Regulatory effects of estrogen on acute lung inflammation in mice

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EVIDENCE IS INCREASING that female hormones play a role in mediating the inflammatory response. Women of reproductive age are less likely than other people to develop rheumatoid arthritis (22), and they experience fewer symptoms of cardiovascular disease (38). Laboratory studies suggest that both testosterone and estrogen may affect outcomes after trauma and sepsis (1, 6, 16, 32, 55). However, clinical studies are limited and have shown conflicting results (13, 30, 47, 39). No studies of hormone replacement therapy (HRT) (5, 46, 51).

In vitro studies have demonstrated the ability of exogenous estradiol to alter cytokine expression. The results of these studies demonstrated a suppressive role for estrogen in IL-6 and IL-1β production both in vivo and in vitro, suggesting a role for ovarian hormones in mediating the inflammatory response. Women of reproductive age are less likely than other people to develop rheumatoid arthritis (22), and they experience fewer symptoms of cardiovascular disease (38). Laboratory studies suggest that both testosterone and estrogen may affect outcomes after trauma and sepsis (1, 6, 16, 32, 55). However, clinical studies are limited and have shown conflicting results (13, 30, 47, 39). No overall mechanism exists to explain the role of female hormones in clinical conditions as diverse as these. The present study was designed to assess whether ovarian hormones modulate lung inflammatory responses in mice.

There is now considerable evidence demonstrating a role for estrogen in mediating the production of proinflammatory cytokines (reviewed in Ref. 42). During estrogen deficiency, increases in IL-1, IL-6, and TNF-α production have been described in ex vivo cultures of unstimulated monocytes and macrophages as well as in osteoblasts (21, 23, 24, 40). Increases in IL-6 and TNF-α have been demonstrated in the circulation of females after natural or surgically induced menopause (15, 17). Serum IL-6 levels were also found to be lower in postmenopausal women undergoing hormone replacement therapy (HRT) (5, 46, 51).

Estrogen deficiency is also associated with increases in cell adhesion molecule expression. Serum levels of ICAM-1 inversely correlate with estradiol levels in women (4), and studies have shown that postmenopausal women treated with HRT or estrogen alone had reduced ICAM-1, E-selectin, and vascular cell adhesion molecule (VCAM)-1 serum levels compared with untreated postmenopausal women (10, 25, 45, 50). In vitro studies have demonstrated the ability of exogenous estrogen to mediate both ICAM-1 and VCAM-1 expression; however, conflicting data exist describing both stimulatory (7, 9, 54) and inhibitory effects (7, 43).

The cytokines TNF-α, IL-6, and IL-1β are important early mediators in acute lung inflammation (52) and are required for expression of selectins and adhesion molecules (35, 36). The selectins related to endothelial cells (P- and E-selectin) are responsible for the initial adhesive interactions with leukocytes, while the adhesion molecules ICAM-1 (28, 34) and VCAM-1 (34, 37) are required for the firm adhesion and ultimate transmigration of leukocytes into the lung interstitium. In addition to selectins and adhesion molecules, both CXC (48) and CC (3, 19, 49) chemokines have been shown to play a role in mediating polymorphonuclear neutrophil (PMN) migration into the inflamed lung. The ability of estrogen to mediate expression of inflammatory cytokines as well as adhesion molecules suggests a role for estrogen in the regulation of lung inflammation. However, very few in vivo studies addressing the effects of estrogen in lung inflammatory injury have been conducted. Therefore, the purpose of the present study was to address this issue.

Accordingly, lung injury was induced in male, female, and ovariectomized (OVX) mice after intratracheal administration of lipopolysaccharide (LPS). PMN migration, lung injury (assessed according to vascular albumin leakage), and cytokine (TNF-α, IL-1α, and IL-1β) and chemokine (CXC and CC) content, as well as adhesion molecule expression (ICAM-1 and VCAM-1), were assessed. In addition, studies using macrophages were performed to determine the in vitro ability of estradiol to alter cytokine expression. The results of these studies demonstrated a suppressive role for estrogen in IL-6 and IL-1β production both in vivo and in vitro, suggesting a role for ovarian hormones in mediating the inflammatory response. Women of reproductive age are less likely than other people to develop rheumatoid arthritis (22), and they experience fewer symptoms of cardiovascular disease (38). Laboratory studies suggest that both testosterone and estrogen may affect outcomes after trauma and sepsis (1, 6, 16, 32, 55). However, clinical studies are limited and have shown conflicting results (13, 30, 47, 39). No overall mechanism exists to explain the role of female hormones in clinical conditions as diverse as these. The present study was designed to assess whether ovarian hormones modulate lung inflammatory responses in mice.
novel pathway by which estrogen suppresses PMN recruitment and tissue damage in acute lung injury.

MATERIALS AND METHODS

Reagents. LPS isolated from Escherichia coli (sterile serotype 026:B6 and 0111:B4), estradiol (stock in ethanol), and myeloperoxidase (MPO) assay reagents were purchased from Sigma (St. Louis, MO). Capture and detection antibodies for TNF-α, IL-1β, IL-6, KC, macrophage inflammatory protein (MIP)-2, and monocyte chemoattractant protein (MCP)-3 were purchased from R&D Systems (Minneapolis, MN), and capture and detection antibodies for mouse albumin were obtained from Bethyl Laboratories (Montgomery, TX). All other antibodies were purchased from Pharmingen (San Diego, CA) unless otherwise specified.

LPS-induced lung injury and experimental design. Wild-type (WT) male and cycling female (with the estrus cycle determined by vaginal smear) mice (4–6 wk old, 18–20 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Because previous studies have shown the greatest protection against inflammation during the proestrus cycle (14), only mice in this phase were used in our experiments.

Fig. 1. Lung vascular permeability and polymorphonuclear neutrophil (PMN) content in bronchoalveolar lavage (BAL) fluids from male and female mice 6 h after airway instillation of lipopolysaccharide (LPS). Mouse albumin levels were measured in BAL fluids (A) as an index of vascular leakage, while BAL fluid PMN content (B) and myeloperoxidase (MPO) content in lungs (C) were measured as an index of PMN influx. Results are means ± SE. For each bar, n = 5 mice. *P < 0.05 compared with uninjured control. #P < 0.05 compared with LPS-treated male mice.

Fig. 2. Lung vascular permeability, BAL fluid content of PMNs, and lung MPO content in female mice with intact ovaries compared with ovariectomized (OVX) mice after LPS-induced lung injury. Mouse albumin levels were measured in BAL fluids (A) as an index of vascular leakage. BAL fluid PMN content (B) and lung MPO content (C) were measured. Results are means ± SE. For each bar, n = 5 mice. *P < 0.05 compared with uninjured controls. #P < 0.05 compared with OVX-treated mice.

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experiments. Mice 6–8 wk of age were anesthetized by intraperitoneal injection of 150 mg/kg ketamine HCl and 65 μg/kg xylazine hydrochloride, and LPS was instilled intratracheally (1 mg/kg in 50 μl of sterile saline) during inspiration. In separate experiments, LPS-induced lung injury was administered in C57BL/6 OVX and cycling female (in estrus) mice (The Jackson Laboratory) at 6–8 wk of age. In another set of experiments, estradiol (50 μg/kg in 400 μl of PBS) or vehicle was administered intraperitoneally 1 h before LPS instillation. Six hours after LPS instillation or at the time points indicated for bronchoalveolar lavage (BAL) fluid cytokine analysis, mice were euthanized and BAL fluid was collected and stored at −80°C. Lungs were perfused, snap frozen in liquid nitrogen, and stored at −80°C.

**BAL fluid collection and cell counts.** BAL was performed three times with 0.8 ml of sterile saline. The recovered BAL fluid was centrifuged at 300 g for 10 min at 4°C. The cell-free supernatant fluids from the first wash were stored at −20°C for further analysis of cytokines/chemokines and mouse albumin content using ELISA. Cells were counted with the aid of a hemocytometer, and PMN populations were found to contain at least 95% PMN as demonstrated by cytospin and differential stain analysis.

**Determination of myeloperoxidase activity.** After BAL, lungs were perfused via the right ventricle with 2 ml of sterile PBS, snap frozen in liquid nitrogen, and stored at −80°C. To measure myeloperoxidase (MPO) activity, whole lungs were homogenized and sonicated in 50 mM KPO4 buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant fluids containing MPO were incubated in a 50 mM KPO4 buffer containing the substrate H2O2 (1.5 M). In the presence of o-dianisidine dihydrochloride (167 μg/ml; Sigma Aldrich), enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm over 3 min using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

**Determination of albumin content in BAL fluid.** Mouse albumin levels in BAL fluid were measured using a mouse albumin ELISA kit purchased from Bethyl Laboratories. The detection limit for this ELISA was 7 ng/ml.

**Morphological assessment of lung injury.** To assess lung injury morphologically, lungs were fixed by intratracheal instillation of 1 ml of buffered formalin (pH 7.2, 10%) 6 h after intratracheal instillation.
of LPS. Histological examination was conducted after tissue sectioning and staining with hematoxylin and eosin.

Isolation of peritoneal macrophages. Macrophages were isolated from the peritoneal cavity of 8- to 10-wk-old C57BL/6 WT male mice 4 days after peritoneal injection with 0.5 ml of 3% thioglycolate medium, yielding >95% macrophages as demonstrated using cyto-spin and differential stain analysis. The cells were seeded at a density of $10^6$ cells/ml in RPMI containing 10% fetal bovine serum and

Fig. 4. Time course analysis of TNF-α (A) and IL-1β (B) levels in BAL fluids from OVX mice compared with ovary-intact mice after LPS-induced lung injury. TNF-α (C) and IL-1β (D) levels in BAL fluids from male and female mice 6 h after LPS instillation. Results are means ± SE. For each value, $n = 5$ mice. *$P < 0.05$ compared with sham-treated mice.

Fig. 5. CXC [macrophage inflammatory protein (MIP)-2 and KC] and CC [monocyte chemoattractant proteins (MCP)-1 and MCP-3] chemokine levels in BAL fluids from OVX mice compared with ovary-intact mice 6 h after LPS-induced lung injury. Results are means ± SE. For each bar, $n = 5$ mice. *$P < 0.05$ compared with uninjured controls.
plated into 24-well plates at 0.5 ml/well. After 2–3 h of incubation, the cells were washed and stimulated with LPS (100 ng/ml) in the presence or absence of estradiol at concentrations considered to be physiological (10^{-8}–10^{-11} M). The plates were washed and blocked for 1 h with PBS containing 3% BSA. Various dilutions of samples with appropriate standards were added to the wells and incubated for 2 h, followed by washing and incubation in appropriate biotinylated secondary antibody (2 μg/ml) for 1 h. Wells were washed twice, and streptavidin peroxidase was added for 30 min, followed by washing and incubation in O-phenylenediamine dihydrochloride substrate (Sigma Aldrich) for 10 min. The reaction was stopped by addition of 0.5 M sulfuric acid. Absorbance was measured at 490 nm using a Molecular Devices plate reader. The detection limit for all cytokines/chemokines ranged between 30 and 120 pg/ml. ICAM-1 levels in whole lung homogenates were determined using an ELISA kit purchased from R&D Systems, with a detection limit of 30 pg/ml.

**Statistical analysis.** All numerical results are expressed as means ± SE. For these assays, statistical analysis was performed using one-way repeated-measures ANOVA, followed by a multiple-comparison procedure using the Student-Newman-Keuls method. A value of $P < 0.05$ was considered significant.
RESULTS

LPS-induced lung injury. Lung injury in response to LPS was determined by measuring albumin leakage into the alveolar compartment and PMN migration into the lungs as determined on the basis of BAL fluid PMN content and MPO content in whole lung extracts. Six hours after LPS challenge, albumin levels in both male and female mice were significantly increased compared with uninjured (control) lungs (Fig. 1A). In male mice, there was an almost fourfold increase in albumin leakage over that found in female mice after LPS instillation. Increases in lung permeability correlated with increases in PMN accumulation in lungs as determined on the basis of BAL fluid PMN content (Fig. 1B) and lung MPO content (Fig. 1C). As with albumin leakage, PMN accumulation in the lungs of male mice administered LPS was significantly higher than levels in similarly treated female mice.

The above data suggest a possible role for ovarian hormones in modulating acute lung injury. To extend these observations, lung inflammatory injury was examined in female mice in which the ovaries had been removed surgically (OVX mice). The comparison group was female mice with intact ovaries. Similar to the above findings, both lung permeability (Fig. 2A) and lung PMN accumulation (Fig. 2B and C) in response to LPS instillation were significantly increased in LPS-treated OVX mice compared with levels observed in ovary-intact mice. Morphological changes in the lungs of these LPS-treated mice correlated with increased MPO activity (Fig. 3). As expected, PMNs were absent from the lungs of sham-treated and OVX mice not otherwise treated (Fig. 3, A–D). After intratracheal administration of LPS, intraalveolar and interstitial accumulation of PMN was evident (Fig. 3, E–H). In the lungs of OVX mice, the LPS-induced inflammatory response was considerably more intense as evidenced by a more enhanced alveolar accumulation of PMN, fibrin deposition, and hemorrhage (Fig. 3, E and F).

Cytokine/chemokine levels in BAL fluid of mice exposed to LPS. The early response cytokines, TNF-α, IL-1β, and IL-6, were measured in the BAL fluids of OVX and female mice administered LPS intratracheally (Fig. 4). TNF-α levels were significantly increased 2 h after LPS administration in both OVX and ovary-intact mice. There was no statistically significant difference in TNF-α levels in the two groups (Fig. 4A). Elevations in IL-1β levels were slightly delayed, reaching a plateau between 4 and 6 h (Fig. 4B). In OVX mice, there was a significant increase in IL-1β levels compared with those in ovary-intact mice. Similar results were obtained in studies comparing male with female treated mice 6 h after LPS administration (Fig. 4, C and D). TNF-α levels were the same, but male mice had higher levels of IL-1β. No detectable levels
Both TNF-α/H9251 male and female mice treated with LPS (Fig. 6) showed a statistically significant difference in VCAM-1 levels between treated and untreated groups. Enhanced VCAM-1 expression during acute lung inflammation was associated with ICAM-1 levels, which were significantly upregulated in the lungs of male mice (Fig. 6).

Effects of ovarian hormones on ICAM-1 and VCAM-1 expression in lungs of mice after LPS-induced lung injury. To assess a possible role for endogenous estrogen in mediating the lung inflammatory response, we determined whether exogenous estradiol could mimic the apparent regulatory function of ovaries in acute lung injury. Administration of estradiol before intratracheal LPS instillation in OVX mice significantly reduced both albumin leakage (Fig. 7A) and BAL fluid PMN accumulation (Fig. 7B) 6 h after LPS administration. OVX mice had more intense albumin leak and buildup of PMN in BAL fluids compared with ovary-intact mice or OVX mice treated with estradiol. This decrease in lung injury in estradiol-treated OVX mice correlated with a significant reduction in BAL fluid IL-1β levels but not in TNF-α levels (Fig. 8, A and B). Compared with OVX mice, ovary-intact mice and OVX mice treated with estradiol showed a significant reduction in lung ICAM-1 and serum IL-6 levels (Fig. 8, C and D). Thus it appears that endogenous estrogen is capable of mediating acute lung inflammation in mice, which may be mediated through its effect on IL-1β, IL-6, and ICAM-1.

Estradiol regulates IL-1β but not TNF-α production in peritoneal macrophages. Because macrophages are known to possess both estrogen receptors-α and -β (33), a possible role for estrogen in mediating TNF-α and/or IL-1β production in macrophages was examined. Stimulation of macrophages with LPS (100 ng/ml) for 18 h caused substantial production of both TNF-α and IL-1β (Fig. 9). Similar to the in vivo response, estradiol had no effect on TNF-α production after exposure of macrophages to LPS (Fig. 9A). However, LPS-induced IL-1β levels were markedly reduced with increasing estradiol concentrations (Fig. 9B).

DISCUSSION

The results of this study demonstrate a suppressive role for intact ovaries or exogenously administered estradiol in OVX mice in regulating acute lung injury and PMN recruitment in response to intratracheal LPS administration. These results are in agreement with those of recent studies in which female rats were found to be more resistant to shock (1, 6) and carrageenan-induced lung injury (14) than their male counterparts. In addition, clinical studies have suggested that females are more resistant to infection than males and that male patients have a higher incidence of sepsis and shock after surgery than females (39, 47, 53). Laboratory studies using rodent models of shock and sepsis have also shown females to be more resistant to infection (2). However, in these studies, the immunoinflammatory response in females was preserved while in males it was depressed, suggesting a possible desensitization of the inflammatory response in males. In those studies as well as in the present report, resistance to injury correlated with the presence of either endogenous or exogenous estrogen, suggesting that estrogens serve a protective role against acute inflammation.

Studies of the mechanism by which estrogen negatively regulates the inflammatory response in vivo have been limited.
In the present study, a suppressive role of estrogen on IL-6 and IL-1β production both in vivo and in vitro was demonstrated, suggesting a novel pathway by which estrogen mediates LPS-induced lung injury. The ability of estrogen to regulate IL-1β production is in agreement with earlier studies demonstrating increased IL-1β production with estrogen deficiency in ex vivo cultures of unstimulated macrophages (23, 24, 41), as well as in vivo in response to carrageenan-induced lung injury (14). In contrast to these findings, some researchers have reported stimulatory effects of estrogen on both IL-1 and IL-6 production in macrophages and/or monocytes (18, 20, 29). However, studies reporting decreases in cytokine activity with menopause are absent, suggesting that stimulatory effects of estrogen on cytokine activity may occur only under nonphysiological conditions.

In the context of lung injury, IL-1β serves as an early mediator of acute lung injury. It is linked to increased production of CXC chemokines in BAL fluid and adhesion molecule expression (ICAM-1, E-selectin) on vascular endothelial cells, each group of which is required for attracting PMN into the lung. In addition to IL-1β, IL-6 is an early inflammatory mediator and has been described as inducing ICAM-1 expression in endothelial cells (44). In the present study, increases in both IL-1β and IL-6 levels corresponded to increased lung content of ICAM-1, which may be a mechanism by which male or OVX mice showed intensified accumulation of PMN and enhanced lung injury.

A role for estrogen in regulating ICAM-1 expression has been demonstrated in this study. ICAM-1 expression in the lungs of LPS-treated OVX and male mice was enhanced compared with ovary-intact mice. These findings correlated with enhanced IL-1β and IL-6 production in BAL fluid and in serum, respectively. Pretreatment of the OVX mice with exogenous estradiol before LPS-induced injury reduced IL-1β, IL-6, and ICAM-1 levels back to those found in ovary-intact mice. Interestingly, injection of estradiol at the same time as LPS instillation had no effect on IL-1β, IL-6, or ICAM-1 levels (data not shown), suggesting transcriptional regulation by estradiol. Because IL-1β and IL-6 both possess NF-κB binding sites in the promoter region, it is likely that the effect of estrogen on these mediators is mediated through estradiol’s effect on NF-κB. In addition, both IL-1β and IL-6 are known activators of NF-κB. IL-1β has been shown to mediate NF-κB activation in the lungs of mice either directly or indirectly after IgG immune complex deposition (27). Therefore, IL-1β and IL-6, by their ability to upregulate NF-κB, may have been responsible for upregulating ICAM-1 expression in the present study. Numerous in vivo studies have supported a role for estrogen in mediating cell adhesion molecule expression. Decreases in serum ICAM-1 and VCAM-1 levels after estrogen or hormone replacement therapy have been described in postmenopausal women (10, 25, 45, 50). In vitro studies also have demonstrated a role for estrogen in mediating ICAM-1 expression; however, the results are conflicting, with both suppressive (7, 43) and stimulatory (7, 9, 54) effects having been reported. Because ICAM-1 is known to mediate PMN migration in acute lung injury (28, 34), the ability of estrogen to suppress ICAM-1 expression in the lungs of LPS treated mice suggests a pathway by which estrogen inhibits PMN migration into the lung.

In addition to its role in mediating chemokine and cell adhesion molecule expression in lung injury, IL-1β is a potent suppressor of apoptosis in a number of cell types, including neutrophils and epithelial cells (11, 12, 26). Apoptosis is a process of controlled cell death that is important in the remodeling of tissues during the repair process. Apoptosis has been shown to play a role in acute lung injury, but whether it is beneficial to the lung repair process is still controversial (31). In the present study, decreases in IL-1β production with estrogen treatment in OVX mice may suggest a beneficial role for estrogen by inducing apoptosis during acute lung inflammation, but this is speculative. This hypothesis is in agreement with a recent study in which estrogen was shown to induce apoptosis in macrophages by interacting with the Fas ligand promotor (33). Further studies into the mechanisms by which estrogen mediates apoptosis and the potential cell types involved may improve the understanding of the role of estrogen in acute lung inflammation.

The results of this study demonstrate a regulatory role for ovarian hormones and exogenous estradiol in modulating lung injury and PMN migration into LPS-injured lungs. In addition, a role for IL-1β, IL-6, and ICAM-1 in enhancing the inflammatory response in OVX and male mice has been demonstrated. Further studies of the mechanisms of estrogen’s actions on these mediators of inflammation are needed to obtain a better understanding of the role of estrogen in suppressing the anti-inflammatory response.

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REFERENCES


9. Cif MC, Kleinman HK, Grant DS, Schnaper HW, Fauzi AS, and Hoffman GS. Estradiol enhances leukocyte binding to tumor necrosis...


