Differential expression of uterine NO in pregnant and nonpregnant rats with intrauterine bacterial infection

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Fang, L., B. Nowicki, and C. Yallampalli. Differential expression of uterine NO in pregnant and nonpregnant rats with intrauterine bacterial infection. Am J Physiol Regulatory Integrative Comp Physiol 280: R1356–R1363, 2001.—Previous studies have demonstrated that nitric oxide (NO) is involved in the uterine host defense against bacterial infection. In nonpregnant rats, NO production in the uterus was shown to be lower, and inducible NO synthase (NOS) expression was undetectable. However, studies in pregnant rats show abundant expression of inducible NOS with significant elevation in NO production in the uterus. We have recently reported that intrauterine Escherichia coli infection caused a localized increase in uterine NO production and inducible NOS expression in the nonpregnant rat. In our present study, we examined whether the uterine NO production, NOS expression, and uterine tumor necrosis factor-α protein are increased in pregnant rats with intrauterine pathogenic Escherichia coli infection. Unlike the nonpregnant state, the NO production in the infected uterine horn of pregnant rats was not significantly elevated after bacterial inoculation compared with the contralateral uterine horn. The expression of uterine NOS (types II and III) also did not show significant upregulation in the infected horn. This is in contrast to that in nonpregnant animals, in which type II NOS was induced in the uterus on infection. Moreover, intrauterine infection induced an elevated expression of tumor necrosis factor-α protein in the infected horn both of nonpregnant and of pregnant rats. These data suggest that the sequential stimulation of NOS expression, especially the inducible isoform, and generation of uterine NO are lacking during pregnancy despite an elevated tumor necrosis factor-α after infection. In summary, NO synthesis response may be maximal at pregnancy, and infection may not further induce the NO system. Present studies, together with our previous report that intrauterine infection-induced lethality in pregnancy rats was amplified with the inhibition of NO, suggest that pregnancy is a state predisposed for increased complications associated with intrauterine infection and that the constitutively elevated uterine NO during pregnancy may help contain or even reduce the risk of infection-related complications.

cytokines; Escherichia coli; gestation; Dr fimbriae; nitric oxide
In addition, NO was considered to be a crucial effector in the host defense in Leishmania-infected mice (5, 36). The microbicidal and microbiostatic activity of NO has been demonstrated against a number of protozoa, viruses, and bacteria (6, 12, 29). Furthermore, an increased NO production induced by infection and endotoxin via cytokines such as TNF-α and IL-6 could also play a role in these host defense mechanisms (33).

Maternal intrauterine infections have been related to several adverse outcomes of pregnancy in human and experimental animals including intrauterine fetal death, premature labor, premature rupture of membranes, and abortion (3, 13, 32). Compared with nonpregnant, pregnant women are more susceptible to the infectious diseases caused by viruses, bacteria, fungi, and protozoa; once women are infected, these diseases tend to become severe. The pathophysiology of the gestation-associated infections is thought to correlate with pregnancy-altered systemic and local host responses. Maternal host defense against these pathogens is primarily cell mediated, and so it is believed that maternal cell-mediated immunity is downregulated to avoid immunological rejection of the fetus. The amount of immunosuppression increases with the duration of the gestation (39).

It is well accepted that the pregnant state is associated with a reduced host response to infection, which may be different from the nonpregnant state. Therefore, the present study was undertaken to evaluate the responsiveness of uterine NOS expression and NO production in pregnant rats with intrauterine pathogenic E. coli infection compared with those from the nonpregnant rat uterus. The levels of uterine TNF-α, a well-known proinflammatory cytokine that can stimulate the upregulation of inducible NOS and high output of NO (21, 24), were also measured in the infected pregnant and nonpregnant animals to assess whether differential uterine NO generation is a consequence of altered proinflammatory cytokine response.

MATERIAL AND METHODS

Animals and Treatment

**Infected pregnant rat model.** Adult timed-pregnant Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Houston, TX). All experiments were approved by the Animal Care and Use Committee at The University of Texas Medical Branch and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Two days before experimental infection, each animal received one dose of streptomycin (7.0 mg/g body wt; Parker-Davis, Morris Plains, NJ)/xylazine (5 mg/kg body wt; Phoenix Scientific, St. Joseph, MO) anesthesia. Infected animals were randomly killed in groups of five using a CO2 inhalation chamber at 12 and 24 h after bacterial infection, and tissues were collected for further study. We used an E. coli strain (IH11128) that expresses Dr fimbriae, a virulent factor that mediates adherence to the mucosal surface, in our experiments because of its association with gestational infections and its capacity to colonize epithelial cells (27) through the attachment to its tissue receptors.

**Infected nonpregnant rat model.** Adult female nonpregnant Sprague-Dawley rats, without regard to the stage of estrus cycle, were bilaterally ovarioctomized (OVX) while they were under anesthesia and treated as described above. Similar to pregnant animal protocol, 2 days before experimental infection, each animal received one dose of streptomycin (7.0 mg/g of weight). Because it was impossible to pass the blunt needle through the cervix in nonpregnant rats, we used laparotomy to inoculate bacteria into the uterine lumen of these animals. Under anesthesia, a laparotomy exposing both uterine horns was performed and an inoculum of Dr E. coli in a volume of 200 μl of PBS (5 × 10⁹ bacterial cells/ml) was injected into the lumen of the exposed left uterine horn through an 18-gauge needle. The cervical end of the horn was ligated to prevent loss of inoculum through the injection site. The right uterine horn, which was injected with the same amount of PBS followed by ligation of the cervical end, served as the control within each animal. The laparotomy incision was closed, and the animals were randomly killed in groups of five using a CO2 inhalation chamber at specific times after infection.

**Tissue collection.** The uterine tissues from all animals were removed for assessment of nitrite production or were either immediately snap frozen in liquid nitrogen or placed in Tissue-Tek OCT embedding compound and frozen in liquid nitrogen. Frozen specimens were stored at −70°C until they were sectioned for immunofluorescence-staining studies and immunoblotting analysis.

**Evaluation of nitrite production by rat uterine tissue with HPLC.** Uterine tissues were cut into 2-mm strips (~100 mg), placed in minimum essential medium (GIBCO, Gaithersburg, MD) containing 1% penicillin and 1% streptomycin, and placed in a CO2 incubator with humidified chamber at 37°C for an initial 1-h equilibration period to eliminate the effect of cutting. Media was replaced with fresh media, and incubation continued for 24 h. Because NO spontaneously autooxidizes to form the stable metabolites nitrite and nitrate, measurement of these products provides an indirect assay for NO production. Nitrates were converted to nitrites, and these were measured as the final product of NO metabolism. We used a novel and highly sensitive HPLC method for the measurement of nitrites released into media from infected and noninfected rat uterus (7).

Nitrite concentrations in the media were measured in triplicate by the HPLC method as described earlier (7). Briefly, an aliquot of media was injected into a spectrophotometric nitrite (NO₂⁻) and nitrate (NO₃⁻) analyzer, which consists of HPLC pumps, a cadmium-reducing column, a postcolumn reactor, and an ultraviolet detector (Beckman System Bold 126, Beckman, Fullerton, CA). The samples were carried through the analyzer in a 0.3% aqueous ammonium chloride buffer containing 0.07% EDTA (pH 8.0). Nitrates were reduced to nitrites on the cadmium-reducing column. The ultraviolet-absorbing derivative was formed by postcolumn reaction with a solution containing 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% HCl. The
Western immunoblotting analysis for NOS II and III in rat uterine tissue. Western blot was performed as previously described (7). Full-thickness uterine tissues containing both endometrium and myometrium were homogenized in 50 mM Tris buffer (pH 7.4) containing 0.1 mM EGTA, 0.1 µl β-mercaptoethanol, 100 nmol phenylmethylsulfonyl fluoride, and 0.2 mg/ml trypsin inhibitor. The homogenate was centrifuged at 1,000 g for 15 min at 4°C, and the supernatant was used for immunoblotting. The concentration of proteins in the supernatant fraction was measured with the biocinchonic acid (BCA) kit (Pierce, Rockford, IL). As positive controls for NOS isoforms, proteins obtained from cytosolic fractions of cytokine-stimulated RAW264.7 cells (NOS II) and membrane fraction of human endothelial cells (NOS III) were used. Equal amounts of protein (40 µg) were size fractionated on 7.5% (wt/vol) SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were allowed to dry in air and placed in blocking buffer [1% BSA (wt/vol) in 10 mM Tris buffer with 100 mmol NaCl, 0.1% Tween 20 (vol/vol)] at pH 7.5, for 1 h at room temperature. The blots were incubated with specific monoclonal antibodies of NOS III and II (Transduction Laboratories Lexington, KY) for 1 h at room temperature. The blots were washed three times for 30 min each with buffer [10 mM Tris, 100 mmol NaCl, 0.1% Tween 20 (vol/vol), pH 7.5] incubated with horseradish peroxidase-conjugated goat antimouse antibody (Transduction Laboratories). The membranes were washed, and proteins were visualized using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The intensity of specific immunoreactive bands on autoradiographic film was quantified using a densitometric image-scanning system (PDI, Huntington Station, NY).

Immunofluorescent staining for the distribution of NOS II in pregnant rat uterus. The immunofluorescent staining method was performed by a modified immunofluorescence protocol (7, 41). Cryosections of 5 µm from rat uterus were cut and fixed in 70% acetone. Five percent normal goat serum and Avidin-Biotin blocking buffer were applied to slides to reduce nonspecific binding. Because polyclonal antibodies against NOS II (Upstate Biotechnology, Lake Placid, NY) and III (Transduction Laboratories) appeared to work better for immunofluorescent studies (7, 41), these antibodies in PBS buffer were added to the sections and incubated for 90 min. After slides were washed in PBS, they were incubated with biotinylated goat anti-rabbit IgG (Vector Lab, Burlingame, CA) for 45 min at 25°C. After washes in PBS, the detection step was performed with fluorescein avidin-D (Vector Lab) for 1 h at room temperature. Slides were then washed four times in PBS, counterstained with propidiod iodide to visualize nuclei, and mounted with Vectashield mounting media (Vector Lab), then they were viewed under a Nikon fluorescence microscope (Nikon, Melville, NY). For negative control sections, rabbit IgG was used instead of primary antibodies.

Western immunoblotting analysis for TNF-α in pregnant and nonpregnant OVX rat uterine tissues. Western blotting was similar to the described above. The procedures for blotting were similar to that described above.

Statistical analysis. One-way ANOVA or Student’s t-test was used to evaluate differences between various treatments. Differences were considered significant at P < 0.05.

RESULTS

Nitrite production by uterine tissue from rats with intrauterine infection. Analysis of HPLC absorbance peaks of nitrite generated by the uterine tissue from nonpregnant and pregnant rats, a quantitative indicator of NO production, is presented in Fig. 1. In nonpregnant rats, increases in nitrite production by the infected uterine horn 12 or 24 h after bacterial inoculation were observed (P > 0.05) compared with those from the noninfected uterine horn. These results are similar to our previous report (7). Interestingly, NO production in the noninfected uterine horns of pregnant rats was significantly higher (3.5-fold) compared with that of nonpregnant OVX rats (Fig. 1). However, in the infected horn of pregnant rat uterus, a significant induction after intrauterine infection was not demonstrable (Fig. 1). Moreover, the baseline uterine NO levels in pregnant rats are similar to the infection-stimulated uterine NO levels in nonpregnant rats. This indicates that the uterine NO production may be at near maximum in pregnant rats and that there is a lack of uterine NO production increase on intrauterine infection in pregnant rats.

Western immunoblotting analysis for NOS II of experimental rats. NOS II protein was undetectable in nonpregnant OVX animals, whereas it is expressed abundantly during pregnancy (Fig. 2A). Protein from murine macrophages was used as a control for NOS II. Densitometric analysis (Fig. 2B) indicated that intrauterine infection of nonpregnant rats caused a significant elevation in the expression of NOS II protein in pregnant rats at different time points after infection. Uterine tissues were obtained from rats killed at 12 and 24 h after bacterial inoculation. Data are presented as µmol/100 mg uterus, calculated from absorbance area. Results are means ± SE (n = 4) for both infected and noninfected horns. *P < 0.05.
infected uterine horn but not in the noninfected horn when examined at 12 and 24 h after inoculation \((P < 0.05)\). In pregnant animals, however, no significant changes were found in NOS II contents between the infected and noninfected horns.

**Western immunoblotting analysis for NOS III in rat uterine tissue.** A specific band corresponding to the size of NOS III protein was present in the homogenates of all uterine horns from every tested group whether or not they received the bacterial inoculum. Densitometric analysis demonstrates that no significant differences are apparent in the homogenates of infected and noninfected uterine horns, either in pregnant or nonpregnant rats (Fig. 3B). Protein from human endothelial cells was used as a control for NOS III.

**Immunofluorescent staining for the distribution of NOS II in pregnant rat uterus.** Abundant staining for NOS II was detected in the uterine stromal layer and in the connective tissue area between myometrial bundles (Fig. 4a) in the infected uterine horn. No detectable staining was visualized in epithelial and smooth muscle cells (Fig. 4a). A similar pattern of immunoreactivity for NOS II was found in sections from the noninfected horn of pregnant rats (Fig. 4c). In the nonpregnant rat uterus, abundant staining for NOS II was detected in the uterine stromal layer in the infected horn, and no detectable staining was visualized in any cells in the noninfected horn (see Ref. 7 for details).

**Western immunoblotting analysis for TNF-\( \alpha \) in rat uterine tissues.** With the use of a similar immunoblotting method, the expression of TNF-\( \alpha \) protein was demonstrable in the uterine tissues of nonpregnant OVX and pregnant rats, irrespective of infection (Fig. 5A). However, the intensity of TNF-\( \alpha \) expression was greater in the infected uterine horn compared with noninfected horn at 12 and 24 h after infection, either in nonpregnant or pregnant rats. In addition, the levels of expression of uterine TNF-\( \alpha \) in response to infection were greater in pregnant rats compared with those from nonpregnant OVX rats (Fig. 5, \( P < 0.05 \)).

**DISCUSSION**

Previous studies have suggested that the uterus is capable of NO generation and that NO may have a role in maintaining uterine quiescence during pregnancy \((8, 17, 25, 34, 40–42)\). Inducible NOS (NOS II) expression is detectable in the pregnant rat uterus by Western blot and immunofluorescence microscopy \((41)\) as well as the presence of mRNA for NOS II \((4)\). Constitutive NOS (NOS III) expression is present both in the nonpregnant \((2, 41)\) and in pregnant rat uterus \((41)\). Furthermore, uterine NO and NOS expression may be important host-response factors to intrauterine infection both in pregnant human and in experimental animals \((7, 16, 26)\).

The major finding of this study is that the pattern of the responsiveness of NOS II to intrauterine bacterial infection.
infection in pregnant rats is different from nonpregnant animals. Unlike the nonpregnant rats challenged with bacteria, there was no significant elevation in uterine NO production or NOS II protein expression in pregnant rats (Fig. 1A, Fig. 2). Lack of changes in uterine NOS II protein expression due to infection observed in pregnant rats was also confirmed by immunofluorescence methods, i.e., no apparent changes in the pattern of distribution of NOS II-expressing cells (Fig. 4). This may be suggestive of a lack of response of the NO system (both NO production and inducible NOS II expression) to intrauterine bacterial infection in the pregnant state. This lack of response could be due to saturation of NOS II expression and NO generation during pregnancy to function as an endogenous smooth muscle relaxation factor. Thus infection cannot induce further NOS II or NO production in the pregnant rat uterus. However, pregnancy appeared to be a state predisposed for increased complications associated with intrauterine infection (1, 11, 14, 20, 33). An elevated uterine NO production (constitutively) during pregnancy may help contain or even reduce the infection-related complications. Therefore, if NO production is reduced during pregnancy, the infection-related complications may become more pronounced. This is further supported by our previous report in which inhibition of NO with L-NAME increased the lethality of pregnant rats on intrauterine infection (26).

In this study, immunoblotting for NOS III demonstrated that intrauterine infection did not change the amount of this constitutive NOS isoform (Fig. 3). This phenomenon was also observed in the uterus of infected nonpregnant OVX rats. This result further confirms that NOS III is a constitutive isoform in rat uterine tissue. In the absence of infection, both uterine NO production and NOS II protein expression were significantly higher in the pregnant rat uterus compared with those from nonpregnant rats (Figs. 1 and 2). These observations are similar to our previous reports (4, 41, 42).

In our previous study, the uterine macrophage and NK cells in nonpregnant uterine tissues were shown to be the NOS II-expressing cells after infection (7). The distribution of NOS II-expressing cells after infection (7). The distribution of NOS II-expressing cells in the current study is similar to our previously published data in pregnant rats (41). No apparent differences were de-

Fig. 4. Immunohistochemical localization of NOS II protein in cross sections from infected horn and noninfected horn from P rats at 24 h after intrauterine infection. Green fluorescence indicates NOS II-containing cells. E, epithelial cells; M, myometrium. No significant change in NOS-II staining is detectable in the infected and noninfected horn. All sections were counterstained with propidium iodide for nuclear viewing (red immunofluorescence). a: infected horn with NOS II antibody; b: infected horn without NOS II antibody; c: noninfected horn with NOS II antibody; d: infected horn without NOS II antibody. Arrows indicate NOS II-positive cells.
 Differential Expression of Uterine NO

Fig. 5. Western immunoblotting of protein for tumor necrosis factor (TNF-α) using a polyclonal antibody in the tissue homogenate of uterus from P and NP OVX rats at 12 and 24 h after intrauterine infection. A: specific bands in the homogenates of infected horn and noninfected horn from 2 representative rats in each group. B: relative densitometric changes in uterine TNF-α expression in the uterus. Data in each are expressed relative to the values from NP OVX rats at 12 h (100%). Values are the means ± SE from 4 animals in each group. *P < 0.05. TNF-α+, positive control band.

We found a significant increase in the expression of uterine TNF-α protein after bacterial infection both in nonpregnant OVX rats and in pregnant rats. TNF-α is a cytokine associated with inflammation and infection. The presence of bioactive TNF-α in human amniotic fluid and placental culture supernatants indicates its possible involvement in normal pregnancy (19). Intrauterine infection-induced increases in TNF-α both in pregnant and in nonpregnant rats observed in the current study are similar to the changes in uterine TNF-α both in humans and in experimental animals with intraterine infection (11). Several studies have shown that lipopolysaccharide (LPS) stimulates TNF-α protein in uterine macrophage and NK cells (18), cultured human fetal membranes, and decidual explants (1). Furthermore, high levels of TNF-α during gestation are associated with intrauterine infection and preterm labor (23, 33).

TNF-α has been shown to induce NOS II expression and subsequently increase NO production during infection. In nonpregnant rats, infection caused elevations in the levels of both TNF-α and NOS II proteins and NO production in the uterus (Figs. 1 and 2). In pregnant rats, however, infection stimulated the expression of TNF-α (Fig. 5) without changes in NOS II protein and NO production (Figs. 1 and 2). This may indicate that the NO expression in pregnant rat uterus is no longer inducible by the increased TNF-α after infection, because the levels of this enzyme are already maximally induced (Fig. 5). When pregnancy-associated elevations in uterine NO are reduced by an inhibitor of NO, 1-NAME, the severity of infection is increased resulting in higher lethality to infection (26). Moreover, NO presumably derived from tissue-associated macrophages and NK cells in the uterus may be an important antimicrobial compound, and high local concentrations of NO may independently have bacteriostatic or bactericidal effects during pregnancy. Thus perhaps the elevated NOS II expression may compensate for other potential immunological defects during pregnancy.

Hirsch's laboratory (14) observed that the increased uterine TNF-α levels in a mouse model with intrauterine E. coli infection were concomitant with the upregulation of inducible cyclooxygenase enzyme. As a major gram-negative bacterial product, LPS activates transcription of TNF-α gene and stimulates massive production of bactericidal molecules such as NO by macrophages and NK cells. Previous experiments from Hunt's lab (18) suggest that LPS may be capable of stimulating TNF-α production by nonlymphocytes as well as by lymphocytes. For example, the human Jar cell (choriocarcinoma cell) was reported to produce TNF-α after the stimulation with LPS (44). In addition, the release of uterine TNF-α and NO in uterine tissue was observed in the LPS-injected rat model. The results from our current study also suggest that uropathogenic E. coli is a strong stimulator for induction of TNF-α and NOS II expression in nonpregnant rat uterine tissue. Previous studies on uropathogenic E. coli with P or type 1 fimbriae also indicate that the fimbrial protein could activate the cytokine network (TNF-α and IL-1β) of local mucosal epithelial cells and immune cells, either with LPS or alone (37). The E. coli strain, IH11128, that we used in this study expresses LPS and two types of fimbriae (type 1 and Dr); together, these factors may serve to induce TNF-α and the NO system.

In summary, we used uropathogenic Dr+ E. coli to initiate an intrauterine bacterial infection in nonpregnant and pregnant rats and observed the responsiveness of the NO system and TNF-α. We found that both NO production and NOS II expression in the uterus were increased in nonpregnant but not in pregnant rats. These studies suggest that the uterine NO system may be maximally activated during pregnancy to function as an endogenous relaxation factor as well as a local antimicrobial agent to protect against uterine infection. This upregulated NO system in the uterus cannot be stimulated further.
Perspectives

Intrauterine infection during pregnancy can result in increased neonatal morbidity and mortality, fetal growth retardation, and sepsismic. It is suggested that immunological responses are dampened during pregnancy to accommodate the fetus. This reduced immunological host-response state could provide ample opportunity for amplification of the severity of potential infection. NO, a gas molecule, has been implicated as an important modulator of infection and immunity through its antimicrobial activity. Our current study provides evidence that local NO synthesis and expression of inducible NOS (NOS II) are increased on intrauterine infection in the nonpregnant rat. However, in pregnant rats, there is a lack of further increase in the NO system indicating that this system is functioning at maximum during pregnancy to protect against infection and also to provide smooth muscle relaxation. When pregnancy-associated elevations in uterine NO are reduced by an inhibitor (L-NAME), the severity of uterine infection is increased, resulting in higher lethality (26). The elevated local concentrations of NO in the uterus may have a bactericidal function, and thus perhaps the increased NOS II expression during pregnancy may be compensatory for other potential immunological deficiencies. Therefore, the uterine NO system during pregnancy may play an important role in maintaining host defense against infection, and disturbance of this system could result in infection-related complications.

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