAUMA CONtributes TO MORE costs and loss of work-time years than malignancies or cardiovascular diseases (39). Most common causes of fatalities following severe trauma are complications in different organs leading to multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF). Despite numerous advances in intensive care medicine, ischemia/reperfusion injury, sepsis, and organ dysfunction leading to MOF remain the major causes of death in trauma patients as well as following major surgery (9, 15, 18, 34, 48). There is increasing evidence that the milieu of sex hormones influences the posttraumatic immune response. Previous studies from our laboratory and others indicate that 17β-estradiol (E2) has beneficial effects in modulating the posttraumatic immune/inflammatory response and prevents neutrophil-mediated tissue damage (43, 47).

Transendothelial migration of immunocompetent cells is a key step in development of tissue damage. Chemokines and their receptors play a pivotal role in mediating leukocyte infiltration into the inflamed tissue. They intervene at every step of transmigration from blood vessels into tissue (2). Chemokines are released from both immunocompetent and intrinsic cells (10, 46). We have previously shown that lung neutrophil infiltration is apparent following trauma-hemorrhage (T-H) (47). Neutrophils release cytokines, enzymes, and oxygen radicals, which leads to tissue damage, organ dysfunction, and ultimately organ failure (1, 6). Although other chemokine receptors, such as CCR1, 2, and 5, are associated with neutrophil infiltration (33), transmigration of neutrophils is primarily mediated via activation of keratinocyte-derived chemokine (KC) receptor CXCR2 by the CXC chemokine keratinocyte-derived chemokine [KC, cytokine-induced neutrophil chemoattractant (CINC), growth-related oncosgene-α (GRO-α), and CXC ligand 1 (CXCL1)] (25, 28).

Since E2 decreased GRO-α RNA expression and protein production in human endothelial cells (21), we hypothesized that E2 inhibits KC production following T-H, which should prevent neutrophil infiltration. To test this hypothesis, we investigated the effect of E2 treatment on KC and cytokine production, neutrophil infiltration, and edema formation in a murine T-H model.

MATERIALS AND METHODS

Animals and experimental groups. All animal studies were carried out in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male C3H/HeN mice 8–12 wk old and weighing 19–23 g were obtained from Charles River Laboratories (Wilmington, MA). Mice were either treated with estrogen or normal saline.

T-H procedure. Mice in the T-H groups were anesthetized with isoflurane (Mirad, Bethlehem, PA) and restrained in a supine position (20). A 2-cm midline laparotomy was performed, which was closed in two layers with sutures (Ethilon 6-0; Ethicon, Somerville, NJ). Both femoral arteries and the right femoral vein were cannulated with polyethylene tubing (Becton Dickinson, Sparks, MD). Blood pressure was measured via one of the arterial lines using a blood pressure analyzer (Micro-Med, Louisville, KY). Upon awakening, animals were bled rapidly through the other arterial catheter to a mean arterial blood pressure of 35.0 ± 5.0 mmHg within 10 min, which was then maintained for 90 min. At the end of the procedure, the animals were resuscitated via the venous line with four times the shed blood volume in the form of Ringer lactate. In the treatment group, E2 (1 mg/kg body wt) or vehicle (cyclodextrin) (Sigma, St. Louis, MO) was administered intravenously following sham operation or at the onset of resuscitation. After removing the catheters, the incisions were closed in two layers with sutures (Ethilon 6-0; Ethicon, Somerville, NJ).

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flushed with lidocaine and closed with sutures. Sham-operated animals underwent the same surgical procedures but were neither hemorrhaged nor resuscitated.

**Tissue harvesting.** The animals were anesthetized with isoflurane at 2 h following sham operation or resuscitation in the T-H groups, and blood was obtained via cardiac puncture using a syringe coated with EDTA (Sigma). Blood was centrifuged (2,500 g, 10 min, 4°C), and the plasma was stored at −80°C. Lungs were removed aseptically, frozen in liquid nitrogen, and stored at −80°C. Frozen tissue samples were thawed and suspended in 1% protease inhibitor cocktail (Sigma). The samples were sonicated on ice (Sonic Dismembrator; Fisher Scientific, Hampton, NH). The samples were then centrifuged at 12,000 g for 10 min at 4°C. The supernatants were frozen and stored at −80°C until further assayed. Aliquots were used to determine protein concentration (DC Protein Assay; Bio-Rad, Hercules, CA).

**Preparation of Kupffer cells.** Kupffer cells were isolated as previously described (40). In brief, the portal vein was catheterized with a 27-gauge needle, and the liver was perfused with 20 ml of Hanks’ balanced salt solution (HBSS; Gibco, Grand Island, NY) at 37°C, which was immediately followed by perfusion with 15 ml of 0.05% collagenase IV (Worthington, Lakewood, NJ) in HBSS with 0.5 mM CaCl2 (Sigma) at 37°C. The liver was then removed and transferred to a Petri dish containing the above mentioned collagenase IV solution. The liver was minced, incubated for 15 min at 37°C, and passed through a sterile mesh stainless steel screen into a beaker containing 10 ml of cold HBSS with 10% FBS. The hepatocytes were removed by centrifugation at 50 g for 3 min. The residual cell suspension was washed twice by centrifugation at 800 g for 10 min at 4°C in HBSS. The cells were then resuspended in complete RPMI 1640 medium containing 10% FBS and antibiotics (50 U/ml penicillin, 50 mg/ml streptomycin, and 20 μg/ml gentamycin, all from Gibco) and layered over 16% Histodenz (Sigma) in RPMI 1640 medium and centrifuged at 2,300 g for 45 min at 4°C. After removing the nonparenchymal cells from the interface, the cells were washed twice by centrifugation (800 g, 10 min, 4°C) in complete RPMI 1640 medium. The cells were then resuspended in complete RPMI 1640 medium and plated in a 96-well plate at a cell density of 5 × 105 cells/ml. After 2 h of incubation (37°C, 95% humidity, and 5% CO2), nonadherent cells were removed by washing with RPMI 1640 medium. We compared the number of adherent cells at the end of 2 h and found no significant difference in the number of adherent cells in sham and T-H animals. The cells were then cultured under the above mentioned conditions for 24 h with or without 1 μg/ml LPS (Sigma). The cell-free supernatants were harvested and stored at −80°C until assayed.

**Isolation of RNA and quantitative real-time PCR.** Total RNA was prepared by TRI Reagent (Life Technologies, Grand Island, NY), and 2 μg of total RNA was then reversed to cDNA by MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed using TaqMan method on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were obtained from Applied Biosystems. The reaction mixture for TaqMan assay contained 5 μl of ×2 TaqMan Universal PCR Master Mix (Applied Biosystems) and 40 ng of cDNA. All samples were tested in triplicate, and average values were used for quantification. GAPDH mRNA was used as an endogenous control. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from the TaqMan probe. Analysis was performed using SDS v2.2 software (Applied Biosystems) according to the manufacturer’s instructions. The comparative cycle threshold (CT) method (ΔΔCT) was used for quantification of gene expression.

**Flow cytometric analysis of chemokine concentration.** KC concentrations in the plasma, Kupffer cell supernatants, and lung tissue were determined with Cytokine Bead Array inflammatory kits using flow cytometry according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA), as described previously (13). Briefly, 50 μl of mixed capture beads were incubated with 50-μl samples for 1 h at 25°C, and then 50 μl of mixed phycoerythrin detection reagent was added. After incubation for 1 h at 25°C in the dark, the complexes were washed twice and analyzed using the LSRII flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was carried out using the accompanying FACSDiva software and FCAP Array software (BD Biosciences). Tissue chemokine content was normalized to protein concentration.

**Myeloperoxidase assay.** The accumulation of neutrophils in the lung tissue was assessed by determination of the myeloperoxidase (MPO) activity as previously described (13). Frozen tissue samples were thawed and suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyltrimethylammonium bromide (Sigma). The samples were sonicated on ice, centrifuged at 12,000 g for 15 min at 4°C, and an aliquot (30 μl) was transferred into 180 μl of phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.005% hydrogen peroxide (Sigma). The change in absorbance at 460 nm was measured spectrophotometrically for 10 min. MPO activity was calculated using a standard curve that was generated using human MPO (Sigma), and values were normalized to protein concentration.

**Determination of wet-dry ratios.** Wet-to-dry weight ratios of lungs were used as a measure of tissue edema. Tissue samples were weighed immediately after removal (wet weight) and then subjected to desiccation in an oven at 80°C (Blue M, Asheville, NC) until a stable dry weight was achieved after 48 h. The ratio of the wet-to-dry weight was then calculated.

**Statistics.** Statistical analysis was performed using SigmaStat computer software (SPSS, Chicago, IL). Statistical significance was assumed where probability values of less than 0.05 were obtained. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by the Student’s t-test or the rank sum test (Mann-Whitney U test). Results are expressed as means ± SE.

**RESULTS**

**Effect of E2 on lung KC gene expression and protein content.** T-H led to a significant increase of KC gene expression and protein content in lung tissue of vehicle-treated animals compared with their respective sham group (P < 0.05). These increases were markedly diminished by E2 treatment. Sham animals treated with E2 did not show any differences compared with vehicle-treated sham animals (P > 0.05) (Fig. 1, A and B).

**Effect of E2 on Kupffer cell KC and cytokine production capacities.** Kupffer cell KC production capacity was increased following T-H in vehicle-treated animals (P < 0.05). Treatment with E2 decreased KC production capacity in T-H animals (P < 0.05) (Fig. 2A). Kupffer cell production capacities of the proinflammatory cytokines TNF-α and IL-6 were elevated 2 h following T-H in vehicle-treated animals (P < 0.05) (Fig. 2, B and C). E2 treatment markedly prevented the increase in Kupffer cell production capacity for TNF-α and IL-6 in T-H mice (P < 0.05). E2 had no effect on KC or cytokine production capacities in sham animals (P > 0.05) (Fig. 2, B and C).

**Effect of E2 on plasma KC and cytokine levels.** Plasma levels of KC were increased following T-H in vehicle-treated animals (P < 0.05). Treatment with T-H mice with E2 decreased plasma KC concentrations following T-H (P < 0.05). E2 had no effect in animals after sham procedure (P > 0.05) (Fig. 3A). Plasma levels of TNF-α and IL-6 were elevated 2 h following T-H in vehicle-treated animals (P < 0.05) (Fig. 3, B and C). E2 treatment markedly decreased plasma concentrations of TNF-α and IL-6 in T-H mice (P < 0.05). No effects of E2 on TNF-α
and IL-6 levels were detected in sham animals ($P > 0.05$) (Fig. 3, B and C).

Effect of E2 on MPO activity and wet-to-dry weight ratio. T-H increased MPO activity in lungs of vehicle-treated animals ($P < 0.05$), which was reduced by treatment of animals with estrogen by about $47\%$ ($P < 0.05$) (Fig. 4). E2 treatment had no effect in sham animals ($P > 0.05$) (Fig. 4). Lung tissue edema formation was increased in animals following T-H treated with vehicle ($P < 0.05$) (Fig. 5). Increase in wet-to-dry weight ratio was reduced by treatment with E2 following T-H ($P < 0.05$) (Fig. 4). Wet-to-dry weight ratios were comparable in both sham groups ($P > 0.05$) (Fig. 5).

**DISCUSSION**

Neutrophil infiltration is a key step in the development of organ dysfunction following major trauma. Chemokines play a pivotal role in mediating extravasation of neutrophils to the site of inflammation (11). Following T-H, neutrophil influx is believed to be responsible for induction of tissue damage (42). In this study, we examined the effect of E2 on KC, a major neutrophil chemoattractant, as well as on proinflammatory cytokines such as IL-6 and TNF-α. Our results indicate that plasma and lung KC levels, as well as Kupffer cell KC and cytokine production capacities, are increased, and KC gene expression is upregulated in the lung following T-H. Additionally, T-H caused an increase in lung neutrophil infiltration and increased the lung wet-to-dry weight ratio, a parameter for tissue damage. Treatment of animals with E2 at the onset of resuscitation diminished or prevented increased Kupffer cell production capacities and decreased lung KC protein and gene expression as well as plasma KC and cytokine levels compared with T-H vehicle-treated mice. Moreover, this was associated with a reduction of neutrophil influx and edema formation in the lung. Thus these findings suggest that upregulation of KC following T-H contributes to neutrophil infiltration and tissue damage, which was decreased by administration of E2 following T-H.

Kupffer cells are the largest pool of tissue-embedded macrophages in the body (5). Due to their localization in the liver sinusoids, they are permanently exposed to gastrointestinal-derived pathogens and represent the first line of defense against infections. Furthermore, they were identified as the major source of inflammatory cytokines following stress conditions and therefore contributing to the systemic levels (13).

KC is a major chemoattractant for neutrophils (35) releasing reactive metabolites, which in turn causes tissue damage and
edema formation (6). Oxygen radicals released by neutrophils have been shown to be the major cause of tissue damage following T-H (42). Furthermore, the influence of KC on neutrophil infiltration was investigated in various studies (3, 24, 30). Whereas silencing KC gene expression had no effect on neutrophil infiltration in a murine two-hit model (25), other investigators described a more profound role for the rat KC homologue CINC (32). Our recent studies indicate that monocyte chemoattractant protein-1 (MCP-1) regulates KC production following T-H. Support for this notion comes from our studies that showed that blocking MCP-1 prevented upregulation of KC, neutrophil infiltration, and tissue damage following T-H. Alternatively, restitution of KC abolished the beneficial effects of blocked MCP-1 (8). After blocking CXCR2, the receptor for KC, attenuation of neutrophil influx was detected following hemorrhage and cecal ligation and puncture, as well as after ozone exposure, and in cigarette smoke-induced lung inflammation (14, 24, 38). Whereas neutrophil influx is associated with locally increased KC levels, no activation or infiltration of neutrophils was detected after injection of recombinant KC in healthy mice (3). These results thus indicate that priming of neutrophils by other factors is necessary to initiate transmigration. This notion is supported by data from a human wound healing model showing elevated levels of GRO-α (human homologue to KC) at the site of injury 7 days after resolution of neutrophil infiltration (7).

Besides reactive metabolites released from neutrophils, KC itself causes tissue damage. Injection of recombinant KC resulted in necrosis and increased synthesis of type I collagen without infiltration of neutrophils, indicating a direct cell toxic effect of KC (37). However, it remains unclear which cells are the main source of KC production. In a model of a local infection, neutrophils were the major source of KC (10), whereas in a model of acute lung injury, depletion of either macrophages or neutrophils reduced lung KC levels (23). In another study, it was shown that CINC production in hepatocytes...
cytes was regulated by IL-β released by Kupffer cells, identifying hepatocytes as another source of KC production (26). After stimulation, peritoneal macrophages also showed an upregulation of KC expression (4). Thus these studies suggest that there is more than one cell type that produces KC.

There is increasing evidence that the sex hormone milieu prevailing at the time of injury influences immune responses (17, 43). Impaired immune functions in ovariectomized females and young males following T-H were restored by E2 treatment (17, 19). In the current study, E2 treatment following T-H decreased cytokine Kupffer cell production capacities and decreased plasma IL-6 and TNF-α, as well as KC concentrations, which was associated with less lung neutrophil infiltration and remote organ damage. An immunomodulating effect of E2 on immunocompetent cells such as T-cells, neutrophils, macrophages, and dendritic cells has been demonstrated in various studies (13, 29, 31, 47). Whereas investigators have shown that E2 inhibits neutrophil infiltration following T-H, ischemia/reperfusion injury, experimental abdominal aortic aneurysm (12), and sepsis, other studies (41) suggest that E2 does not influence neutrophil infiltration following exercise. Nonetheless, our study (13) has also shown that treatment of animals with E2 following T-H prevents upregulation of MCP-1, which was associated with less neutrophil infiltration and edema formation in the lung under those conditions.

During pregnancy in mice, E2 and progesterone induce an increase in intrauterine KC production, suggesting that chemokines play a role in accumulation of immunocompetent cells in the uterus during pregnancy (44). Moreover, in cultured human umbilical vein endothelial cells, exposure to E2 decreased GRO-α (rat homologue to murine KC) mRNA expression and protein levels (21). However, there is only one study (36) investigating the effect of E2 on KC production under stress conditions. In a model of LPS-induced acute lung inflammation, KC levels were comparable in the bronchoalveolar lavage fluid from male, female, and ovariectomized mice. So far, the effect of E2 on KC under stress conditions has not yet been completely investigated.

Although E2 normalized the Kupffer cell KC production capacity, the KC plasma levels were decreased but not normalized. This suggests that cells other than Kupffer cells may also contribute to the circulating levels. It is also possible that the clearance of KC takes longer than the 2-h time period examined in this study. Further studies examining the time course of KC production and clearance will provide additional insight into this question.

Although chemokine and cytokine levels were nearly normalized after treatment with E2 in T-H mice, neutrophil infiltration and edema formation were not completely restored under such conditions. Previous studies (47) have shown that neutrophil infiltration and tissue damage occur later in the posttraumatic course than the 2-h period examined in the current study. The time period of 2 h used in this study may in part explain the less than optimal effect of E2 observed in entirely alleviating neutrophil infiltration. It is also possible that a higher dose of E2 than that used in this study may be required to produce a more prominent effect. Another explanation would be the redundancy of the chemokine system, suggesting that blocking one ligand can be compensated by other ligands. In particular, the KC receptor CXCR2 seems to be first primed by KC and thereafter activated by macrophage inflammatory protein-2 (CXCL2) (22). Since systemic and lung KC levels were not restored to the levels observed in sham animals following T-H and E2 treatment, CXCR2 may still be primed by the slightly elevated KC levels. Moreover, E2 treatment in ovariectomized mice downregulated adhesion molecules in a model of vessel injury-inhibiting neutrophil chemotactic activity (27). However, blocking of KC after treatment with E2 was associated with a reduction of lung neutrophil infiltration and edema formation, suggesting that KC contributes to these pathological changes following T-H. It should, however, be noted that definitive proof that KC is the major attractant for neutrophils following T-H has still not been obtained. In view of this, studies in KC-deficient mice or treatment with KC-blocking agents are necessary to confirm this notion.

It is tempting to speculate the mechanism by which estradiol alters the posttraumatic immune response. Previous studies have shown that estradiol blocks NF-κB activation during stress conditions (45), which in turn can lead to prevention of cytokine production (16). Since all of the investigated cytokines are regulated by NF-κB, it is likely that this is the common mechanism by which estradiol prevents upregulation of cytokines following T-H.

Since free radicals and proteases released by infiltrating neutrophils are responsible for producing tissue damage during the posttraumatic immune response, understanding of neutrophil trafficking is fundamental for any therapeutic approach in this field. Our data demonstrate that E2 downregulates production and systemic levels of KC, a neutrophil chemoattractant, following T-H. Moreover, this was associated with attenuated neutrophil infiltration and less edema formation. Although there is only limited knowledge about how E2 modulates neutrophil infiltration following T-H. Moreover, this was associated with attenuated neutrophil infiltration and less edema formation. Although there is only limited knowledge about how E2 modulates neutrophil trafficking following T-H. Despite this limitation, however, the present study supports the concept that modulating chemokine production could serve as a novel therapeutic target for the treatment of posttraumatic immunological alterations.

ACKNOWLEDGMENTS

We thank Bobbi Smith for editing.

GRANTS

This work was supported by National Institutes of Health Grant R01-GM-37127. M. Schwacha is supported in part by National Institutes of Health Grant K02-AI-49960.

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