The female intestine is more resistant than the male intestine to gut injury and inflammation when subjected to conditions associated with shock states

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Homma, Hiroshi, Erik Hoy, Da-Zhong Xu, Qi Lu, Rena Feinman, and Edwin A. Deitch. The female intestine is more resistant than the male intestine to gut injury and inflammation when subjected to conditions associated with shock states. Am J Physiol Gastrointest Liver Physiol 288: G466–G472, 2005.—Having documented that proestrus female rats are more resistant to shock-induced acute gut and hence lung injury than male rats, we tested the hypothesis that the female gut is more resistant to injury and produces less of an inflammatory response than the male gut when exposed to conditions associated with shock states (hypoxia and acidosis) utilizing the ex vivo Ussing chamber system. Ileal mucosal membranes harvested from normal male and female rats mounted in Ussing chamber systems were exposed to normoxia or 40 min of hypoxia at a normal pH (pH 7.3) or acidosis (pH 6.8). Cytokine and nitric oxide levels in the serosal compartment of the Ussing chamber were measured at the end of the 3-h experimental period to assess the immunoinflammatory response, whereas FITC-dextran (mol wt 4,300) was employed to assess barrier function. Histomorphological changes were used to quantitate gut mucosal injury. Hypoxia, acidosis, or hypoxia plus acidosis was associated with a significant increase in proinflammatory cytokine production [interleukin (IL)-6, tumor necrosis factor, and macrophage inflammatory protein (MIP)-2] by the male compared with the female intestinal segments. In contrast, the female gut manifested a higher anti-inflammatory response (nitric oxide and IL-10) and improved intestinal barrier function as well as less evidence of mucosal injury than the male intestinal segments. Administration of estradiol or the testosterone receptor antagonist, flutamide, to male rats abrogated the increase in gut injury and the increased IL-6 and MIP-2 response observed after hypoxia plus acidosis. These results suggest that gender differences in the ex vivo intestinal response to stresses, such as hypoxia and acidosis, exist and that the administration of estradiol or blockade of the testosterone receptor to male rats mitigates these gender differences.

Ussing chamber; gender; ileal membrane; hypoxia/low pH; nitric oxide

ALTHOUGH MULTIPLE ORGAN DYSFUNCTION syndrome (MODS) is the leading cause of death in intensive care units today (14), effective therapies have been slow to be developed, at least in part, because of an incomplete understanding of its basic biology (14). Consequently, studies directed at elucidating the pathophysiology of MODS have assumed major clinical importance. Over the years, we and others have focused on the role of gut ischemia/injury as a factor in the subsequent development of MODS (7, 13). Most recently, these studies have documented that, after trauma-hemorrhagic shock (T/H/S), the gut becomes a cytokine-generating organ (17) and that factors exiting the gut via the mesenteric lymphatics contribute to lung injury, endothelial cell dysfunction, and neutrophil activation (2, 14–16, 19, 41). Although these studies illustrate the concept that the gut plays a role in the pathogenesis of T/H/S-induced lung injury, they were conducted with male rats exclusively. Based on recent studies documenting that proestrus rats are relatively resistant to T/H/S-induced immune depression (4), we tested whether female rats were more resistant to T/H/S-induced acute lung injury than male rats. In this study (1), T/H/S caused gut and lung injury in the male but not the female rats, and the mesenteric lymph from the male but not the female rats subjected to T/H/S caused endothelial cell injury. From these results, we concluded that the resistance of female rats to hemorrhagic shock-induced lung injury appears to be secondary to their resistance to shock-induced gut injury. Although the reasons why the female gut is more resistant to T/H/S-induced intestinal injury than the male remains to be fully determined, there are several possible explanations. One possibility is that intestinal microcirculatory blood flow is better preserved in females than males during low-flow states, such as hemorrhagic shock. Another possibility is that the female gut is more resistant to intestinal injury than the male gut when exposed to similar levels of intestinal ischemia. Last, the inflammatory response of the intestine to low-flow states and/or ischemia may be less in female than male rats, thereby potentially limiting the extent of gut injury. Consequently, the aim of this study was to test the hypothesis that the intestine of proestrus female rats is more resistant to injury and produces less of an inflammatory response than the male gut when exposed to conditions associated with shock states (hypoxia and acidosis). The rationale for testing the intestinal segments at an acidicotic and a normal pH is based on clinical studies indicating that, during periods of shock or decreased splanchnic perfusion, the pH of the gut is acidic (22) and animal models showing that loss of intestinal barrier function is greater when the extracellular intestinal pH is acidic (36).

Because of the inherent complexities of performing these types of studies in vivo, we have utilized the ex vivo Ussing chamber model system. In this system, intestinal segments harvested from male or female rats can be exposed to shock-like conditions (hypoxia and reoxygenation with or without acidosis) without the confounding variables associated with in vivo studies (18). Thus, using this model system, we compared the effects of hypoxia/reoxygenation and acidosis on intestinal injury and barrier function and the intestinal production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, macrophage inflammatory protein (MIP)-2, and nitric oxide (NO)
using intestinal segments harvested from male or proestrus female rats. We measured IL-6, TNF-α, and MIP-2, since proinflammatory cytokines and chemokines are elevated in trauma patients and in animal models of shock, injury, and inflammation and have been implicated in the pathogenesis of MODS (14, 20, 26). Likewise, because there is substantial evidence indicating that bioactive NO production plays a complex but important role in gut injury with modest increases in NO being protective and larger increases being deleterious (37), we also measured NO production and the anti-inflammatory cytokine IL-10.

MATERIALS AND METHODS

Animals. Specific pathogen-free Sprague-Dawley rats (weight: male, 315–385 g; female: 275–350 g) were used. Animals were housed for at least 1 wk before use and were maintained according to the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were fed standard laboratory chow (Diet 5001;Ralston Purina, St. Louis, MO) and water ad libitum. The experiments were approved by the New Jersey Medical School Animal Care and Use Committee.

Experimental design and Ussing chamber system. Ileal mucosal membranes were harvested from normal male and proestrus female rats (estrus stage verified by vaginal smears) and were mounted in Ussing chambers as previously described (18). Briefly, once the rats were anesthetized with pentobarbital sodium (50 mg/kg) given intraperitoneally, the viscera was exposed, and 2-cm Peyer’s patch-free segments of the distal ileum were harvested. The ileal segments were opened along the mesenteric border and washed in cold medium to remove the luminal contents, after which the serosa and the external longitudinal muscle layers were removed by blunt dissection. The ileal mucosal membranes then were mounted between the two halves of the Ussing chamber (1.12 cm² opening). Two calomel voltage-sensitive electrodes and two Ag-AgCl current-passing electrodes (World Precision Instrument, Sarasota, FL) were connected to the Ussing chamber via agar bridges. Both the mucosal and serosal sides of the chamber were connected to circulating reservoirs containing DMEM, supplemented with 20 mM l-glutamine (Sigma). Ports were present in each of the reservoirs for sample collection and the addition of reagents. The temperature of the reservoirs was maintained at 37°C by a jacketed circulating water bath. The fluid within the reservoirs was oxygenated and driven by a gas lift column of 95% N₂-5% CO₂. After baseline PD and R were measured, fluorescein isothiocyanate (FITC)-dextran (mol wt 4,300) was used to evaluate the permeability of the intestinal segments as follows. Samples of the medium from the serosal compartments were collected at the end of each 180-min experimental period and placed in a 96-well plate. FITC-dextran concentrations were determined by fluorescein detection (FL-500 Microplate Fluorescence Reader; BIO-TEK, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

IL-6, IL-10, TNF-α, and MIP-2 levels in medium from the serosal compartments were measured using ELISA kits specific for rat IL-6, IL-10, TNF-α, and MIP-2 (Biosource, Camarillo, CA).

NO production was assessed by measuring total nitrite and nitrate levels in 100-μl samples collected from the serosal compartment using the nitrate reductase and Griess reagent, as previously described (30). The samples were read at 543 nm with a spectrophotometer. Sodium nitrite (Sigma) was used as the standard.

Ileal mucosal membrane histology. At the end of the 180-min experimental period, the ileal mucosal membranes mounted in the Ussing chamber were harvested and fixed in 10% buffered formalin. The tissue was then dehydrated and embedded in paraffin, as previously described (30). The samples were read at 543 nm with a spectrophotometer. Sodium nitrite (Sigma) was used as the standard.

Assays. FITC-dextran (mol wt 4,300) was used to evaluate the permeability of the intestinal segments as follows. Samples of the medium from the serosal compartments were collected at the end of each 180-min experimental period and placed in a 96-well plate. FITC-dextran concentrations were determined by fluorescein detection (FL-500 Microplate Fluorescence Reader; BIO-TEK, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

In both the proestrus female and the male rats, the resistance values (Table 1) of the ileal membranes of all the groups decreased in a similar fashion over time, a response that is consistent with the literature (18). The resistance values of the membranes exposed to hypoxia were significantly lower at the end of the 40-min hypoxic period than the normoxic membranes at this time point. This response was true for both genders, although the resistance was decreased to a greater
degree in the male than the female ileal membranes of the normoxia-acidosis group at the end of the 40-min hypoxic period (Table 1). The PD of male and female ileal membranes, even under normoxic conditions, had decreased after 40 min in the Ussing chamber (Fig. 1A). However, the PD of the female ileal membranes remained stable, whereas the PD of the male ilea continued to significantly \( (P < 0.05) \) decrease over the 180-min experimental period. Thus, at 40, 120, and 180 min, the PD of the female ileal membranes incubated under normoxic conditions was significantly higher than the male membranes (Fig. 1A). Similar trends were observed in the ileal membranes exposed to normoxia plus acidosis (Fig. 1B). Thus the PD was better preserved in the female than the male groups.

Compared with the normoxic groups, ileal membranes exposed to a 40-min hypoxic insult manifested a lower PD that persisted throughout the 180-min experimental period (Fig. 1, A and B). In both the male and female hypoxia pH 7.3, as well as in the female hypoxia plus acidosis groups, the PD of the ileal membranes partially recovered after the 40-min hypoxia period but never reached the levels observed in the normoxia groups. However, at the end of the 40-min hypoxic period and at 120 and 180 min, the PD of the female ileal membranes subjected to hypoxia plus acidosis was higher than the comparable male membranes.

Hypoxia combined with acidosis increased the mucosal permeability of both the male and female intestinal segments, with the male intestinal segments being increased to a greater extent than the female ileal membranes (Fig. 2). Additionally, the permeability of the intestinal membranes to the dextran permeability probe appeared to be greater in the male than the female rats under nonstressed conditions (normoxia) and during normoxia or hypoxia plus acidosis (Fig. 2). However, after a pure hypoxic insult, this gender difference was lost (Fig. 2). Morphologically, the male, but not the proestrus female, intestinal segments subjected to hypoxia or acidosis or the two combined developed evidence of mucosal injury (Fig. 3). Although the incidence of villus injury was higher in the male rats, the magnitude of villus injury, when it occurred, was similar between the male and female rats (Fig. 4).

Gender differences in the proinflammatory cytokine response to hypoxia and/or acidosis were observed with an increase in IL-6 production by the ileal membranes from the males but not the females (Fig. 5). A similar trend was...
observed for TNF-α and MIP-2, where the levels in the serosal chambers of the male intestinal segments were higher than the females, although these differences only reached statistical significance in the hypoxia plus acidosis groups (Figs. 6 and 7). In contrast to proinflammatory cytokine and chemokine production, IL-10 levels were higher in the serosal chambers of the female than the male rats under all conditions (Fig. 8). NO production was also higher in female intestinal segments under normoxic conditions and under conditions of normoxia plus acidosis or hypoxia plus acidosis, but not hypoxia alone (Fig. 9).

Next, to further evaluate the hypothesis that the differences between the male and female intestinal response to hypoxia plus acidosis was related to sex hormones, we tested whether the administration of estradiol and/or the blockade of testosterone, utilizing the testosterone receptor antagonist flutamide, would limit gut injury and reduce the proinflammatory response in male rats. Consistent with the earlier results, intestinal permeability and gut injury were increased in the male rat intestinal segments exposed to hypoxic and acidic conditions (Table 2). However, the administration of estradiol or flutamide largely abrogated these changes. Likewise, the increased proinflammatory response (IL-6 and MIP-2) observed when the male rat intestinal segments were subjected to hypoxia and acidosis was prevented by estradiol and flutamide pretreatment (Table 3). Last, estradiol and flutamide pretreatment increased the IL-10 response of the male intestinal segments exposed to hypoxia and acidosis (Table 3).
DISCUSSION

Based on our recent work in male rats, it appears that, after an episode of splanchnic ischemia, gut-derived factors are critical for the development of shock-induced distant organ and cellular dysfunction (2, 6, 16, 27, 41, 43). However, some human (8, 23, 32, 37) and animal (4, 5) studies suggest that females may tolerate sepsis and shock better than males, and recent animal work suggests that the reduction in shock-induced acute lung injury is the result of the relative resistance of the female rat to T/HS-induced gut injury (1, 9). Although there is some evidence that female rats have an improved hemodynamic response after trauma (3) and that the administration of estradiol during the resuscitative phase of hemorrhagic shock improves intestinal perfusion (24), the mechanisms by which the female gut is more resistant to T/HS-induced injury than the male gut remain to be elucidated.

One possible explanation for why the female gut is more resistant to T/HS-induced injury is that the female gut tolerates an ischemic episode better than the male gut. Thus the goal of the current study was to test the hypothesis that female rats have less shock-induced acute injury than male rats because their guts are more resistant to injury and their proinflammatory response is blunted. Because of the potential confounding effects of differences in vasomotor tone and the immunoinflammatory responses observed in vivo when male and proestrus female rats are subjected to T/HS (3–5, 24), we used the ex vivo Ussing chamber model system to test this hypothesis. In the Ussing chamber system, these confounding variables are avoided, and it is possible to ensure that the hypoxic insult to which the male and female guts are exposed is identical. Because in vivo (35) and in vitro (40) studies indicate that at an acidotic pH the response of the gut to an insult is magnified and clinical studies document that intestinal acidosis is common during periods of hemorrhage or hypotension (22), to more accurately model the in vivo effects of gut ischemia in the Ussing system, we added experimental arms where the intestinal mucosal segments were tested under acidic conditions.

**Fig. 6.** Tumor necrosis factor (TNF)-α concentrations in serosal fluid samples at 180 min after exposure to normoxia or hypoxia with or without acidosis. Data are expressed as means ± SD, with n = 6 rats/group. *P < 0.05 vs. male or female normoxia pH 7.3 groups.

**Fig. 7.** Macrophage inflammatory protein (MIP)-2 concentrations in serosal fluid samples at 180 min after exposure to normoxia or hypoxia with or without acidosis. Data are expressed as means ± SD, with n = 6 rats/group. *P < 0.05 vs. male or female normoxia pH 7.3 groups.

**Fig. 8.** IL-10 concentrations in serosal fluid samples at 180 min after exposure to normoxia or hypoxia with or without acidosis. Data are expressed as means ± SD, with n = 6 rats/group. *P < 0.05 vs. comparable female groups. #P < 0.01 vs. all other groups.

**Fig. 9.** Nitric oxide concentrations in serosal fluid samples at 180 min after exposure to normoxia or hypoxia with or without acidosis. Data are expressed as means ± SD, with n = 6 rats/group. *P < 0.05 vs. comparable male group.
The results of this study indicate that there are functional and physiological differences between the proestrus female and the male intestine that may contribute to the resistance of the proestrus female to hemorrhagic shock-induced gut injury. Specifically, the PD, morphology, and barrier function (as reflected in permeability to dextran) of the female gut was better preserved than the male gut during ex vivo normoxic conditions (i.e., 3-h normoxic period) and when the intestinal segments were exposed to acidosis under normoxic or hypoxic conditions. Likewise, there were gender differences in the intestinal inflammatory response, as reflected by an augmented IL-6 response in the male but not the female intestinal segments under conditions of acidosis, hypoxia, or hypoxia plus acidosis. A similar trend was observed for TNF-α and MIP-2, where their levels of production were higher in the male than the female intestinal segments exposed to hypoxia plus acidosis. In contrast to the proinflammatory cytokine response, production of the anti-inflammatory cytokine IL-10 increased to a greater extent in the female than the male intestinal segments. Additionally, NO production by the female intestinal segments during normoxia and during acidosis and hypoxia plus acidosis was greater than that observed in the male intestinal segments. It appears that these differences in gut function, morphology, and inflammatory response between the proestrus female and the male intestinal segments subjected to hypoxia plus acidosis were related to the effects of both estradiol and testosterone, since the pretreatment of male rats with estradiol or the testosterone receptor antagonist flutamide limited gut injury and dysfunction and the intestinal proinflammatory response. These results are consistent with studies showing that estradiol inhibits cytokine production via a nuclear factor-κB-mediated mechanism (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (cNOS), while at the same time limiting excessive NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive NO synthase (25) and that estradiol potentiates modest increases in NO production by stimulating constitutive 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FEMALE RAT GUT IS MORE RESISTANT TO SHOCK-INDUCED INJURY

GRANTS

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REFERENCES


