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# Effects of Female Hormones (17$\beta$-Estradiol and Progesterone) on Nitric Oxide Production by Alveolar Macrophages in Rats

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**The effect of female sex hormones on nitric oxide (NO) production was studied in alveolar macrophages (AMs). Male rats were treated with endotoxin (LPS) intratracheally or saline as control. AMs were obtained by bronchoalveolar lavage 90 min later and were cultured in the presence or in the absence of LPS and 17$\beta$-estradiol or progesterone ($10^{-9}$ to $10^{-4}$ M). NO production was assessed by measurement of nitrites in the medium. In some experiments, NO production by AMs was measured in intratracheally LPS-treated orchidectomized rats or in female control and ovariectomized rats. Both spontaneous and stimulated NO production were higher in AMs from female than from male rats, but without statistical significance. However, ovariectomy induced significant inhibition in spontaneous production of NO by AMs. In orchidectomized rats, the NO response by AMs to LPS stimulation relative to spontaneous NO production was significantly downregulated. Female sex hormones in physiological concentrations seem to be necessary for spontaneous NO production in female rats. Pharmacological doses of estradiol inhibited *in vitro* LPS-stimulated NO production in AMs of both saline- and LPS-treated rats, and basal NO production only in LPS-treated male rats. Progesterone at $10^{-4}$ M inhibited basal and *in vitro* LPS-stimulated NO generation by AMs of both saline- and LPS-treated male rats. In LPS-treated female rats *in vitro* LPS-stimulated NO production was not affected by estradiol treatment. In ovariectomized LPS-treated female rats progesterone at $10^{-5}$ M significantly inhibited NO production by *in vitro*-stimulated AMs. Thus female sex hormones may contribute to the gender-related differences in the immune response.** 

***Key Words:*** **nitric oxide; gender difference; estradiol; progesterone; endotoxin.**

Several lines of evidence support the concept that both humoral and cell-mediated immunity are more active in females than in males and steroid sex hormones may play an important role in regulating this responsiveness (1, 2). Sex-linked hormonal factors may influence the immune response and modify the expression of autoimmunity in animals as well as in humans (1). Whether in large clinical studies on sepsis (3) or experimental studies with mice, females have been reported to have better tolerance of sepsis than males (4, 5). Additionally, endotoxin had been shown to induce changes in sex steroid hormone levels in male rats with an early and dramatic increase in 17$\beta$-estradiol (E2) and progesterone (P4) levels (6). Similar results have been found in human septic shock (7, 8).

Numerous cell inflammatory functions, e.g., neutrophil chemoattractant generation (9), phagocytic response of PMNs in the blood and the liver and tumor necrosis factor $\alpha$ (TNF$\alpha$) production by Kupffer cells (10), phagocytosis by alveolar macrophages (AMs) and its modulation by tyrosine kinase (11), or

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splenocyte interleukin-2 and interleukin-3 release (5) have been found to exhibit gender differences, supporting the contention that gender and sex hormones influence inflammatory responses. Chemotaxis of PMNs from peripheral blood of healthy adults is enhanced by progesterone, while it is reduced by estradiol (12). Estradiol and progesterone stimulate superoxide release by rat macrophages (13). Depending on their concentrations, estradiol and progesterone can either stimulate or inhibit interleukin-1 (IL-1) production (14–16). Similarly, the production of TNF$\alpha$ or interleukin-6 (IL-6) by macrophages can be modified by female hormones (17, 18).

Nitric oxide (NO) is produced by several different cell types including vascular endothelial cells, smooth muscle cells, neurons, mononuclear phagocytes, and hepatocytes. Alveolar macrophages, as recruited PMN and other phagocytic cells, synthesize NO in response to bacteria, endotoxin, or cytokines and humoral mediators of inflammation (19). NO has an important role in host defense (20) and a protective effect has been demonstrated in liver cells (21–23). Female hormones can modulate NO production from constitutive NOS in aortic rings (24), endothelial cells (25, 26), and inducible NOS in monocyte-like cell lines (27) or peritoneal rat macrophages (13), indicating that the sensitivity of macrophages to variations in concentrations of female sex hormones may contribute to the gender-related differences in the immune response.

The purpose of this study was to investigate the modulating effect of female sex hormones on NO production by AMs in rats.

## METHODS

*Animals.* Male Sprague–Dawley rats (300–380 g), Hilltop Lab Animals, Scottsdale, PA) were anesthetized with ether and given intratracheal injections of *Escherichia coli* endotoxin, B26:06 (Difco, Detroit, MI) (LPS 300 $\mu$g/kg in 0.1 ml of normal saline) or saline (0.1 ml/rat). For this procedure, a midline incision was made above the sternum. The trachea was exposed by dissection, a 28-gauge needle was inserted into the trachea above the carina, and LPS or isotonic saline was instilled. Ninety minutes later, the rats were anesthetized with an intramuscular injection of ketamine–xylazine (90 and 9 mg/kg, respectively) and the trachea was catheterized. Lung lavage was performed with 20 ml of Hepes buffer (140 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 10 mM KCl, 0.1 mM $CaCl_2$, 0.2 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 5 mM glucose, pH 7.30). The lavage fluid was centrifuged at 200*g* for 10 min and the cell pellet was collected. In some experiments naive male rats were anesthetized with an intramuscular injection of ketamine–xylazine and then orchidectomized. In another set of experiments, female rats age-matched with the male rats (200–250 g) were studied. Some of them were anesthetized with an intramuscular injection of ketamine–xylazine (45 and 4.5 mg/kg, respectively) and then ovariectomized. Orchidectomized and ovariectomized animals were allowed to recover from surgery for a period of 1 week before bronchoalveolar lavage. The use of animals conformed to National Institutes of Health guidelines.

*Preparation of alveolar macrophages.* The cell pellet collected from lung lavage was resuspended in Hepes buffer and subjected to discontinuous Ficoll–Hypaque density gradient centrifugation to separate AMs and lung-infiltrated PMNs. The viability of the purified AMs was greater than 95% as determined by trypan blue exclusion. Then AMs were plated on 23-mm plastic 12-well tissue culture plates in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cell density was $2.5 \times 10^5$ cells/0.85 ml medium/well. Cells were maintained for 20 h in primary culture in the absence or in the presence of LPS (8.5 $\mu$g) and without or with various concentrations of 17$\beta$-estradiol or progesterone. Nitrite was determined by the Griess reaction as described previously (27) and was taken as an index of NO production. Dose–response curves of $10^{-4}$, $10^{-5}$, $10^{-7}$, and $10^{-9}$ M 17$\beta$-estradiol or progesterone, or $10^{-5}$, $10^{-7}$, and $10^{-9}$ M 17$\beta$-estradiol + progesterone were compared to controls, containing cells in medium alone or in medium containing the appropriate concentration of ethanol as vehicle.

*Statistical analysis.* All the data are reported as mean ± SE, with the number of independent samples specified in each instance. Data were analyzed by Student's two-tailed *t* test or analysis of variance followed by a Student–Newman–Keuls test, with significance set at $P < 0.05$.

**TABLE I**

Cell Recovery from BAL Fluid from Male and Female Rats Following Various Treatments

| | Total cell count | AMs |
|---|---|---|
| Control male ($n = 12$) | 12.4 ± 1.5 | 7.5 ± 1.3 |
| Orchidectomized ($n = 5$) | 13.2 ± 2.5 | 9.5 ± 2.6 |
| Control female ($n = 4$) | 13.8 ± 3.7 | 7.8 ± 1.5 |
| Ovariectomized ($n = 4$) | 10.2 ± 2.3 | 9.4 ± 1.5 |

*Note.* The numbers represent $\times 10^6$ cells. Numbers in parentheses refer to the number of rats in each group.

## RESULTS

The body weight of male rats was 331 ± 4 g ($n = 17$). The body weight of age-matched (10–15 weeks) female rats was 246 ± 4 g ($n = 8$). The number of cells recovered by bronchoalveolar lavage from male and female rats following various treatments is shown in Table I.

*Spontaneous (basal) and in vitro LPS-stimulated NO production by AMs of male rats after endotracheal administration of saline and endotoxin.* As can be seen in Fig. 1, intratracheally administered endotoxin caused a significant increase in spontaneous NO production. *In vitro* LPS stimulation induced a higher (563 ± 18%) increase in NO production in saline than in LPS-treated rats (222 ± 77%) ($P < 0.001$) (Fig. 1).

*Effect of in vitro treatment with estrogen and progesterone on basal and in vitro LPS-stimulated NO generation by AMs from intratracheally saline and LPS-treated male rats.* The highest concentration of *in vitro* 17$\beta$-estradiol administration ($10^{-4}$ M), decreased NO production by *in vitro* LPS-stimulated and nonstimulated AMs from animals receiving LPS endotracheally. With the same concentration of 17$\beta$-estradiol, the production of NO was also reduced in LPS-stimulated AMs from saline-treated animals. Lower doses of 17$\beta$-estradiol did not significantly modify NO production in any of the groups (Fig. 2). In both saline and LPS-treated rats, high concentrations ($10^{-4}$ M) of progesterone nearly totally suppressed NO production by AMs. In LPS-treated animals, $10^{-5}$ M progesterone induced significant inhibition in spontaneous and LPS-stimulated NO production by AMs, and with the same concentration of progesterone, the production of NO was also reduced in LPS-stimulated AMs from saline-treated animals. Lower concentrations of progesterone did not induce any significant change in NO production by these cells (Fig. 3).

A potential additive effect between progesterone and estradiol was tested for three concentrations ($10^{-9}$, $10^{-7}$, $10^{-5}$ M) in some intratracheally LPS-treated rats. The simultaneous *in vitro* administration of both hormones did not modify the results obtained by estrogen or progesterone alone (data not shown).

The production of NO by AMs after intratracheal administration of LPS from control male rats was compared to that from orchidectomized rats. The spontaneous production of NO was not different in orchidectomized (11.8 ± 1.7 nmol/ml) and in control (11.3 ± 1.1 nmol/ml) rats. However, the percentage of increase relative to basal after *in vitro* stimulation by LPS was significantly reduced in orchidectomized rats (152 ± 19 vs 222 ± 18%, $P < 0.05$).

*NO production in intratracheally LPS-treated female rats with and without ovariectomy.* Ovariectomy induced a significant reduction in spontaneous NO production by AMs. In the presence of all concentrations of estradiol or progesterone, NO production by AMs tended to be lower in ovariectomized than

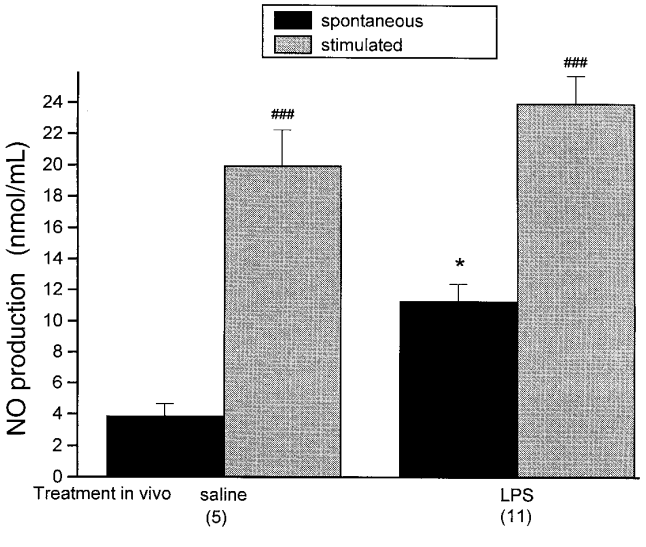


**FIG. 1.** The effects of intratracheally administered saline and LPS on NO production by AMs of male rats. *$P < 0.05$, saline vs LPS; ###$P < 0.001$, spontaneous vs stimulated. Numbers in parentheses refer to the number of animals in each group.

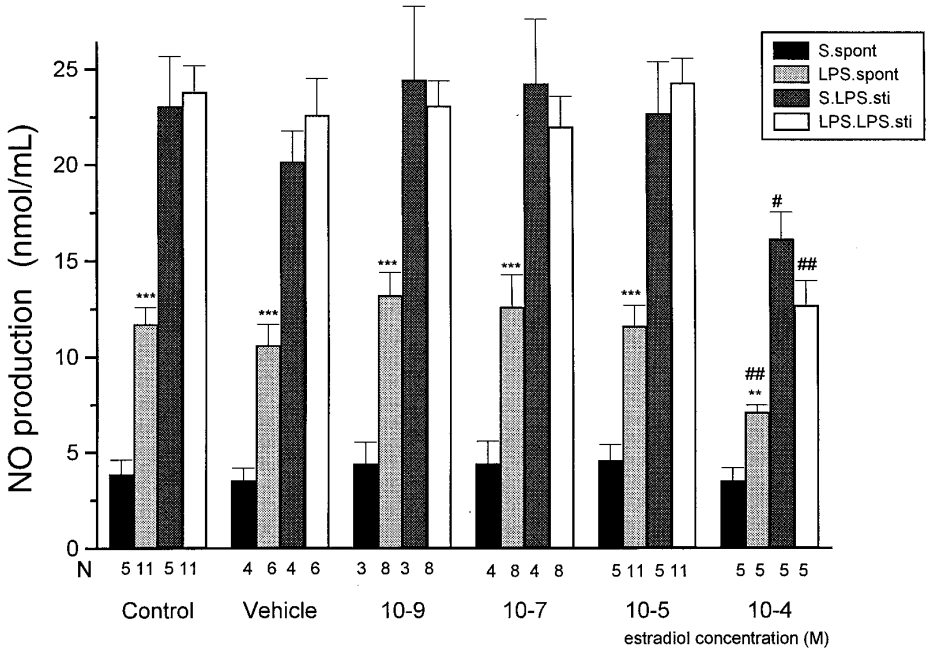


**FIG. 2.** Modulation by estradiol of spontaneous (spont) and *in vitro* LPS-stimulated (LPS.sti) NO production by AMs of intratracheally saline (S) or LPS-injected male rats. $^{**}P < 0.01$, $^{***}P < 0.001$ LPS.spont vs S.spont; $\#P < 0.05$, $\#\#P < 0.01$ estradiol vs control. (Under both *in vivo* conditions, the differences between spontaneous and *in vitro* LPS-stimulated values are highly significant.)

in control animals. With estradiol treatment the difference in spontaneous NO production reached statistical significance at $10^{-9}$ and $10^{-7}$ M (Fig. 4), with progesterone treatment only at $10^{-7}$ M concentration (Fig. 5). *In vitro* LPS-stimulated NO production was not affected by estradiol treatment (Fig. 4), and it was significantly reduced only at $10^{-5}$ M progesterone concentration in ovariectomized rats relative to controls (Fig. 5).

*Comparison of the production of NO by AMs from intratracheally LPS-treated male and female rats.* The spontaneous and LPS-stimulated production of NO by AMs after intratracheal administration of LPS from control and orchidectomized male rats, and control and ovariectomized female rats is summarized in Fig. 6. The spontaneous and *in vitro* LPS-stimulated production of NO by AMs tended to be higher in control (i.e., intact) female than in intact male rats, but without reaching statistical significance. AMs of LPS-treated control male rats produced significantly less NO in response to *in vitro* LPS in the presence of $10^{-9}$ (23.2 ± 1.3 nmol/ml) and $10^{-7}$ M (22.0 ± 1.55 nmol/ml) estradiol than AMs of identically treated control female rats (26.4 ± 0.4 and 29.4 ± 2.0 nmol/ml, respectively) ($P < 0.05$).

## DISCUSSION

*Spontaneous (basal) and in vitro LPS-stimulated NO production by AMs of male rats after endotracheal administration of saline and endotoxin.* NO is one of the effector molecules produced at high levels by activated murine and human macrophages and involved in host defense mechanisms (19, 20). Alveolar macrophages synthesize NO in response to cytokines and humoral mediators of infection (29, 30). The capacity of LPS to induce mRNA for nitric oxide synthase had been demonstrated (31). Our studies also show that intratracheal LPS administration induces an early increase in spontaneous NO production by rat AMs; however, the release of NO by these primed cells in response to *in vitro* LPS stimulation was downregulated. These results differ from those reported by Shellito, showing no spontaneous release of NO by AMs from mice after intratracheal LPS administration, but a marked upregulation in *in vitro* response to interferon gamma or LPS (32). This can

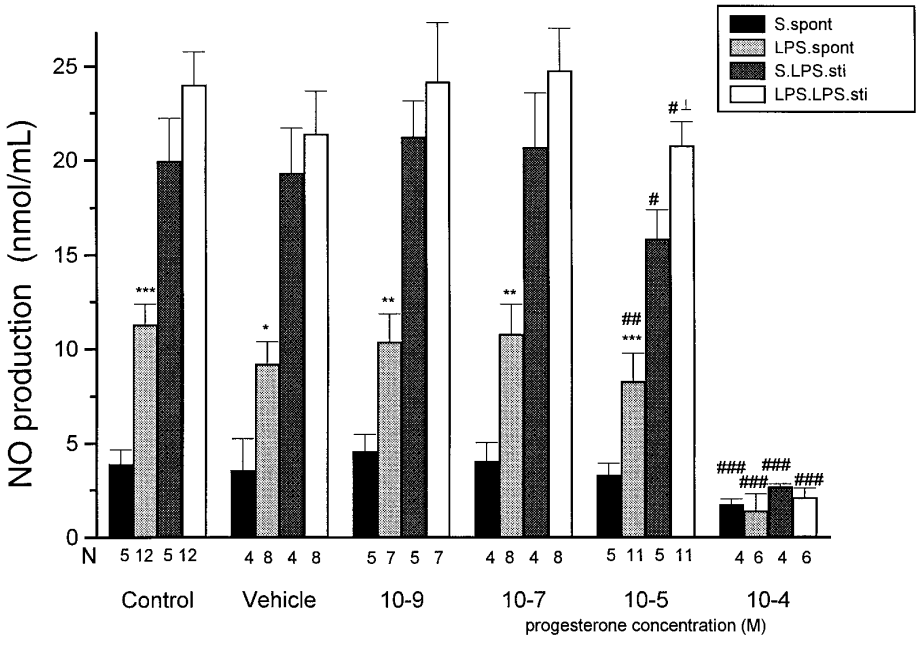


**FIG. 3.** Modulation by progesterone of spontaneous (spont) and *in vitro* LPS-stimulated (LPS-sti) NO production by AMs of intratracheally saline (S) or LPS-injected male rats. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ LPS.spont vs S.spont; $\perp P < 0.05$ LPS.LPS.sti vs S.LPS.sti; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ progesterone vs control. (Under both *in vivo* conditions, the differences between spontaneous and *in vitro* LPS-stimulated values are highly significant.)

be explained by different administered doses of LPS and different *in vivo* and *in vitro* time courses between the two studies, as well as the different species used. Furthermore, the temporal sequence of inflammatory cell influx after intratracheal LPS is delayed in mice in comparison to rats (32).

Interestingly, our results with intratracheal LPS administration are different than those observed 90 min after iv LPS administration in which the spontaneous production of NO was lower than in iv control saline-administered rats (33). In this latter study the *in vitro* stimulation by LPS was highly upregulated in LPS-treated male rats. This suggests that the increased gene expression of iNOS may require more time after intratracheal than intravenous administration. Similarly, iv administration of LPS induced IL-1 mRNA expression earlier than using the intratracheal route (34). Furthermore, macrophage activation and the cytokine network after LPS stimulation may be different in these two situations. Intravenous infusion of LPS induces early margination of leukocytes in microvessels, which interact with the endothelial cells, whereas intratracheal administration of LPS causes progressive influx of leukocytes into the alveolar space.

In other macrophage type cells as in Kupffer cells, spontaneous NO production was low in both saline and iv LPS-treated rats, and *in vitro* stimulation by LPS upregulated NO production only in Kupffer cells from LPS-treated animals (28). This suggests that the cytokine network can also vary from one macrophage cell type to another, leading to differences in NO production.

*Effect of in vitro treatment with estrogen and progesterone on basal and in vitro LPS-stimulated AMs from intratracheally saline- and LPS-treated rats.* Sex hormones exert important actions in immune responses of the acute phase of the inflammatory process. Chemotaxis of PMNs from peripheral blood of healthy adults was enhanced by progesterone, while it was reduced by estradiol (12). Female steroid hormones have been shown to interact with cytokines in several cell systems. These interactions include both regulation of cytokine production by gonadal steroids and regulation of steroid production and response to sex hormones by cytokines. Estra-

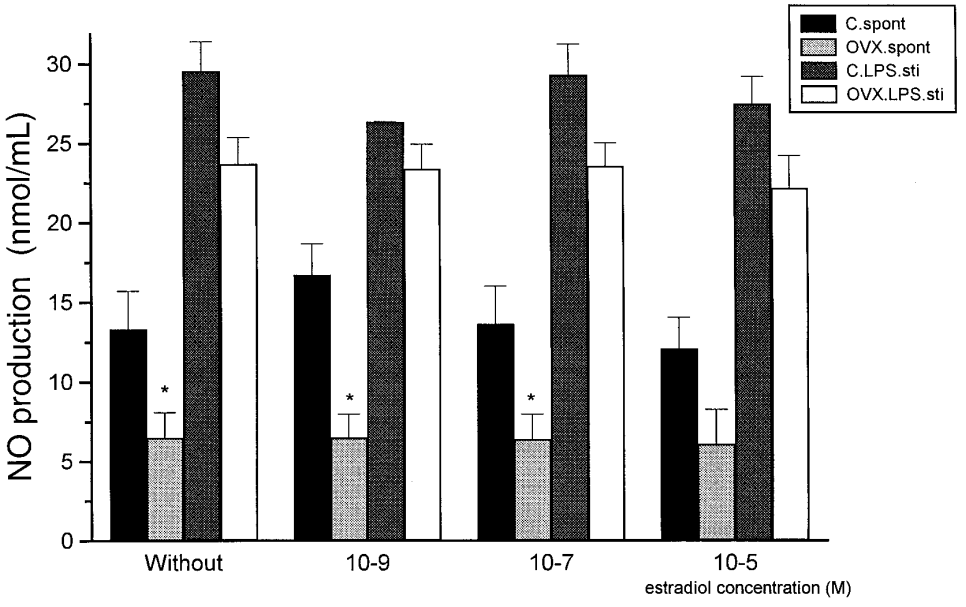


**FIG. 4.** Modulation by estradiol of spontaneous (spont) and *in vitro* LPS-stimulated (LPS.sti) NO production by AMs of intratracheally LPS-injected control (C) and ovariectomized (OVX) female rats ($n = 4$ in each group). *$P < 0.05$, OVX.spont. vs C.spont. (In both control and OVX groups, the differences between spontaneous and *in vitro* LPS-stimulated values are highly significant.)

diol increased TNF-induced adhesiveness of endothelial cells for leukocytes via increased expression of the endothelial adhesion molecules E-selectin, ICAM-1, and VCAM-1 (35).

The pretreatment of mice with estriol enhanced the peak serum level of TNF, modified the kinetics of IL-6 increase after LPS administration (18), and reduced IL-6 mRNA in peritoneal macrophages (17).

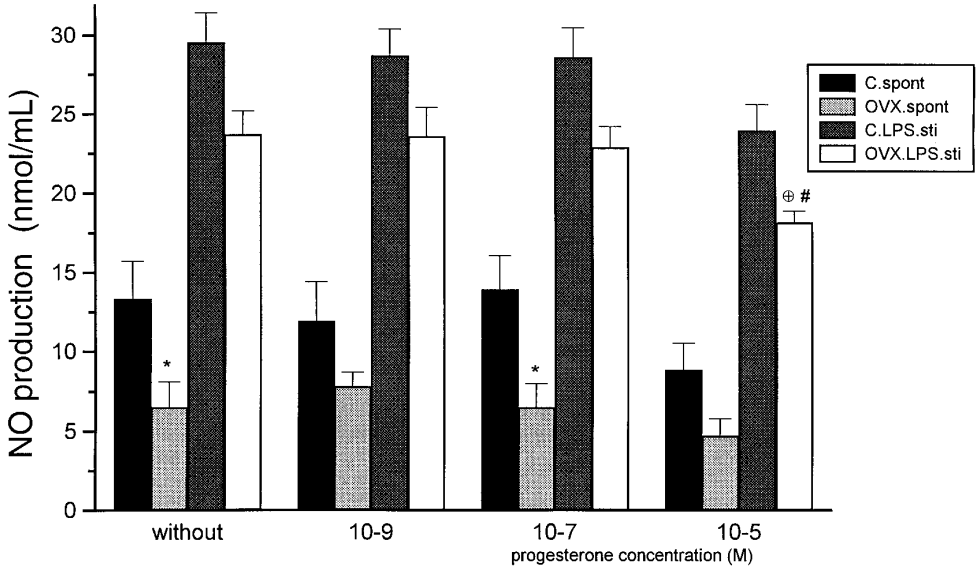


**FIG. 5.** Modulation by progesterone of spontaneous (spont) and *in vitro* LPS-stimulated (LPS.sti) NO production by AMs of intratracheally LPS-injected control (C) and ovariectomized (OVX) female rats ($n = 4$ in each group). *$P < 0.05$, OVX.spont vs C.spont, $\oplus P <$ 0.05, OVX.LPS.sti vs C.LPS.sti; #$P < 0.05$, with vs without progesterone. (In both control and OVX groups, the differences between spontaneous and *in vitro* LPS-stimulated values are highly significant.)

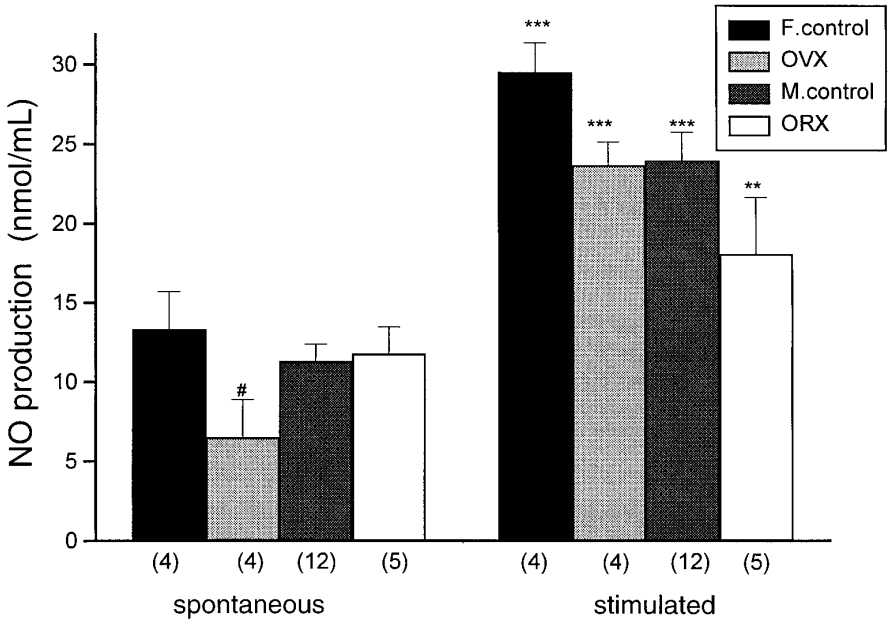


**FIG. 6.** Spontaneous and LPS-stimulated NO production by AMs from control (M.control) and orchidectomized (ORX) and control (F.control) and ovariectomized (OVX) intratracheally administered LPS rats. Numbers in parentheses refer to the number of animals in each group. $^{**}P < 0.01$; $^{***}P < 0.001$ stimulated vs spontaneous; $\#P < 0.05$ OVX control vs F.control.

The *in vitro* data on cytokine expression have been contradictory. At low concentrations, estrogen stimulated IL-1 release from human monocytes (15, 16), whereas high doses inhibited IL-1 production (16). Similar results were reported with progesterone (16). Furthermore, *in vitro* administration of estradiol decreased the ability of peritoneal macrophages from mice to produce IL-6 without any significant effect on TNF production (17).

The regulation of both constitutive (cNOs) and inducible (iNOs) nitric oxide synthase activities has also been shown to be modulated by female sex hormones. The administration of estradiol increased endothelium-dependent relaxation (36), and increased NO production in endothelial cells (24–26) via an estrogen receptor-mediated system (25).

In this study we showed that high concentrations of estradiol or progesterone induced significant inhibition of NO production by both *in vivo*-treated and *in vitro* LPS-stimulated AMs, but lower concentrations of the hormones did not induce any significant change. Female sex hormones have been shown to modify NO formation in nonpulmonary macrophage cells. Both low and high dosages of estradiol 17$\beta$ and mainly progesterone have been demonstrated to decrease nitrite release by nonseptic rat peritoneal macrophages (13). Miller *et al.* showed that high dosages of progesterone strongly inhibited iNOS gene activity in IFN-$\gamma$ + LPS-stimulated mouse macrophage-like cells, whereas estradiol did not modulate NO production (27). However, *ex vivo* peritoneal macrophage cultures from estradiol-treated mice failed to demonstrate any significant increase in NO production in response to an *in vitro* LPS challenge (18). The concentrations of female sex steroids used in our study resulting in significant inhibition of NO production are at pharmacological levels, and therefore the physiological significance needs to be interpreted with caution. However, under pathological conditions, e.g., in septic patients or in experimental animals, such high concentrations have been noted (6–8) and may influence outcome.

The mechanism of modulation of the immune response of macrophages by sex hormones is not well understood. Estradiol could act on macrophages *in vitro* via the estradiol receptor, since estrogen receptors have been reported on murine and rat macrophages as well as in monocytic cell lines (37–39). However, no progesterone receptors were identified. Progesterone's inhibitory effect might be mediated

through binding to the glucocorticoid receptor (27), but this has not been demonstrated. Female sex hormones could also modulate NO production by macrophages indirectly since they modulate the production of cytokines (14–17). Indeed, pretreatment of animals with anti-TNF or anti-IFN-$\gamma$ induced moderate (32) or pronounced (13) decrease in NO production by macrophages.

*Gender differences.* Several gender differences have been demonstrated in macrophage functions: AMs of LPS-injected female rats produce significantly more TNF$\alpha$ and are less dependent on protein tyrosine kinase activity for modulation of TNF$\alpha$ secretion than AMs of equally treated age-matched male rats (11). Phagocytic activity in AMs after LPS treatment is upregulated in male rats only (11). Gender differences have also been shown in LPS-stimulated TNF$\alpha$ secretion in Kupffer cells of acutely ethanol-intoxicated rats challenged by LPS (10), and in neutrophil function and cytokine-induced neutrophil chemoattractant generation in endotoxic rats (9).

High plasma levels of female hormones have been found in septic male rats (6) and in male septic ICU patients (7, 8). Orchidectomy and not adrenalectomy suppressed this increase in endotoxic male rats, indicating that during sepsis, estrogen and progesterone increases are partially produced by the testes, probably via activation of testicular aromatase which can convert androgen hormones to estrogen (6). The significance of elevated plasma female hormones during sepsis remains unknown. However, a protective action of estrogen has been shown in experimental endotoxic shock (4, 5).

In this study, both spontaneous and stimulated NO production were higher in AMs from female than in male rats, but without statistical significance. Ovariectomy induced significant inhibition in spontaneous production of NO by AMs, a finding which is in concert with the known tendency of estrogens to enhance immune responses, albeit through still unidentified mechanisms (40). Furthermore, in orchidectomized rats, the LPS-stimulated NO production by AMs was significantly downregulated. A lower production of female hormones in orchidectomized than in control LPS-treated animals might explain this inhibition in NO production. However, modifications in the production of female sex hormones by the testes have not been demonstrated after intratracheal administration of LPS. On the other hand, AMs of LPS-treated control male rats produced significantly less NO in response to *in vitro* LPS in the presence of $10^{-9}$ and $10^{-7}$ M estradiol than AMs of identically treated control female rats ($P < 0.05$), suggesting that the sensitivity of NO production by AMs to estradiol inhibition is also gender related. This hypothesis is supported by the fact that high concentrations of estrogens are associated with severe sepsis or endotoxemia in males (6–8) and may be a hallmark of a concomitant compromised host defense system.

In summary, intratracheal administration of LPS induces early changes in NO production by AMs, which are different from those observed after iv LPS challenge. Female hormones seem to participate in the modulation of NO production in this model, in both female and male rats and may contribute to the gender-related differences in the immune response. Physiological concentrations of female sex hormones are needed for spontaneous NO production as demonstrated by the decrease in NO production in ovariectomized animals. However, pharmacological doses of estradiol and mainly progesterone can inhibit this production.

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