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

L. FANG,1,2 B. NOWICKI,1,3 AND C. YALLAMPALLI1,2
1Department of Obstetrics and Gynecology, 2Anatomy and Neuroscience, and 3Microbiology and Immunology, The University of Texas Medical Branch, Galveston, Texas 77555–1062

Received 21 March 2000; accepted in final form 8 January 2001

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

INTRAUTERINE INFECTION DURING pregnancy is a serious problem with significant neonatal morbidity and mortality, including low birth weight, high-risk pregnancy, fetal growth retardation, and septicemia (1, 11, 14, 20, 33). The underlying predisposition to intrauterine infection during pregnancy is poorly understood. Suggested mechanisms include a decrease in the immune response during pregnancy and pregnancy-induced hormonal changes (19). Recently, much emphasis has been placed on the role of inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), prostaglandins, and nitric oxide (NO) in the pathophysiology of intrauterine infection and infection associated with uterine immune response (1, 14, 15, 22, 26, 31, 33).

NO, a simple gas molecule, is generated from L-arginine by NO synthase (NOS) and was found to be involved in a variety of physiological functions, including neurotransmission, vasodilation, and infection (9, 10, 24). Three different isoforms of NOS (I, II, and III) have been identified in mammalian cells (9, 24, 41). Types I and III NOS are constitutive isoforms originally isolated from neurons and endothelial cells, respectively. NOS II is an inducible form originally found in mouse macrophages that has been shown to participate in the immune response to infection (9, 24). Several reports indicate that NOS III, but not II, is expressed in nonpregnant rat uterus, whereas both NOS II and III are expressed in pregnant rat uterus (4, 41, 42). Uterine NO production is substantially elevated during pregnancy (41–43). Furthermore, the inhibition of NO synthesis with nitro-L-arginine methyl ester (L-NAME; an inhibitor of NOS) increased the bacterial invasion and the infection-induced lethality in pregnant rats (26, 28). NOS II is upregulated in the nonpregnant rat uterus on intrauterine bacterial inoculation, and the NOS II expression is restricted to uterine macrophages and natural killer (NK) cells (7).

Several lines of evidence indicate that NO may be involved in the host defense mechanisms against infection (6, 29). Inhibition of NOS activity significantly decreased the survival time of mice infected with Salmonella typhimurium or Escherichia coli (6). Furthermore, the mortality rate was increased in endotoxemic rats from 33% to 74% when NO synthesis was inhibited

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 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 fimbriae, a virulent factor that mediates adherence to the mucosal surface, 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). The nitrites 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
areas of the absorbance peak at 546 nm were determined with an integrator.

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, 0.14 mM β-mercaptoethanol, 100 mM 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 biocinchoninic 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 mM 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 mM 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 propidium 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 in place of primary antibodies.

Western immunoblotting analysis for TNF-α in pregnant and nonpregnant OVX rat uterus. Full-thickness uterine tissues from OVX rats were homogenized as described above. Because TNF-α has a molecular weight of 18, the homogenates of uterine tissue were size fractionated on 10%–20% (wt/vol) gradient SDS-PAGE gels. The polyclonal antibody of TNF-α raised against purified recombinant rat TNF-α was used at a dilution of 1:10,000. Both the antibody and the rat TNF-α for positive control were from Biosource International (Camarillo, CA) and horseradish peroxidase-conjugated goat anti-rabbit antibody was from Santa Cruz Biotech (Santa Cruz, CA). 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 absorbence peaks of nitrate generated by the uterine tissue from nonpregnant and pregnant rats, a quantitative indicator of NO production, is presented in Fig. 1. In non-pregnant 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 horns from OVX rats were homogenized as described above. Because TNF-α has a molecular weight of 18, the homogenates of uterine tissue were size fractionated on 10%–20% (wt/vol) gradient SDS-PAGE gels. The polyclonal antibody of TNF-α raised against purified recombinant rat TNF-α was used at a dilution of 1:10,000. Both the antibody and the rat TNF-α for positive control were from Biosource International (Camarillo, CA) and horseradish peroxidase-conjugated goat anti-rabbit antibody was from Santa Cruz Biotech (Santa Cruz, CA). 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 absorbence peaks of nitrate generated by the uterine tissue from nonpregnant and pregnant rats, a quantitative indicator of NO production, is presented in Fig. 1. In non-pregnant 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

Fig. 1. Nitrite generated by uterine tissues from infected and noninfected horns of nonpregnant (NP) ovariectomized (OVX) rats and pregnant (P) 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 homogenate 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-α in rat uterine tissues. With the use of a similar immunoblotting method, the expression of TNF-α protein was demonstrable in the uterine tissues of nonpregnant OVX and pregnant rats, irrespective of infection (Fig. 5A). However, the intensity of TNF-α 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-α 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 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 after infection (7). The distribution of NOS II-expressing cells after infection (7). The distribution of NOS II-expressing cells after infection (7). The distribution of NOS II-expressing cells after infection (7).
In the current study, we initiated 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 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.

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 septicaemia. 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-NNAME), 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.

For editorial and graphic assistance, we thank Obstetrics/Gynecology Publications director and staff. R. G. McConnell, Kristi Barrett, John Helms, and Traci Smith.

We gratefully acknowledge support received through grants from the National Institutes of Health: HD-30273 and HL-58144 to C. Yallampalli and DK-42029 to B. J. Nowicki.

REFERENCES


Downloaded from: http://ajpregu.physiology.org/ by 10.220.32.246 on June 29, 2017


