Severe hypoxemia in the absence of blood loss causes a gender dimorphic immune response

MARKUS W. KNÖFERL,1 DORAID JARRAR,2 MARTIN G. SCHWACHA,2 MARTIN K. ANGELE,3 WILLIAM G. CIOFFI,4 KIRBY I. BLAND,2 AND IRSHAD H. CHAUDRY2

1Department of Surgery, Center for Surgical Research, Brown University School of Medicine and Rhode Island Hospital, Providence, Rhode Island 02903; 2Department of Surgery, School of Medicine, University of Alabama-Birmingham, Birmingham, Alabama 35294; 3Department of Trauma-Surgery, University of Ulm, 89075 Ulm, Germany; and 4Department of Surgery, Klinikum Grosshadern, 81377 Munich, Germany

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Knöferl, Markus W., Doraid Jarrar, Martin G. Schwacha, Martin K. Angele, William G. Cioffi, Kirby I. Bland, and Irshad H. Chaudry. Severe hypoxemia in the absence of blood loss causes a gender dimorphic immune response. Am J Physiol Cell Physiol 279: C2004–C2010, 2000.—A gender dimorphic immune response has been observed after trauma and severe hemorrhage, a condition believed to be associated with tissue hypoxia. Although studies have shown that hypoxemia per se in males causes a systemic inflammatory response, it is unclear if the inflammatory response to hypoxemia exhibits gender dimorphic characteristics. To study this, male and female C3H/HeN mice in the proestrus state of the estrous cycle were subjected to hypoxemia (95% N2-5% O2) or sham hypoxemia (room air) for 60 min. Later (2 h), plasma interleukin (IL)-6 and tumor necrosis factor (TNF) may be marked with 'advertisement' in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and that tissue hypoxia is considered to be an important contributing factor in the pathophysiology of severe hemorrhagic shock, the present study was undertaken to test the hypothesis that severe hypoxemia in the absence of blood loss and tissue trauma would also produce a gender dimorphic inflammatory/immune response.

MATERIALS AND METHODS

Animals. Inbred male and female 8- to 9-wk-old (24–26 g body wt) C3H/HeN mice (Charles River Laboratories, Wilmington, MA) 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 published by the National Institutes of Health. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and Brown University.

Experimental groups. Male and proestrus female mice were randomly assigned to either a hypoxemia group or a sham group. The animals in groups 1 and 2 served as sham controls and underwent all manipulations necessary for induction of hypoxemia; however, they were exposed to room air. Animals in groups 3 and 4 were subjected to hypoxemia. Groups 1 and 3 consisted of male mice, and animals in groups 2 and 4 consisted of female mice in the proestrus state of their estrous cycle. The estrous cycle phase was determined from the cytology of vaginal smears obtained daily at 7:00 to 8:30 AM. Proestrus was defined when both leukocytes and nucleated epithelial cells were observed in approximately equal numbers. Eight to nine mice were included in each group.

Hypoxemia model. The hypoxemia model previously described by Ertel et al. (8) in mice was employed. In brief, mice 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 5 l/min for a period of 60 min. Control (sham) animals were kept in the second chamber, which was flushed with room air for 60 min at the same time. The animals were constantly monitored during this period, and no immediate or late mortality was observed with the use of this model of hypoxemia.

To confirm the capacity of this murine model to induce systemic hypoxemia and to examine the effects of hypoxemia on arterial blood pressure, a separate cohort of male and proestrus female mice was lightly anesthetized with methoxyflurane overdose at 2 h after hypoxemia or sham hypoxemia, and the spleen, peritoneal macrophages, and whole blood were harvested as described below.

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

Preparation of splenocyte and splenic macrophage cultures. The spleens were removed aseptically and were placed in separate petri dishes containing 4°C PBS solution. Splenocytes were isolated as previously described in detail (22). Viability was tested by trypan blue exclusion and was found to be ~95% irrespective of the group. The splenocytes were suspended in RPMI 1640 (GIBCO-BRL, Grand Island, NY) containing 10% heat-inactivated FBS (GIBCO-BRL) at a final concentration of 1 × 106 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 with 5% CO2 and 90% humidity in the presence of 2.5 μg/ml concanavalin A (ConA; Pharmacia/LKB Biotech, Piscataway, NJ). After incubation, the cell-free supernatants were harvested and stored at −80°C until analysis for IL-3, IL-10, and interferon (IFN)-γ. A second portion of the splenocyte suspension was placed in a 96-well microtiter plate (Corning Glass, Corning, NY) in aliquots of 100 μl/well. The cell's ability to proliferate in response to mitogenic stimulation with 0 (negative control) or 2.5 μg/ml ConA (Pharmacia/LKB Biotech) was measured by the [3H]thymidine incorporation technique previously described (22).

Splenic macrophages were purified by plastic adherence, as previously described in detail (20). The monolayers of splenic macrophages (~106 cells/well) were stimulated with 10 μg lipopolysaccharide W (from Escherichia coli 055:B5; Difco Laboratories, Detroit, MI) in 1 ml of Click's medium containing 10% heat-inactivated FBS (GIBCO-BRL) for 48 h at 37°C with 5% CO2 and 90% humidity. At the end of the incubation period, cell-free supernatants were collected and subsequently stored at −80°C until analysis for cytokine and PGE2 levels.

Assessment of cytokine release. The capacity of the mixed splenocyte culture to produce IFN-γ was assessed by determining the amount of IFN-γ in the collected culture supernatant according to the method of Migliorini et al. (13). In brief, serial dilutions of the splenocyte supernatants were added to RAW 264.7 cells (1 × 105 cells/ml) in duplicate and were incubated for 24 h at 37°C with 5% CO2 and 90% humidity. Nitrite levels in cell-free supernatants were determined using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H3PO4). Relative units per milliliter of IFN-γ activity present in the unknown samples were determined by comparison of the curves produced by dilutions of the samples with those generated by a dilution of recombinant murine standard (IFN-γ standard: 400 U/ml; Genzyme, Cambridge, MA).

For measurement of IL-3 release, serial dilutions of the supernatants and standards were added to IL-3-dependent FDC-P1 cells (2.5 × 105 cells/ml) and were incubated for 24 h at 37°C with 5% CO2 and 90% humidity. At the end of this period, 1 μCi of [3H]thymidine (specific activity 6.7 Ci/mmol; New England Nuclear, Wilmington, DE) was added to each well, and the cultures were incubated for an additional 16 h. The cells were then harvested on glass-fiber mats, and the β-decay was detected by liquid scintillation radiography, as previously described (12). IL-1β and IL-10 in supernatants were determined using the sandwich enzyme-linked immunoabsorbent assay technique according to the manufacturer's
instructions [IL-1β (Genzyme); IL-10 (Pharmingen, San Diego, CA)].

IL-6 and TNF-α activity was determined by assessing either the 72-h proliferation of the IL-6-dependent murine hybridoma 7TD1 or the 24-h cytotoxicity induced in WEHI 164 clone 13 cells stimulated by serial dilutions of plasma, as previously described (4). For the last 3 h of incubation, 20 μl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/ml in RPMI 1640; Sigma Chemical, St. Louis, MO) were added to each well. The amount of dark blue formazan crystal formation was then measured spectrophotometrically at 570 nm. The units of IL-6 or TNF-α activity were determined by comparison of curves produced from dilutions of a recombinant mouse IL-6 standard (200 U/ml; Genzyme) or murine TNF-α (100 U/ml; Genzyme), respectively, according to the methods of Mizel (14).

**Measurement of macrophage PGE₂ release.** PGE₂ levels in splenic macrophage supernatants were determined using an enzyme-linked immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

**Statistical analysis.** The results are presented as 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. A P value < 0.05 was considered significant.

**RESULTS**

Arterial Po₂ and mean arterial blood pressure. The hypoxemia model was initially evaluated in a separate set of males and proestrus females. The results indicate that arterial Po₂ decreased from baseline values of 120 mmHg to values of ~40 mmHg at 20 min after the beginning of hypoxemia and remained at that level throughout the duration of hypoxemia (Fig. 1A). At 20 min after the end of hypoxemia, Po₂ returned to values slightly higher than baseline and remained elevated at 120 min after hypoxemia. There were no differences in arterial Po₂ between male and proestrus female mice that were subjected to hypoxemia. Mean arterial blood pressure slightly decreased during the course of hypoxemia in both males and proestrus females (Fig. 1B; P < 0.05). After hypoxemia, mean arterial blood pressure rapidly increased to values somewhat lower than that observed before the induction of hypoxemia. In males and females subjected to sham hypoxemia, blood pressure slightly increased, presumably as a result of restraint stress.

**Plasma levels of IL-6 and TNF-α.** Plasma concentrations of IL-6 were markedly increased (~6-fold) in male mice at 2 h after hypoxemia (P < 0.05; Fig. 2A). In contrast, circulating IL-6 levels in proestrus females subjected to hypoxemia were similar to sham values (Fig. 2A). Plasma TNF-α levels increased approximately twofold in males subjected to hypoxemia compared with sham males (P < 0.05; Fig. 2B). In proestrus females, however, no increase in plasma TNF-α was observed after hypoxemia (Fig. 2B).

**Splenocyte responses.** Splenocyte IFN-γ release was depressed in male mice at 2 h after hypoxemia (P < 0.05; Fig. 3A), whereas no significant change in IFN-γ production was observed in proestrus females. Similarly, the production of IL-3 by splenocytes harvested from male mice after hypoxemia was increased (P < 0.05), whereas in proestrus females splenocyte IL-3 release was unaltered under those conditions (Fig. 3B). In contrast, IL-10 production by splenocytes harvested from male mice after severe hypoxemia was increased (P < 0.05 compared with male sham animals; Fig. 3C). In proestrus females, splenocyte IL-10 was unaffected by hypoxemia.

Splenocyte proliferative capacity was significantly depressed after hypoxemia (Fig. 4). In proestrus females, however, no depression of splenocyte proliferative capacity was observed after hypoxemia compared with female sham animals (Fig. 4).

**Spleenic macrophage responses.** Splenic macrophage IL-1β release was depressed in male mice subjected to hypoxemia (P < 0.05 compared with male shams; Fig. 5A). In females that underwent hypoxemia, splenic macrophage IL-1β production was maintained at sham levels. Similarly, splenic macrophage IL-6 production was also significantly depressed in males that underwent hypoxemia (P < 0.05 compared with male shams) and was maintained in proestrus females under those conditions (Fig. 5B). Splenic macrophage IL-10 release was depressed in males after hypoxemia (P < 0.05 compared with male shams). In females, however, splenic macrophage IL-10 production was maintained.
There is increasing evidence that hormonal mechanisms, specifically sex steroids, are involved in regulating immune functions after trauma and hemorrhagic shock. In this regard, it has been shown that testosterone plays a crucial role in producing the immunosuppression observed in males after trauma-hemorrhage and also contributes to the increased susceptibility to subsequent sepsis under those conditions (7). In contrast, studies have shown that immune functions after trauma-hemorrhage were maintained or even enhanced in females in the proestrus state of the estrous cycle (21). The maintained immunocompetence in females after trauma-hemorrhage was also associated with significantly decreased mortality from subsequent sepsis compared with in males subjected to similar insults (7). Because systemic concentrations of female sex hormones (i.e., estrogen, prolactin) are increased in the proestrus state (16), it has been specu-

(Fig. 6A). IL-12 release by splenic macrophages from male mice was also significantly depressed after hypoxemia ($P < 0.05$ compared with male shams; Fig. 6B). IL-12 production by splenic macrophages harvested from proestrus females after hypoxemia was comparable to sham values.

In male mice, PGE$_2$ production was maintained at sham levels in splenic macrophages harvested from animals that underwent hypoxemia (Fig. 7). PGE$_2$ production by splenic macrophages from proestrus females after hypoxemia was slightly, but significantly, suppressed compared with female sham animals ($P < 0.05$).

**DISCUSSION**

Previous studies from our laboratory have demonstrated a systemic inflammatory response with elevated IL-6 and TNF-α in male mice subjected to hypoxemia in the absence of blood loss or severe hypotension (8). Comprably increased circulating concentrations of proinflammatory cytokines have been observed after trauma-hemorrhage (2, 5), a condition that is associated with tissue hypoxia. In this regard, tissue hypoxia is considered to be an important factor for producing the changes in cell and organ metabolism and in immune responsiveness after hemorrhagic shock (6).
lated that those hormones are involved in maintenance of immunocompetence in females after hemorrhagic shock. Recent studies from our laboratory have shown that ovariectomy performed in females 2 wk before trauma-hemorrhage reduced systemic levels of sex hormones and significantly depressed splenic and peritoneal macrophage functions after trauma-hemorrhage (10). This depression in macrophage functions in ovariectomized females after trauma-hemorrhage was also associated with a significantly increased mortality from subsequent sepsis compared with females in the proestrus state. Although these studies suggest the importance of sex hormones in regulating immune functions after trauma-hemorrhage, it remains unknown whether or not such mechanisms are involved in the inflammatory response to severe hypoxemia in the absence of tissue trauma and blood loss. The results presented here indicate that hypoxemia increased plasma concentrations of IL-6 and TNF-α as compared with male controls. In females, however, no differences in plasma levels of IL-6 and TNF-α were evident between animals subjected to hypoxemia or normoxemia. The observation that plasma concentrations of proinflammatory cytokines significantly increased in males at 2 h after hypoxemia are in accordance with previous studies in rats (8, 18). In those studies, the elevation of circulating proinflammatory cytokines was shown to be associated with a marked activation of peritoneal macrophages and Kupffer cells to release those cytokines in vitro (8). Although Kupffer cell and peritoneal macrophage cytokine production was not determined in the present study, it appears likely that the increased plasma concentrations of proinflammatory cytokines after the hypoxic insult are the result of activation of these macrophage populations in males due to the hypoxic insult. The findings that IL-6 and TNF-α were not increased in females at 2 h after hypoxemia suggest that, in females, the release of those cytokines by Kupffer cell peritoneal macrophages was not enhanced. Nonetheless, additional studies are required to confirm this.

Our results also indicate that splenic T lymphocyte proliferative capacity was significantly depressed in male mice at 2 h after hypoxemia, whereas no depression of the lymphoproliferative responses was observed in proestrus females. A significant depression of splenocyte IFN-γ and IL-3 release was also observed in males subjected to hypoxemia but not in females in the proestrus state. These findings are consistent with the results from studies performed in a murine model of trauma-hemorrhage which indicate that splenic T lymphocytes responded to hypoxemia with a marked activation of peritoneal macrophages and Kupffer cells to release those cytokines in vitro (8). Although Kupffer cell and peritoneal macrophage cytokine production was not determined in the present study, it appears likely that the increased plasma concentrations of proinflammatory cytokines after the hypoxic insult are the result of activation of these macrophage populations in males due to the hypoxic insult. The findings that IL-6 and TNF-α were not increased in females at 2 h after hypoxemia suggest that, in females, the release of those cytokines by Kupffer cell peritoneal macrophages was not enhanced. Nonetheless, additional studies are required to confirm this.

Fig. 4. Proliferative capacity of splenocytes harvested from male and proestrus female C3H/HeN mice at 2 h after sham hypoxemia or hypoxemia. Splenocytes were stimulated with 2.5 μg/ml ConA for 48 h, and proliferation was measured by the [3H]thymidine incorporation technique. Data are means ± SE of 8–9 animals in each group. *P < 0.05 vs. male sham hypoxemia and #P < 0.05 vs. male hypoxemia by ANOVA.

Fig. 5. Release of IL-1β (A) or IL-6 (B) by splenic macrophages harvested from male and proestrus female C3H/HeN mice at 2 h after sham hypoxemia or hypoxemia. Splenic macrophages were cultured in the presence of 10 μg/ml lipopolysaccharide (LPS) for 48 h, and cytokine levels in cell free supernatants were determined. Data are means ± SE of 8–9 animals in each group. *P < 0.05 vs. male sham hypoxemia and #P < 0.05 vs. male hypoxemia by ANOVA.
phocyte functions were significantly depressed in males after trauma-hemorrhage while proestrus females had maintained immune responses (21). In contrast, production of the anti-inflammatory Th-2 cytokine IL-10 by splenocytes was significantly increased in males after hypoxemia. In proestrus females, however, IL-10 production was not altered after hypoxemia compared with female sham animals. IL-10 has profound suppressive effects on cell-mediated immune responses (9) and has been implicated in the suppression of splenocyte immune functions after hemorrhage (3). In this regard, our results indicate that the increased production of IL-10 by splenocytes from males after hypoxemia was associated with a significantly depressed T lymphocyte proliferative capacity as well as splenocyte IL-3 and IFN-γ production. These findings suggest that, in males, the increased IL-10 production by splenocytes after hypoxemia might contribute to the depression of splenocyte immune functions under those conditions.

In addition to splenocytes, gender differences were observed in the response of splenic macrophages to severe hypoxemia. Although splenic macrophage IL-1β and IL-6 release was significantly depressed in males at 2 h after hypoxemia, no differences in splenic macrophage function were observed in females that underwent hypoxemia. The depression of splenic macrophage cytokine productive capacity observed in males after hypoxemia suggests that cell populations other than splenic macrophages are responsible for the increased systemic concentrations of proinflammatory cytokines observed in males under such conditions. The production of IL-12 by splenocytes was also significantly depressed in males after hypoxemia, whereas in females the release of this cytokine was unaltered. IL-12 is a well-characterized stimulant of certain aspects of T lymphocyte function and is known to facilitate the conversion to a Th-1 phenotype, which is associated with elevated IL-2 and IFN-γ release (15).

Because splenocyte cultures contain splenic macrophages, as well as T lymphocytes, it could be suggested that the depressed production of IL-12 in males after hypoxemia contributes to the expression of an immunosuppressive Th-2 cytokine profile characterized by increased splenocyte IL-10 and depressed IFN-γ production. Conversely, it appears that, in proestrus females, the maintenance of splenic immune function might not solely be the result of the direct actions of sex hormones on a specific cell population (i.e., T lymphocytes or macrophages). Rather, sex hormones may modulate macrophage T lymphocyte interactions, possibly at the level of antigen presentation.

Our results also indicate that splenic macrophage PGE₂ production was maintained in male mice after hypoxemia, whereas in females PGE₂ production slightly decreased under those conditions. Previous studies from our laboratory performed in a rat model of severe hypoxemia have shown that, immediately after hypoxemia, PGE₂ plasma concentrations were significantly increased in male animals but returned to sham levels by 4 h after the hypoxic insult (18). Studies have also shown that PGE₂ upregulates IL-10 production and suppresses IL-12...
production (17). Therefore, the observation that macrophage PGE\(_2\) release was unaltered in males after hypoxemia suggests that the modulation of these cytokines in males is independent of this eicosanoid.

Although our findings indicate that the inflammatory response to severe hypoxemia exhibits a gender-specific pattern, the mechanisms responsible for this observation remain unclear. In view of the results from studies performed in a murine model of trauma-hemorrhage (1, 21), it could be suggested that hormonal mechanisms might also be involved in regulating immune responses after hypoxemia. In summary, the data presented here demonstrate that severe hypoxemia results in significantly increased plasma concentrations of proinflammatory cytokines in males but not in females. Furthermore, 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. In males, splenic macrophage cytokine release was significantly depressed after hypoxemia, whereas in proestrus females splenic macrophage functions were unaltered compared with female shams. These findings indicate that splenocyte and splenic macrophage immune functions as well as circulating proinflammatory cytokines exhibit a gender-specific pattern after severe hypoxemia. Thus, unlike males, females in the proestrus state do not appear to manifest immunosuppression after severe hypoxemia.

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