Estrogen pretreatment protects males against hypoxia-induced immune depression

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Received 24 September 2001; accepted in final form 9 December 2001

Knöferl, Markus W., Martin G. Schwacha, Doraid Jarrar, Martin K. Angele, Keith Fragoza, Kirby I. Bland, and Irshad H. Chaudry. Estrogen pretreatment protects males against hypoxia-induced immune depression. Am J Physiol Cell Physiol 282: C1087–C1092, 2002. First published December 12, 2001; 10.1152/ajpcell.00454.2001.—Hypoxemia depresses cell-mediated immune functions in males, whereas proestrous females do not show such a depression. We hypothesized that elevated systemic estradiol levels in proestrous females prevent hypoxia-induced immune depression. To study this hypothesis, male C3H/HeN mice were pretreated with 17β-estradiol (E2, 40 μg/kg body wt sc) or vehicle for 3 days before induction of hypoxemia and again immediately before induction of hypoxia. The mice were subjected to hypoxemia (95% N2-5% O2) or sham hypoxemia (room air) for 60 min, and plasma and spleen cells were collected 2 h later. In vehicle-treated mice, splenocyte proliferation and interleukin-2 and interleukin-3 production were depressed after hypoxemia. E2-pretreated animals, however, displayed no such depression in splenic T cell parameters after hypoxemia. Splenic macrophage cytokine production was also depressed in vehicle-treated mice subjected to hypoxia, whereas it was normal in E2-pretreated mice. In summary, these findings indicate that administration of E2 before hypoxemia prevents the depression of cell-mediated immune functions. Thus administration of 17β-estradiol in high-risk patients before major surgery might decrease hypoxia-induced immune depression under those conditions.

There is a wealth of information indicating that hormonal mechanisms play an important role in regulating immune functions under stressful conditions, such as trauma-hemorrhage and septic shock (2). Studies have also demonstrated that severe hypoxemia in males, in the absence of blood loss or tissue trauma, results in a profound systemic inflammatory response and depressed immune responses similar to those observed after trauma-hemorrhage, a condition associated with regional tissue hypoxemia (10, 18). Similarly, although depressed cell-mediated immune functions have been observed in males after trauma-hemorrhage, no depression has been observed in proestrous females under such conditions (15, 34). Several studies have provided evidence that the hormonal environment is responsible for the gender-specific inflammatory response after adverse circulatory conditions. Testosterone has been shown to exert immunosuppressive effects on immune functions, while estrogens appear to be immunoprotective (1, 33). Recently, administration of 17β-estradiol has been shown to exhibit beneficial effects on posttraumatic immune responses in males after trauma-hemorrhage (16). Additional support for the protective role of estrogens comes from studies in cardiovascular research indicating gender differences in the susceptibility to hypoxia-induced or oxidant-mediated organ and cell dysfunction (12, 22, 25, 31). In light of those findings, we hypothesized that administration of 17β-estradiol in males should prevent the depression of cell-mediated immune responses after severe hypoxemia. The aim of the present study, therefore, was to determine whether pretreatment of males with 17β-estradiol has any protective effects on immune responses after hypoxemia.

Materials and Methods

Animals. Inbred male C3H/HeN mice (Charles River Laboratories, Wilmington, MA), 7–8 wk of age (24–27 g body wt), were used in this study. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This project was conducted at Rhode Island Hospital and was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and Brown University.

Experimental groups. Male mice were randomly assigned to the following two treatment groups: mice in group 1 received a subcutaneous injection of 200 μl of corn oil vehicle (Sigma Chemical, St. Louis, MO), and animals in group 2 received a subcutaneous injection of 17β-estradiol (40 μg/kg dissolved in corn oil). In preliminary studies, this dose of 17β-estradiol was found to increase plasma 17β-estradiol concentrations in male mice comparable to levels observed in}

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female mice in the proestrous state of the estrous cycle. Animals were treated daily between 8 and 9 AM for 3 days before experimentation and again immediately before the induction of hypoxemia or sham hypoxemia. Animals from each treatment group were then randomly assigned to the hypoxemia or sham hypoxemia group (n = 7–8/group).

**Murine model of hypoxemia.** The hypoxemia model used in our experiments was previously described by Ertel et al. (10). Animals were placed in two plastic chambers (20 × 10 × 8 cm), each with an inlet and an outlet, through which the hypoxic gas mixture or room air flowed. Hypoxemia was induced by flushing one of the chambers with a gas mixture of 95% N₂-5% O₂ at a flow rate of 10 l/min for 60 min. At the same time, control (sham) animals were kept in the second chamber, which was flushed with room air for 60 min. The animals were constantly monitored during this period, and no immediate or late mortality was observed with the use of this hypoxemia model. Previous studies using this murine hypoxemia model have shown that, in male mice, arterial Po₂ decreased significantly to ~40 mmHg throughout the duration of hypoxemia and returned to a baseline of 120 mmHg within minutes after the end of hypoxemia (10, 18). The mice were symptomatic for hypoxia, displaying rapid shallow breathing and minimal physical activity. The animals were killed by methoxyflurane overdose 2 h after hypoxemia to obtain the spleen and whole blood.

**Plasma collection and storage.** Whole blood was obtained by cardiac puncture and placed in microcentrifuge tubes (Microtainer, Becton Dickinson, Rutherford, NJ). The tubes were then centrifuged at 16,000 g for 15 min at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored at −80°C until assayed.

**Preparation of splenocyte cultures.** At 2 h after hypoxemia or sham hypoxemia, the spleens were removed aseptically, and splenocytes were isolated as previously described in detail (35). Splenocyte viability was tested using trypan blue exclusion and found to be ~95% in all groups. The splenocytes were then resuspended in RPMI 1640 (GIBCO-BRL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO-BRL) at a final concentration of 1 × 10⁷ cells/ml. The ability of the splenocyte cultures to produce cytokines in response to a mitogenic challenge was assessed by incubation for 48 h at 37°C, 5% CO₂, and 90% humidity in the presence of concanavalin A (2.5 μg/ml; Pharmacia/LKB Biotech, Piscataway, NJ). After incubation, the cell suspension was centrifuged at 300 g for 15 min, and the supernatants were harvested and stored at −80°C until assayed. The cells’ ability to proliferate in response to mitogenic stimulation with 0 (negative control) or 2.5 μg/ml concanavalin A was measured by incorporation of [³H]thymidine, as previously described (30).

**Preparation of splenocyte macrophage cultures.** Splenocyte macrophage cultures were established by adherence, as previously described in detail (36). The monolayers of splenocyte macrophages (1 × 10⁶ cells/ml) were stimulated with 10 μg of lipopolysaccharide from *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, MI) per milliliter of Click’s medium containing 10% heat-inactivated fetal bovine serum for 48 h at 37°C, 5% CO₂, and 90% humidity to assess the cells’ ability to release cytokines. At the end of the incubation period, the culture supernatants were removed, centrifuged at 300 g for 15 min, divided into aliquots, and stored at −80°C until assayed for cytokine concentrations.

**Assessment of cytokine and prostaglandin E₂ production.** The capacity of splenocyte cultures to produce interleukin (IL)-2 (CTLL-2) and IL-3 (FDC-P1) was assessed by determining the amount of respective cytokines in the collected culture supernatant using specific bioassays as previously described in detail (18, 20). IL-6 activity was determined by assessing the 72-h proliferation of the IL-6-dependent murine hybridoma 7TD1 cells stimulated by serial dilutions of plasma or supernatants, as described in detail elsewhere (24). IL-1β (DuoSet, Genzyme Diagnostics, Cambridge, MA) and IL-10 (OptEIA Set, Pharmingen, San Diego, CA) levels in the supernatants were determined by ELISA according to the manufacturer’s recommendations. Prostaglandin (PG) E₂ levels in splenic macrophage supernatants were determined using an ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

**Determination of plasma 17β-estradiol concentrations.** 17β-Estradiol concentrations were measured using a commercially available radioimmunoassay (ICN Biomedicals, Costa Mesa, CA) as recommended by the manufacturer.

**Statistical analysis.** Values are means ± SE. One-way ANOVA, followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons, was used to determine the significance of the differences between experimental means. P < 0.05 was considered significant.

## RESULTS

**Effect of 17β-estradiol pretreatment on plasma estradiol and IL-6 levels.** At 2 h after hypoxemia (i.e., 3 h after the last administration of vehicle or 17β-estradiol), plasma concentrations of 17β-estradiol were significantly increased in male mice pretreated with 17β-estradiol in both treatment groups compared with vehicle-pretreated male mice (P < 0.05; Table 1). Plasma concentrations of IL-6 were also markedly increased in vehicle-pretreated male mice (P < 0.05); however, circulating IL-6 levels in 17β-estradiol-pretreated male mice subjected to hypoxemia were similar to those observed in sham animals (Table 1).

**Splenocyte responses after hypoxemia.** Splenocyte proliferative capacity was significantly depressed in male mice pretreated with vehicle compared with the corresponding sham-treated animals (P < 0.05; Fig. 1A). In male mice pretreated with 17β-estradiol for 3 days before hypoxemia, however, no depression of splenocyte proliferative capacity was observed. At 2 h after hypoxemia, splenocyte IL-2 and IL-3 release was significantly depressed in vehicle-pretreated male mice compared with those observed in 17β-estradiol-pretreated male mice.

**Table 1. Plasma 17β-estradiol and IL-6 concentrations 2 h after sham hypoxemia or hypoxemia.**

<table>
<thead>
<tr>
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<th>17β-Estradiol, pg/ml</th>
<th>IL-6, U/ml</th>
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<tbody>
<tr>
<td>Sham hypoxemia</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>24.5 ± 1.7</td>
<td>0.15 ± 0.12</td>
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<tr>
<td>17β-Estradiol</td>
<td>69.1 ± 4.2†</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>Hypoxemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>24.7 ± 4.0</td>
<td>4.25 ± 1.38*</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>69.5 ± 6.3†</td>
<td>0.67 ± 0.43†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7–8 animals in each group. Plasma 17β-estradiol and interleukin-6 (IL-6) concentrations in male C57H/HeN mice were measured 2 h after sham hypoxemia or hypoxemia. Male mice received corn oil vehicle or 17β-estradiol (40 μg/kg body wt) sc and were subjected to hypoxemia or sham hypoxemia. Plasma samples were harvested 2 h thereafter. *P < 0.05 vs. sham hypoxemia-vehicle; †P < 0.05 vs. hypoxemia-vehicle (ANOVA).
compared with the corresponding sham-treated animals ($P < 0.05$; Fig. 1, B and C). In male mice pretreated with 17β-estradiol, splenocyte IL-2 and IL-3 production after hypoxemia was comparable to that observed in sham animals.

**Splenic macrophage responses after hypoxemia.** After hypoxemia, splenic macrophage IL-1β production was decreased in vehicle-pretreated male mice compared with corresponding sham-treated animals; however, this decrease was not statistically significant (Fig. 2A). In male mice receiving 17β-estradiol before the experiment, IL-1β release after hypoxemia was comparable to values in sham-treated animals. At 2 h after hypoxemia, splenic macrophage IL-6 release was significantly depressed in vehicle-pretreated male mice compared with vehicle-pretreated sham animals ($P < 0.05$; Fig. 2B). However, in male mice pretreated with 17β-estradiol, splenic macrophage IL-6 release was maintained at sham levels after hypoxemia. Similar to the production of IL-6, splenic macrophage IL-10 release was significantly depressed after hypoxemia in vehicle-pretreated mice ($P < 0.05$; Fig. 3A). Pretreatment with 17β-estradiol prevented the depression of splenic macrophage IL-10 production in male mice subjected to hypoxemia. In splenic macrophages harvested from mice pretreated with vehicle, PGE$_2$ production was maintained at sham levels after hypoxemia (Fig. 3B). PGE$_2$ production by splenic macrophages from 17β-estradiol-pretreated male mice was significantly reduced after hypoxemia compared with 17β-estradiol-pretreated sham animals ($P < 0.05$).

**DISCUSSION**

Previous studies have shown that the inflammatory response to severe hypoxemia exhibits a gender-specific pattern (18). In those studies, hypoxemia led to significantly increased plasma concentrations of proinflammatory cytokines in males, but not in females. Furthermore, splenic macrophage cytokine productive capacity was significantly depressed in males, but not in females, after hypoxemia. Additionally, a shift toward an immunosuppressive Th-2 cytokine profile was found in males after hypoxemia, whereas no such shift was observed in females under those conditions. These results indicate that, unlike males, females in the proestrous state do not manifest immunosuppression after severe hypoxemia. Because circulating levels of female sex hormones are increased in the proestrous
has salutary effects on depressed splenocyte immune functions comes from studies that have shown restoration of the depressed splenocyte immune responses in males treated with 17β-estradiol after trauma-hemorrhage (16). Furthermore, trauma-hemorrhage led to immune depression in ovarioctomized females with decreased plasma levels of estrogens; however, treatment with 17β-estradiol during fluid resuscitation normalized splenocyte immune functional parameters (16). In those studies, in vitro addition of 17β-estradiol to splenocyte cultures from animals subjected to trauma also resulted in stimulation of splenocyte proliferation and cytokine production, supporting the notion that estradiol administration before hypoxemia had direct effects on splenocytes. However, it is possible that the beneficial effects of 17β-estradiol pretreatment on splenocyte immune functions are not solely direct actions of this hormone on splenocytes but are also mediated via indirect mechanisms, such as macrophage-splenocyte interactions. In this regard, 17β-estradiol administration altered splenic macrophage functions after severe hypoxemia. Although splenic macrophage IL-1β, IL-6, and IL-10 release was depressed in vehicle-pretreated male mice after hypoxemia, no differences in the release of these cytokines were observed between 17β-estradiol-pretreated male mice subjected to hypoxemia or sham hypoxemia. These results, therefore, suggest that 17β-estradiol prevented the depression of splenic macrophage cytokine productive capacity after hypoxemia. Our results further indicate that splenic macrophage PGE2 production was maintained in vehicle-pretreated male mice after hypoxemia, while in 17β-estradiol-pretreated male mice the production of PGE2 was significantly lower under those conditions. These results are in line with the results from previous studies which indicate that, in proestrous females with increased circulating estrogens, splenic macrophage PGE2 production was significantly lower after hypoxemia than in sham animals (18). Furthermore, studies have shown that, after hypoxemia, plasma concentrations of PGE2 were significantly increased in male animals (32).

Studies have shown that hypoxemia led to significantly increased plasma concentrations of IL-6 in males, whereas in females no differences in plasma IL-6 levels were observed between animals subjected to hypoxemia and those subjected to normoxemia (10, 18). Our observation that plasma concentrations of IL-6 were significantly increased in vehicle-pretreated male mice 2 h after hypoxemia is in accordance with those studies. Regarding the depression of splenic macrophage IL-6 productive capacity observed in vehicle-pretreated males after hypoxemia, it appears likely that cell populations other than splenic macrophages are responsible for the increased systemic concentrations of IL-6. Previously, studies have also shown that the elevation of circulating proinflammatory cytokines was associated with a marked activation of Kupffer cells to release those cytokines in vitro (10). Although Kupffer cell cytokine production was not determined in the present study, it appears likely that the increased

state of the estrous cycle (29), we hypothesized that these hormones are involved in maintaining immunocompetence in females after hypoxemia. The aim of the present study, therefore, was to determine whether pretreatment of male mice with 17β-estradiol has any salutary effects on splenocyte and splenic macrophage immune functions after severe hypoxemia.

The results presented here indicate that, at 2 h after sham hypoxemia or hypoxemia, plasma concentrations of 17β-estradiol were significantly increased in male mice pretreated with 17β-estradiol compared with vehicle-pretreated male mice. Furthermore, the plasma concentrations of 17β-estradiol measured in pretreated males were similar to those described in the literature in proestrous females, suggesting physiological relevance of the treatment regimen used (29).

Hypoxemia resulted in markedly depressed splenocyte proliferation as well as splenocyte IL-2 and IL-3 release capacity in vehicle-pretreated male mice. These results are comparable to the results of our previous studies (18). However, in male mice that were pretreated with 17β-estradiol before the induction of hypoxemia, the splenocyte functional parameters were unaltered compared with those in mice subjected to sham hypoxemia. After hypoxemia, the pattern of splenocyte and splenic macrophage function observed in 17β-estradiol-pretreated males closely resembled that in proestrous females under such conditions (18). Additional support for the notion that 17β-estradiol

Fig. 3. LPS-stimulated splenic macrophage IL-10 (A) and prostaglandin E2 (PGE2, B) production. Male mice received corn oil vehicle or 17β-estradiol (40 µg/kg body wt sc) and were subjected to hypoxemia or sham hypoxemia. After 2 h, splenocytes were harvested and splenic macrophage cultures were established. Values are means ± SE of 7–8 animals in each group. *P < 0.05 vs. hypoxemia-vehicle; #P < 0.05 vs. sham hypoxemia-vehicle; +P < 0.05 vs. sham hypoxemia-estradiol (ANOVA).
IL-6 plasma levels after the hypoxic insult result from activation of these macrophage populations. The finding that IL-6 was not increased in 17β-estradiol-pretreated males 2 h after hypoxemia further suggests that 17β-estradiol prevented the increased release of this cytokine by Kupffer cells. Nonetheless, studies also support other tissues (i.e., gut) as an important source of IL-6 after injury (8, 23). In this regard, Nelson et al. (21) showed that inhibition of gut-derived IL-6 with pentoxifylline improved survival after sepsis. Thus the beneficial effects of 17β-estradiol after hypoxia may also be related to attenuation of the cytokine (IL-6) response of the gut.

Although our findings suggest several possibilities by which 17β-estradiol might contribute to maintain immune functions after hypoxemia, the target level of immunoenocrine interactions remains unclear. Because estrogen receptors have been identified in macrophages (3, 13) as well as splenic T lymphocytes (28), it appears likely that these cells may be prone to functional modulation by exogenous estrogens administered before hypoxemia. Although estrogen’s interactions with lymphocytes (4, 27) and macrophages (6, 7, 26) have been reported in various experimental settings, the exact effects of estrogens on immune cells under hypoxic conditions remain to be determined. It is possible that the beneficial modulatory effects of 17β-estradiol pretreatment on immune functions after hypoxemia might, in part, be the indirect result of the hormone’s effects on other organ systems. In this regard, Razandi et al. (25) showed that 17β-estradiol via membrane-bound estrogen receptor rapidly activates p38 mitogen-activated protein kinase in endothelial cells, thereby preserving endothelial cell structure and function and preventing apoptosis under hypoxic conditions. Griffin et al. (12) demonstrated that although female cardiac fibroblasts are resistant to hypoxia-induced inhibition of DNA synthesis, male fibroblasts are susceptible, and estrogen partially reversed the proliferative response in male cells via estrogen receptor-dependent mechanisms. Furthermore, it is possible that other effects of 17β-estradiol, such as vasodilatation due to changes in ion channel function (11, 14) or increased endothelial nitric oxide production (5, 19), also indirectly contribute to the beneficial effects on immune functions by improving the microcirculation during hypoxic conditions. Recent studies have demonstrated that proestrous females (with elevated estrogen levels) subjected to trauma-hemorrhage (a condition associated with regional tissue hypoxia) do not display an increased mortality after subsequent sepsis, whereas the mortality rate in males was markedly increased (9). These findings suggest that 17β-estradiol may play a critical role in the improved outcome in proestrous females under such conditions. Thus it can be speculated that the 17β-estradiol-induced improvement in immune functions after hypoxia would likely translate into reduced mortality after a subsequent septic insult.

In summary, the data presented here demonstrate that, in vehicle-pretreated males, splenocyte immune functional parameters and splenic macrophage proinflammatory cytokine production were significantly depressed after severe hypoxemia. Pretreatment with 17β-estradiol over 3 days before hypoxemia prevented this depression of splenocyte and splenic macrophage immune functions. At 2 h after hypoxemia, plasma IL-6 levels were significantly increased in vehicle-pretreated males, and 17β-estradiol pretreatment significantly attenuated the increase in circulating IL-6. These findings indicate that pretreatment with 17β-estradiol prevented the adverse effects of hypoxemia on splenocyte and splenic macrophage immune functions as well as increased plasma IL-6 levels and suggest that the low levels of 17β-estradiol in male animals contribute to the depressed immune responses after hypoxemia. Because 17β-estradiol pretreatment attenuated immune responses after hypoxemia, administration of this sex hormone before major surgery might be a useful approach for preventing the depression in cell-mediated immune responses in patients at risk of hypoxemia.

This study was supported by National Institute of General Medical Sciences Grant R01 GM-57127 (I. H. Chaudry) and Deutsche Forschungsgemeinschaft Fellowship Kn 475/1-1 (M. W. Knöferl). Present address of M. W. Knöferl: Dept. of Trauma-Surgery, University of Ulm, Steinhövelstr. 9, 89075 Ulm, Germany.

REFERENCES


11. Freay AD, Curtis SW, Korach KS, and Rubanyi GM. Mechanism of vascular smooth muscle relaxation by estrogen in