Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries

Edwin A. Deitch, Preya Ananthakrishnan, David B. Cohen, Da Zhong Xu, Eleonora Feketeova, and Carl J. Hauser

Department of Surgery, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey

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STUDIES in this area have been performed with male animals, as recently reviewed (3–5), there is an increasing body of literature indicating that females are more resistant to shock-, trauma-, and sepsis-induced immune dysfunction and organ injury than are males. Consequently, the goal of this study was to investigate whether sex hormones act as modulators of acute shock and injury-induced neutrophil activation and, if so, to begin to investigate the mechanisms involved in that modulation. Because most polymorphonuclear neutrophil (PMN) inflammatory responses depend on prolonged increases in cytosolic calcium concentration ([Ca^{2+}]_i), and such prolonged elevations of neutrophil [Ca^{2+}]_i depend on calcium entry from the environment (11, 18, 32), we also studied the ability of estrogen and testosterone to modulate calcium signaling. Additionally, because we had previously shown that attenuation of PMN [Ca^{2+}]_i by inhibiting Ca^{2+} entry through store-operated calcium entry (SOCE) pathways decreased inflammatory PMN responses to trauma and hemorrhagic shock (26), we investigated the effects of estrogen and testosterone on this pathway.

METHODS AND MATERIALS

Animals. Adult female and male pathogen-free Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 250–350 g were used in all experiments after a minimal acclimation period of 5 days. The castration and ovarioectomy procedures were done a minimum of 2 wk before the rats were subjected to sham or actual injury. Animals had free access to food [Teklad 22/5 Rodent Diet (W) 8640, Harlan Teklad, Madison, WI] and water and were maintained under barrier-sustained conditions with 12:12-h light-dark cycles. Female estrus cycle stage was determined by examining the cytology of vaginal smears as described by Baker (6), with only proestrus females being used in this study. All animals were maintained in compliance with the recommendations of the New Jersey Medical School Animal Use and Care Committee, as described in Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996). All experiments were approved by the Animal Use and Care Committee.

Experimental design. The primary goal of this study was to test the hypothesis that sex hormones modulate the acute neutrophil response after a major injury. This hypothesis was tested utilizing both a trauma-hemorrhagic shock (T/H/S) and a thermal injury model. Two distinct models were used to more rigorously test this hypothesis. To isolate the effects of sex hormones from other sex-related factors, neutrophil activation in castrated, and ovariectomized rats were compared with normal male and proestrus female rats in these two model systems. The T/H/S model consisted of a laparotomy (trauma) and...
hemorrhage (30 mmHg × 90 min). The burn model consisted of a 40% total body surface area third-degree scalp burn. These models were chosen for several reasons. First, each model is associated with neutrophil activation, and there is evidence in male rats that T/HS-induced lung injury (27) and burn-induced acute lung injury (28) are mediated by factors liberated from the intestine. Secondly, we have recently documented that female rats are protected from T/HS-induced lung and gut injury (2, 9).

To test the role of sex hormones as modulators of the neutrophil response to injury, in the first group of experiments, the effect of T/HS on neutrophil activation was measured in male and female rats who had and had not undergone gonadectomy. In addition, the ability of plasma from these rats to activate naive neutrophils was tested. In these experiments, four groups of male and four groups of female rats were subjected to T/HS or trauma-sham shock (T/SS). These groups included male rats subjected to T/HS or T/SS, castrated male rats subjected to T/HS or T/SS, proestrus female rats subjected to T/HS or T/SS, and ovariectomized female rats subjected to T/HS or T/SS. To evaluate neutrophil activation, small aliquots of blood (0.5 ml) were obtained for measuring CD11b expression at three time points: before the induction of T/HS or T/SS and at 1 and 3 h after the end of the 90-min shock or sham shock period. Additionally, to evaluate the ability of postinjury plasma to activate naive neutrophils, at 3 h after resuscitation from T/HS or T/SS, plasma samples were obtained and tested for their ability to activate naive neutrophils from donor rats as reflected by upregulation of neutrophil adhesion molecule expression (CD11b) and augmentation of the respiratory burst.

In the second group of experiments, similar groups of male and female rats were subjected to a burn or sham burn injury. As described above, blood samples for in vivo neutrophil CD11b expression were obtained at two time points (preburn and 3 h postburn) and plasma was obtained at 3 h postburn for in vitro testing.

To test the hypothesis that differences in neutrophil activation between male and female rats were related to a sex-related differential resistance to T/HS-induced gut injury and the production of biologically active mesenteric lymph, mesenteric lymph was collected from male and proestrus female rats subjected to T/HS or T/SS, and the neutrophil-activating capacity of these lymph samples was tested on naive donor neutrophils. Thus, in this set of experiments, nongonadectomized male and proestrus female rats were subjected to T/HS or T/SS, and postnodal mesenteric lymph was collected as previously described (27). This group of experiments was considered necessary because proestrus female rats are relatively resistant to T/HS-induced gut injury (2, 9), and thus the neutrophil-activating potential of female T/HS lymph may be much less than male T/HS lymph and could help explain any observed differences in the in vivo neutrophil activation between the male and female rats.

Las, to begin to test the hypothesis that sex hormones can modulate normal neutrophil activation, neutrophils collected from naïve donor rats were stimulated in the presence or absence of estrogen or testosterone. In this experiment, PMN were isolated from naïve male or proestrus female rats. Male PMN were evaluated for their ability to mobilize [Ca\(^{2+}\)] in response to depletion of microsomal Ca\(^{2+}\) stores by the calcium-ATPase inhibitor thapsigargin (TG) in the presence of the synthetic estrogen diethylstilbestrol. The role of estrogens in modulating calcium influx through specific and nonspecific channel mechanisms was assessed by comparing the inhibition of Ca\(^{2+}\) influx by diethylstilbestrol to the inhibition of strontium (Sr\(^{2+}\)) influx (20).

Next, the effects of the major naturally occurring estrogen 17β-estradiol (17β-E2) on male and proestrus female PMN were assessed by using the area under the curve for calcium influx as our measure of SOCE over time. Last, the effect of testosterone was tested on PMN from naïve castrated male rats to determine whether testosterone would augment [Ca\(^{2+}\)] mobilization. Castrated male rats were used because our pilot studies indicated that female PMN appeared to be inherently more resistant to activation than male PMN.

**T/HS model.** Rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg) and subjected to T/HS as previously described (27). With the use of aseptic techniques, the right jugular vein and left femoral artery were isolated and cannulated with polyethylene (PE-50) tubing or 50-gauge silicone catheter containing heparinized saline (10 U), respectively. Next, a 3-cm midline laparotomy (trauma) was performed, followed by closure with a running 3-0 silk suture. The femoral artery catheter was then attached in line to a blood pressure monitor (BP-2 digital blood pressure monitor, Columbus Instruments, Columbus, OH) for continuous blood pressure monitoring. Venous blood was withdrawn into a heparinized 10 ml syringe at a rate of 1 ml/min and mean arterial pressure was maintained at 30 mmHg for 90 min. At the end of the shock period, animals were resuscitated with their shed blood at a rate of 1 ml/min. The mean arterial pressure returned to normal within a few minutes after resuscitation. Rectal temperature was monitored throughout the experiment and maintained at ~37°C by using an electric heating pad under the surgical platform. Our previous studies (16) indicated that the long-term mortality rate of this model ranges from 14 to 25%, with most deaths occurring during or just after the shock period. The T/SS animals were instrumented, underwent a laparotomy, but were not subjected to hemorrhagic shock. After the end of the T/HS or T/SS period, the rats were maintained under anesthesia for the duration of the experiment by the administration of pentobarbital sodium (25 mg/kg) every 2 h as needed.

**Burn model.** The rats underwent a 40% total body surface area burn by using the technique originally described by Walker and Mason (36). The rats were anesthetized with pentobarbital sodium (50 mg/kg) given intraperitoneally. The hair was shaved from the back and abdomen, and a depilatory agent was applied. The internal jugular vein was then cannulated with a 50-gauge silicone catheter by using aseptic technique, and a preburn blood sample was obtained. The rats were then placed in a template containing an opening exposing 20% of the body surface area. The back burn was produced by immersing the back of the animal through the template into boiling water (100°C) for 10 s, followed by an abdominal scald burn for 5 s. All the rats were resuscitated with an additional 3 ml of normal saline intraperitoneally before the abdominal burn and then 2 ml normal saline intravenously to prevent shock. Previous studies using this model have shown that it is not associated with abdominal visceral injury and that the burns are third degree (28). Rectal temperature was maintained at ~37°C with a heat lamp. The control (sham burn) groups were anesthetized, had their jugular vein catheterized, and were then placed in the plastic template and exposed to room-temperature water. After the burn or sham burn injury, the rats were administered the analgesic agent buprenorphine (0.2 mg/kg) for pain control and then allowed to awaken in their cages. At 3 h after the burn or sham burn, the rats were reanesthetized with pentobarbital sodium, and samples were harvested as described in Experimental design.

**In vivo whole blood PMN adhesion molecule assay.** Whole blood samples obtained preinjury, 1 h postinjury, and 3 h postinjury were divided into equal three 100-μl aliquots as previously described (8). The samples were incubated with 10 μl of anti-rat CD11b fluorescein isothiocyanate-labeled monoclonal antibody (BD Pharmingen, San Diego, CA). The samples were vortexed and then placed on ice for 45 min in the dark. They were then lysed by using PharmLyse (BD Pharmingen) for 15 min on ice and then centrifuged and washed three times with calcium and magnesium-free 1% phosphate-buffered saline at 400× g for 15 min at 4°C. Fluorescein isothiocyanate-conjugated mouse immunoglobulin G antibodies (BD Pharmingen) were used as isotype controls for nonspecific antibody binding.

Cells were analyzed for adhesion molecule expression using a FACSscan flow cytometer (Becton-Dickinson, San Jose, CA) and CellQuest software. Daily instrument calibration was performed using Calibrate beads (BD Pharmingen) and Rainbow calibration particles (BD Pharmingen) according to the manufacturer’s recommendations.
Neutrophils were identified by forward and side scatter analysis. Each sample was run in the setup mode until a granulocyte acquisition gate was established. Only gated events were acquired and viewed in the FL1 histogram without marker settings. Five thousand events were collected in granulocyte gate in all studies. Data were expressed as mean fluorescence intensities (MFI) of the FL1 channel.

**In vitro neutrophil respiratory burst activity.** Neutrophils were isolated as previously described (1). Briefly, heparinized whole blood samples (100 μl) from naive control male or proestrus female rats were placed into 5-ml polystyrene round-bottom tubes containing an equal volume of DMEM. The red blood cells were lysed with 1% PharmM lysing solution as previously described. The tubes were spun at 1,135 rpm for 5 min at 25°C. The supernatants were discarded, and the cells were washed twice with HBSS.

The white blood cell pellets were resuspended in 400 μl of HBSS. Plasma samples (25% vol/vol) from the burn, sham burn, T/HS, or T/SS groups were then added, and the tubes were placed into the incubator for 5 min, after which the dihydrorhodamine (15 ng/ml) was added to the tubes. Five minutes after dihydrorhodamine was added, the PMN were stimulated with PMA. After 15 min at 37°C, the PMN respiratory burst was measured by flow cytometry as described above.

**In vitro PMN CD 11b adhesion molecule assay.** Heparinized whole blood samples (100 μl) from control male and proestrus female rats were placed into 5-ml polystyrene round-bottom tubes containing an equal volume of DMEM. The red blood cells were lysed with 1% PharmM lysing solution as previously described (1). The tubes were spun at 1,135 rpm for 5 min at 25°C. The supernatants were discarded, and the cells were washed twice with HBSS. The white blood cell pellets were resuspended in 400 μl of HBSS to which the plasma samples at 25% vol/vol were added. The cells were then processed and analyzed for CD11b expression as described in In vivo whole blood PMN adhesion molecule assay.

**Neutrophil isolation and cell calcium studies.** Our methods are described in detail elsewhere (19). Briefly, anesthetized animals are exsanguinated by cardiac puncture into heparinized syringes (25 U/ml). Blood is centrifuged on Polymorphoprep media (Robbins, Sunnyvale, CA) adapted for use with rat PMN (13). The PMN layer is removed, and the osmolality is restored. Cells were washed and resuspended in HEPES buffer without BSA. PMN are counted and analyzed for purity by flow cytometry. Each of these methods yield PMN of 96–99% purity and >98% viability by trypan blue exclusion.

Cells are incubated with 5 μg/ml fura-2 AM (Molecular Probes, Eugene, OR) for 30 min in the dark at 37°C. PMN were then divided into aliquots (2 × 10⁶ PMN) and placed on ice in the dark. Immediately before study, the cells are centrifuged 5 s at 4,500 rpm and supernatants are removed. Cells are resuspended in HEPES buffer for study. [Ca²⁺]i is determined at 37°C with constant stirring by measuring fura-2 AM fluorescence at 505 nm using 340/380 nm dual wavelength excitation in a Fluoromax-2 spectrofluorometer (Jobin-SPEX, Edison, NJ). Sr²⁺ is used as a marker for divalent cation entry through nonspecific cation channels. The Kₐ for Sr²⁺ association with fura is not precisely known, so [Sr²⁺]: is reported as “apparent [Ca²⁺]:.” The presence of Sr²⁺ in the media has no effect on the Rₘₐₓ/Rₘᵢₙ measurements performed in the presence of 1 mM Ca²⁺ at the end of each experiment (20). The order of study of PMN isolates is alternated to avoid bias related to duration of dye loading or time of cell study. In these experiments, the isolated PMN were exposed to estradiol or diethylstilbestrol for 1 min before being assayed.

**Statistical analysis.** Data comparing differences across time or between multiple groups were analyzed by two-way analysis of variance with the Tukey-Kramer multiple comparisons or the Holm-Sidak tests. Comparisons of differences between the sexes were done using the unpaired t-test. Data are expressed as means (SD). Statistical significance was considered to be a P value ≤0.05.

**RESULTS**

There was no difference in the blood pressure response to T/HS between the male or female rats or the rats subjected to castration or ovariectomy. Overall, the mean arterial blood pressure preshock was 116 mmHg (SD 14); it returned to 123 mmHg (SD 14) immediately after resuscitation and was 92 mmHg (SD 5) at the end of the postresuscitative period (data not shown for individual T/HS groups). The mean arterial blood pressure for the T/SS groups was 121 mmHg (SD 12) before sham shock injury and 113 mmHg (SD 7) at the time the animals were killed. None of the sham-shocked rats died during the experimental procedure, and the overall survival rate of the rats subjected to T/HS was 88% (36 of 41 rats survived).

Neutrophil activation after T/HS, as reflected in upregulation of CD11b expression, was greater in male rats than in proestrus female rats at both 1 h (311 ± 45 vs. 235 ± 50 MFI; P < 0.01) and 3 h (521 ± 120 vs. 261 ± 38 MFI; P < 0.001) after the end of the 90-min shock period. This increase in post-T/HS PMN activation appeared to be related to the presence of male sex hormones, because castration abrogated the increase in T/HS-induced CD11b upregulation as compared with the T/SS group (Fig. 1A), whereas ovariectomy did not reverse the protective effect of the proestrus state (Fig. 1B). In fact, CD11b expression was similar between the T/HS and the T/SS female groups at each time point (Fig. 1B). Nonetheless, the level of CD11b expression was increased post-T/HS or post-T/SS in both sexes, indicating that the stresses associated with the trauma and instrumentation portions of the model were capable of systemic neutrophil activation (P < 0.01 preshock or sham shock vs. postshock or sham shock). Furthermore, at the 3-h time point, but not at the preinjury or 1 h postinjury period, the level of CD11b expression was higher in each of the male groups (T/SS, T/HS, T/HS + castration) than in the comparable female group (Fig. 1; P < 0.05), further indicating that the male rats have an increased CD11b response after stress.

The mortality rate for the burned animals was 7% (3 of 39 rats died), whereas none of the sham-burned rats died. As was observed in the T/HS arm of the study, the process of anesthesia and handling of the rats was associated with an increase in CD11b expression in all of the groups of both sexes, as compared with the preburn CD11b values (Fig. 2). However, in contrast to T/HS, neutrophil CD11b expression was increased above the sham level in both the male and proestrus female rats at 3 h postburn (Fig. 2). However, PMN CD11b expression was still greater in the male than in the proestrus female rats at 3 h postburn (394 ± 42 vs. 294 ± 43 MFI; P < 0.01). Castration partially abrogated the magnitude of the postburn increase in CD11b expression, but ovariectomy had no effect (Fig. 2).

Because the increase in PMN activation after T/HS or burn injury in the male rats as compared with the proestrus female rats could be related to an increased susceptibility of male PMN to activation and/or a greater increase in the PMN-activating properties of male plasma, we next tested the ability of plasma from the male and female T/HS and burn groups to activate normal naive male and female PMN. Plasma from both the male and female T/HS rats increased CD 11b expression (Fig. 3A) and the respiratory burst (Fig. 3B) of naive control male PMN; however, the increase in CD11b expression...
and the respiratory burst was greater in the naive male PMN incubated with male than female T/HS plasma \((P < 0.01)\). Consistent with these in vivo observations, castration was associated with a decrease in the PMN-activating ability of male plasma, whether measured as CD11b upregulation or priming for an augmented respiratory burst (Fig. 3, A and B). In contrast to the in vivo observations in the female rats subjected to T/HS, plasma from the proestrus female rats increased PMN CD11b expression as well as the respiratory burst, and this increase in PMN activation was significantly augmented after ovariectomy (Fig. 3, A and B).

Similar trends were observed after thermal injury, where the plasma of the burned male and female rats increased naive male PMN CD11b expression (Fig. 4A) and primed for an augmented respiratory burst (Fig. 4B). Just as was observed with T/HS plasma, plasma from the male burned rats increased CD11b expression and the respiratory burst of naive male PMN to a greater extent than did proestrus plasma \((P < 0.01)\). Likewise, castration abrogated and ovariectomy increased the ability of burn plasma to activate naive PMN (Fig. 4, A and B).

Next, because the in vivo CD11b was not increased in the female rats after T/HS, we tested whether neutrophils from proestrus females may be more resistant to activation after T/HS than male neutrophils. Consistent with this notion, male, but not proestrus female neutrophils, were activated by both male and female T/HS plasma (Table 1). A similar response was observed when burn plasma was tested, with the control male, but not the proestrus female, neutrophils manifesting an increased CD11b response \((417 \pm 14 \text{ vs. } 258 \pm 8 \text{ MFI}; P < 0.001)\).

Having documented that both the neutrophil-activating ability of T/HS plasma from proestrus rats is less than that of male rats and that proestrus neutrophils are more resistant to activation than male neutrophils, we next examined whether the lack of neutrophil-activating activity of the proestrus female T/HS plasma was related to a lower level of neutrophil-activating factors into the mesenteric lymph of the female than the male rats. This appeared to be the case, because T/HS mesenteric lymph from male rats was able to upregulate CD11b expression and augment the respiratory burst (Table 2) of naive male neutrophils more than T/HS lymph collected from proestrus female rats. In contrast to male naive neutrophils, neutrophils from naive proestrus female rats were not activated by female T/HS lymph, and, although they were activated by male T/HS lymph, the degree of activation was less than that observed in male neutrophils (Table 2).

Because sex hormone levels change over the estrus cycle, we next examined the ability of neutrophils obtained from female rats during metestrus, diestrus, and proestrus to be activated by male T/HS lymph. Male T/HS lymph was used, because it has greater neutrophil-activating ability than female T/HS lymph and our previous work indicates that the plasma neutrophil-activating activity derives from factors in the mesenteric lymph \((4)\). The results of this study show that the ability of male T/HS lymph to activate female neutrophils varies.
during the various stages of the estrus cycle, with the greatest resistance being seen during the proestrus phase of the cycles, where estradiol and other female sex hormones are at their greatest levels (Fig. 5). However, at no stage of the estrus cycle are female neutrophils able to be activated by male T/HS lymph to the same degree as male neutrophils (Fig. 5).

To further evaluate the role of estrogen as a mediator of the neutrophil response, we evaluated the effects of estrogens on neutrophil calcium signaling in vitro. These studies showed that the synthetic estrogen diethylstilbestrol caused a dose-dependent suppression of TG-mediated calcium entry (Fig. 6A) in male rat PMN. Most of this suppression reflected decreased cation entry through the calcium-specific rather than the non-specific (i.e., Sr2+/H+ permeable) pathways (Fig. 6B). We then evaluated the effects of the naturally occurring estrogen 17β-E2 over a pharmacological dose range. We found that it, too, caused dose-dependent suppression of TG-mediated calcium influx in male rat PMN (Fig. 7A). We finally compared TG-stimulated SOCE in male and proestrus female rat PMN and compared the effects of increasing levels of 17β-E2 on their SOCE responses. As seen in Fig. 7B, basal SOCE responses to TG of untreated male rat PMN are significantly greater than those of PMN from untreated proestrus females.

Table 1. Comparison of effects of male versus female plasma on male and proestrus female neutrophil CD11b expression

<table>
<thead>
<tr>
<th>Plasma Group</th>
<th>Male PMN-CD11b Mean (SD)</th>
<th>Female PMN-CD11b Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female T/SS</td>
<td>216 (SD 21)</td>
<td>221 (SD 13)</td>
</tr>
<tr>
<td>Male T/SS</td>
<td>218 (SD 10)</td>
<td>211 (SD 10)</td>
</tr>
<tr>
<td>Female T/HS</td>
<td>277 (SD 32)*</td>
<td>219 (SD 15)</td>
</tr>
<tr>
<td>Male T/HS</td>
<td>397 (SD 49)*</td>
<td>249 (SD 48)</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. female T/SS plasma group; †P < 0.001 vs. all other plasma groups and female PMN exposed to male T/HS plasma.
When male and female PMN were tested in the presence of external 17\beta-E2, we saw that both groups showed significant suppression of SOCE by estrogens ($P < 0.001$) but that the male PMN responses started higher and were significantly more suppressed. Eventually, in the presence of increasing levels of environmental estrogen, the SOCE responses of male and proestrus female PMN became indistinguishable (Fig. 7B). However, in contrast, when testosterone was tested on PMN from naive castrated male rats, it had no effect on TG- or ionomycin-mediated calcium entry. This was true when testosterone was tested at physiological as well as pharmacological levels (Fig. 8).

**DISCUSSION**

Emerging data have described a sexual dimorphism in the response to injury or illness, with differences in immune function manifested as both altered susceptibility to organ injury and to infection. These differences have been attributed to the effects of sex hormones, with the female sex hormone estradiol conferring protection and the male sex hormone testosterone increasing susceptibility to injury (3–5). Although much of the data supporting sex hormones as modulators of responses to shock, trauma, or sepsis are based on animal studies, more than 20 clinical studies investigating the role of sex in trauma or septic patients have appeared since 1998 (3). Although not all of these clinical studies found a clinical difference between male and female patients, many of the prospective studies documented that females had a better outcome than males (14, 15, 30, 33). This advantage of the female sex appeared to be observed primarily in premenopausal females and in patients with more severe injuries (i.e., injury severity scores of $\geq 15$).

Because preclinical animal studies and some clinical studies indicated that females respond differently to shock, trauma, or sepsis than do males, the current study was designed to inves-

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**Table 2. Comparison of effects of male versus female mesenteric lymph on male and proestrus female neutrophil CD11b expression and respiratory burst**

<table>
<thead>
<tr>
<th>Lymph Group</th>
<th>Male PMN-CD11b</th>
<th>Female PMN-CD11b</th>
<th>Male PMN-RB</th>
<th>Female PMN-RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female T/SS</td>
<td>217 (SD 18)</td>
<td>202 (SD 24)</td>
<td>222 (SD 18)</td>
<td>206 (SD 15)</td>
</tr>
<tr>
<td>Male T/SS</td>
<td>222 (SD 5)</td>
<td>189 (SD 27)</td>
<td>271 (SD 37)</td>
<td>219 (SD 11)</td>
</tr>
<tr>
<td>Female T/HS</td>
<td>282 (SD 37)*</td>
<td>242 (SD 49)</td>
<td>304 (SD 36)*</td>
<td>239 (SD 40)</td>
</tr>
<tr>
<td>Male T/HS</td>
<td>538 (SD 13)†</td>
<td>362 (SD 14)†</td>
<td>825 (SD 107)†</td>
<td>427 (SD 24)†</td>
</tr>
</tbody>
</table>

Values are means (SD) with 4–6 lymph specimens per group. CD11b and respiratory burst (RB) activity expressed as mean fluorescence intensity. Lymph tested at 5% final concentration. *$P < 0.01$ vs. female T/SS lymph group; †$P < 0.001$ vs. all other lymph groups and opposite sex male T/HS lymph group.

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Fig. 5. Ability of T/HS lymph from male rats to activate naive female neutrophils varied over the estrus cycle, but response of naive female neutrophils was never as great as that of naive male neutrophils. Data are expressed as means (SD); $n = 4–6$ rats per group. *$P < 0.01$ vs. proestrus neutrophils; †$P < 0.01$ vs. all other neutrophil groups.

Fig. 6. A: diethylsilbestrol (DES) causes dose-dependent suppression of thapsigargin (TG)-mediated calcium influx in rat neutrophils. Fura-loaded rat PMN were suspended in cuvettes in Ca$^{2+}$-free buffer in the presence of DES at concentrations shown for 1 min. Experiment was started and cells were treated at time ($t$) = 30 s with 1 mM TG. Calcium (1 mM) was added at $t = 400$ s to visualize influx. B: effect of DES on TG-mediated strontium (Sr$^{2+}$) (1 mM) and Ca$^{2+}$ movement is seen. There is some suppression of nonspecific (i.e., Sr$^{2+}$ permeable) entry mechanism but more marked suppression of entry through calcium specific mechanisms. Thus DES predominantly suppressed divalent cationic entry through Ca$^{2+}$-specific channels. $n = 4$ Representative experiments.
tigate the role of sex hormones as modulators of acute neutrophil activation. We used two distinct models of neutrophil activation, thermal injury and trauma-hemorrhagic shock, because neutrophil activation and neutrophil-mediated organ injury have been documented in both conditions (11). Additionally, the use of two different trauma models would potentially increase the robustness of the findings. Our observations that in vivo neutrophil activation is greater in male than proestrus female rats after T/HS or burn injury are consistent with studies investigating other aspects of the immunoinflammatory response (4, 24). Furthermore, our observations that castration abrogated T/HS and burn-induced in vivo neutrophil activation while ovariectomy had no effect indicate that testosterone has a greater in vivo effect on neutrophils than the female sex hormones. This observation is somewhat different from the macrophage, lymphocyte, and cytokine responses reported in the literature after T/HS, where both estradiol and testosterone exert modulatory effects on the immunoinflammatory response (4, 24).

Studies of calcium signaling in these cells suggest that estrogen-mediated alterations in PMN calcium entry through SOCE channels play a role in the differential capacity of male and female PMN to participate in inflammation. This key proinflammatory signaling pathway shows relative attenuation in proestrus female rat PMN as well as in male rat PMN subjected to estrogen treatment. These effects were seen both with estrogenic drugs and with authentic estrogen agonists. In the presence of high external estradiol levels, SOCE was suppressed to a very similar extent in both male and female PMN. Thus the effects of estrogens on calcium entry may underlie some of the observed phenotypic differences in male and female PMN responses observed after T/HS or burn injury. Furthermore, the fact that the estrogenic effects occurred so quickly (i.e., 1 min) suggests that these effects were being mediated through estrogen receptor signaling rather than through the more traditional genomic pathway. On the other hand, one limitation of these results is that, because of experimental constraints associated with the SOCE channel studies, only very short PMN incubation times could be tested, and supraphysiological doses of agents had to be employed to

Fig. 7. A: 17β-Estradiol (17β-E2) caused dose-dependent suppression of TG-mediated Ca²⁺ influx in male rat PMN at physiological concentrations (representative traces shown). B: analysis of effects of 17β-E2 on store-operated calcium entry (SOCE) induced by TG in male and female rat PMN (n = 3–6 per group). AUC, area under the cytosolic calcium concentration curve for 100 s after recalcification of the medium. *P < 0.001 for all comparisons among male PMN except 50 vs. 100 nM 17β-E2, which were not significant; **P < 0.01 for all comparisons except 0 nM (control) vs. 25 nM and 50 vs. 100 nM 17β-E2, which were not significant. Thus both male and female PMN show suppression of SOCE by 17β-E2. In the absence of 17β-E2, control male PMN responses were significantly greater than control proestrus female PMN responses (P = 0.029), but no significant differences are seen between male and female PMN SOCE in the presence of high concentrations of external 17β-E2.

Fig. 8. Effects of testosterone on store-operated calcium influx into PMN from castrated male rats. A: cells were kept in calcium-free buffer (−BSA) in cuvettes and incubated for 3 min with testosterone at doses shown or its vehicle (veh) methanol. Experiments were started, and TG (1 μM final concentration) was added at t = 30 s. Calcium was readded to media at 400 s to visualize influx. B: we studied ionomycin-induced calcium influx. PMN were again treated with testosterone at doses shown or vehicle for 3 min. PMN were then stimulated with ionomycin (Iono; 100 nM final concentration) at t = 30 s. Calcium was readded to media at 200 s. Testosterone had no noticeable effect on calcium influx generated by either store depletion agent at any dose. n = 3–4 Representative traces for each condition in the two sets of experiments.
observe an estrogenic effect. Because of these in vitro constraints, the degree to which estrogen has a direct effect on neutrophil function in vivo cannot be answered definitively. Although the in vitro calcium studies were not definitive, because estrogens did modulate the ability of neutrophils to respond and a difference in the response of naive male and female neutrophils to different doses of estrogen was observed, these results are still highly supportive of the notion that estrogens can modulate in vivo neutrophil activation.

The reasons for the failure of ovariectomy to modulate the in vivo neutrophil response in the current study, when it has been reported to influence other arms of the immunoinflammatory response and our in vitro observations indicate that estrogen decreased SOCE, are unclear. However, our other in vitro studies testing the neutrophil-activating effects of plasma from male and proestrus females subjected to castration or ovariectomy prior to trauma-hemorrhage or burn injury on naive male neutrophils is consistent with castration having an anti-inflammatory and ovariectomy having a proinflammatory effect. In these studies, castration abrogated the neutrophil-activating capacity of male plasma, as would be expected from the in vivo results. However, in contrast to the in vivo response, ovariectomy augmented the in vitro neutrophil-activating capacity of T/HS and burn plasma when tested on naive male neutrophils. The endocrine results of ovariectomy and castration are not, of course, entirely parallel, because testosterone can be metabolized further to form estrogens in the presence of aromatase (31). Clearly though, clarification of the differential responses of PMN to hormonal ablation maneuvers will require much further study. This future work will require studies evaluating specific receptor-mediated activation of PMN, because our studies were performed with PMA, which is a nonspecific PMN activator. Thus it is possible that sex-based PMN responses to specific receptor agonists, such as endotoxin, different cytokines, and chemokines, may differ from what was observed with PMA. Furthermore, our in vitro calcium studies failing to find a direct effect of testosterone on neutrophil activation, whereas castration had such a protective effect in vivo, highlights the potential complexities of the in vivo state and suggests that at least some of the neutrophil-modulatory effects of testosterone may be indirect.

The in vivo increase in PMN activation after T/HS or burn injury in the male rats as compared with the proestrus female rats appears to be related to both an increased susceptibility of male PMN to activation as well as to male plasma having greater neutrophil-activating activity than proestrus female plasma. This notion is supported by several of our observations. First, both male and female T/HS and burn plasma samples activated naive control male neutrophils, and the neutrophil-activating activity of the plasma samples collected from the males was greater than that of the proestrus female rats. Second, in contrast to naive male neutrophils, naive proestrus female neutrophils were not activated by either male or female T/HS plasma or burn plasma. Thus, consistent with the in vivo response, the plasma from the male rats subjected to trauma-hemorrhage or thermal injury increased naive male rat PMN activation to a greater extent than plasma from proestrus females, whereas naive female proestrus neutrophils were resistant to activation.

One possible explanation for why the neutrophil-activating activity of the plasma from the proestrus females was less than that of the males subjected to trauma-hemorrhage could be related to our previous observation that proestrus female rats are more resistant to trauma-hemorrhage-induced gut injury, because gut injury is associated with biologically active mesenteric lymph (9). This appears to be the case, because the neutrophil-activating activity of T/HS lymph from male rats was much greater than that of proestrus female rats when tested in vitro on naive male neutrophils. Furthermore, when male and proestrus T/HS lymph was tested on naive female rats, only the male lymph activated the proestrus female neutrophils. This observation that lymph collected from proestrus females has less biological activity than lymph from male rats subjected to T/HS is consistent with other information showing that acute changes in organ function, neutrophil activation, and endothelial cell function after an episode of T/HS are related to factors liberated from the ischemic intestine into the mesenteric lymph, which reach the systemic circulation through the thoracic duct that empties into the subclavian vein (1, 2, 8, 9, 12, 27, 28, 37).

Taken together, the results of the current study indicate that male neutrophil activation in vivo after T/HS or burn injury is greater than in proestrus female neutrophils for at least two reasons. First, neutrophils from male rats appear to be activated to a greater extent by factors contained in post-T/HS and postburn plasma as well as T/HS lymph than are neutrophils from proestrus female rats. Second, male rat plasma and intestinal lymph appear to have greater neutrophil-activating capacity than proestrus female plasma after T/HS or thermal injury. Additionally, although sex hormones appear to modulate the acute neutrophil response to T/HS or burn injury, other sex-independent factors also appear to be important in the pathogenesis of neutrophil activation after T/HS or burn injury. This notion is based on several observations. First, castration did not completely abrogate neutrophil activation in vivo or the in vitro neutrophil-activating ability of post-T/HS or postburn plasma. Second, resistance to in vivo neutrophil activation was not affected by ovariectomy in proestrus female rats after trauma-hemorrhage or burn injury.

In considering the potential mechanisms by which sex hormones modulate neutrophil activation, it is important to point out that ovariectomy affects more than estrogen levels. Although the testes are the primary source of testosterone in the male and thus castration reduces testosterone, the hormonal response to ovariectomy is more complex. Although the ovaries are the primary source of estrogens in females, ovariec-

Tomy also decreases progesterone, prolactin, and dehydroepiandrosterone (DHEA) production (17, 29, 34). In fact, the plasma levels of estrogen, progesterone, and prolactin all peak during the proestrus stage of the reproductive cycle. Thus, although there are data to suggest that estrogen plays an important role in the sex-dimorphic response to T/HS (4, 21, 22), prolactin and DHEA have also been demonstrated to have immunomodulatory effects, and both have been shown to preserve organ function after injury (10, 23, 25, 38). Thus it is also possible that these hormones act in combination to provide the beneficial effects on neutrophil activation seen in the proestrus female. Additionally, the cellular mechanisms by which the sex hormones modulate PMN function after shock and/or trauma remain to be determined. However, there is increasing data suggesting that many of the immunoinflammatory effects of sex hormones, especially estrogens, act via
activation of cell surface receptors and not via traditional genomic pathways (5). Consequently, future studies employing estrogen receptor knockout mice as well as specific estrogen receptor agonists and antagonists will be of importance in beginning to clarify the mechanisms by which sex hormones are signaling PMN to modulate their response.

In summary, the results of the current study indicate that a sexual dimorphism in the in vivo response of neutrophils to trauma exists, with male neutrophils being activated to a greater extent that proestrus female neutrophils. This sex-related difference appears to be related to both cellular and humoral factors. Evidence for a cellular difference between male and female neutrophil activation is based on the observation that naive female neutrophils are more resistant to activation after exposure to burn plasma or T/HS plasma or lymph than naive male neutrophils and that this resistance to T/HS lymph-induced activation varies across the estrus cycle. Additionally, the neutrophil-activating capacity of burn and trauma-hemorrhage plasma and trauma-hemorrhage lymph was much greater in male than in proestrus female rats, indicating that the humoral environment after trauma is more proinflammatory in male than in proestrus female rats. Last, it appears that estrogen is capable of abrogating calcium signaling via its effect on SOCE channels, whereas the fact that plasma and lymph from male, but not female, rats can activate naive female neutrophils suggests that the observed in vivo resistance of proestrus neutrophils to trauma-induced activation is based on both cellular as well as humorally based differences between the sexes. The exact clinical significance of these observations will require patient-based studies, because increased PMN activation can be beneficial as well as deleterious, depending on the exact clinical situation. For example, although increased PMN activation during noninfectious inflammatory or ischemia-reperfusion states, such as trauma, hemorrhage shock, or burn injury, may lead to PMN-mediated bystander tissue injury, failure of PMN activation during an infectious episode could be equally deleterious.

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