Nitric oxide synthase isoforms in the rat uterus: differential regulation during pregnancy and labour

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Production of nitric oxide in the uterus is increased during pregnancy and decreased during delivery. In this study the isoform of nitric oxide synthase which may be responsible for the changes in nitric oxide production was investigated in relation to pregnancy and delivery. Monoclonal antibodies were used to measure changes in neuronal nitric oxide synthase (NOS I), macrophage nitric oxide synthase (NOS II) and endothelial cell nitric oxide synthase (NOS III) protein in the rat uterus by densitometric scanning of specific bands. Results show that: (1) NOS II protein concentrations in the uterus were substantially increased during pregnancy and were decreased during delivery, both at term and preterm (induced by RU486); (2) NOS III protein was present at all stages examined but the concentrations were unchanged; (3) NOS I was present in the rat uterus during the nonpregnant stage but not during pregnancy and delivery. The changes in uterine NOS II protein concentrations during pregnancy and delivery were further confirmed by the changes in the Ca^{2+} -independent, but not Ca^{2+} -dependent, nitric oxide synthase activity. Therefore, an increase in NOS II, thus in nitric oxide production during pregnancy, may play a role in maintaining uterine quiescence.

Introduction

Nitric oxide (NO) is a multifunctional molecule that mediates a number of diverse physiological processes, including vasodilation, neurotransmission, and platelet antiaggregation (Moncada *et al.*, 1991). Relaxation of smooth muscle by nitrovasodilators is thought to be mediated by the increase of guanosine 3',5'-cyclic monophosphate (cGMP) in smooth muscle cells (Jackson and Busse, 1991). We have recently demonstrated that NO is a myometrial relaxant and proposed that NO, generated in the gravid uterus, plays a role in maintaining uterine quiescence during pregnancy. Studies from our laboratory (Yallampalli *et al.*, 1993, 1994a, b) and others (Natuzzi *et al.*, 1993; Sladek *et al.*, 1993) provide strong evidence that NO generation is upregulated during pregnancy and downregulated during delivery and postpartum. In rats, we have demonstrated that the production of NO, measured as total nitrites, is increased during mid-gestation and is markedly decreased during spontaneous delivery and postpartum (Yallampalli *et al.*, 1994a). Others have also reported a decrease in nitric oxide synthase (NOS) activity in rat (Natuzzi *et al.*, 1993) and rabbit (Sladek *et al.*, 1993) uterine tissues at term. An increase in NOS content in the uterus may also be important in the maintenance of pregnancy, and a decrease in NOS content in this tissue at term may play a role in initiation of labour.

The NOS isoforms share a common overall catalytic scheme for the oxidation of L-arginine to form NO and L-citrulline and can be divided into two functional classes based on the

dependence of calcium for activity (Moncada *et al.*, 1991). The cytokine inducible isoform, NOS II, binds calmodulin tightly and its activity is Ca^{2+} -independent. The constitutive forms, NOS I and NOS III, bind calmodulin in a reversible and Ca^{2+} -dependent manner. Although all three isoforms of NOS show a considerable degree (overall 50%) of similarity (Sessa, 1994), these proteins can be readily identified by their molecular size, antigenicity and their dependency on Ca^{2+} for activity.

In the present studies, the rat uterus was examined for the types of isoforms of NOS by western blotting using isoform-specific monoclonal antibodies and by NOS activity. Further, the pregnancy-associated changes in different isoforms in the uterus were characterized to ascertain the isoform(s) involved in the changes we observed in the NO production (Yallampalli *et al.*, 1994a). All three isoforms are present in the rat uterus and changes in NOS II may be responsible for the changes in NO production by the uterus during pregnancy.

Materials and Methods

Animals

Pregnant and nonpregnant (adult cycling, 180–200 g body weight; prepubertal, 60 g body weight) Sprague–Dawley rats were purchased from Harlan Sprague–Dawley (Houston, TX) and were maintained on a 12 h light:12 h dark schedule. Six rats were used in each group. Normal duration of gestation for rats is 22 days. Pregnant animals were killed in a CO_2 inhalation

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chamber either on day 18 of gestation (day 1 of gestation being day of a sperm positive vaginal smear), at the time of spontaneous delivery at term (1–3 pups delivered) or on day 1 postpartum. In a separate experiment, preterm delivery in six pregnant animals was induced by intraperitoneal injection of an antiprogesterone, RU486 (Biomol, Plymouth Meeting, PA, 10 mg per rat in mineral oil). This treatment, given on day 17 of gestation, induced preterm delivery on day 18. RU486-induced labour has been extensively used as a model of preterm labour and this model was used in this study. The nonpregnant animals during dioestrus and prepubertal rats at 24 days old were also killed in a CO₂ inhalation chamber. The uteri from all animals were removed immediately, cleaned and quickly frozen in liquid nitrogen and stored at -70°C until used.

Tissue homogenization and preparation of subcellular fractions

Full thickness uterine tissues were homogenized in 50 mmol Tris l^{-1} buffer (pH 7.4) containing 0.1 mmol EGTA l^{-1} , 0.14 μl β -mercaptoethanol ml^{-1} , 100 mmol phenylmethanesulfonyl fluoride (PMSF) l^{-1} and 0.2 mg trypsin inhibitor ml^{-1} . The homogenate was centrifuged at 1000 g for 15 min at 4°C and the supernatant was centrifuged at 102 000 g for 30 min at 4°C . The supernatant (cytosolic fraction) was decanted from the pellet and the pellet (membranous fraction) was resuspended in buffer. The concentration of proteins in all subcellular fractions was measured with the BCA kit (Pierce, Rockford, IL). As positive controls for different isoforms of NOS, proteins obtained from cytosolic fractions of rat cerebellum (NOS I) and cytokine stimulated RAW264.7 cells (NOS II) and membrane fraction of human endothelial cells (NOS III) were used.

Western blotting

Equal amounts of protein (30 μg) were size fractionated on 7.5% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were allowed to dry in air and were then placed in blocking buffer (1% (w/v) BSA in 10 mmol Tris buffer l^{-1} with 100 mmol NaCl l^{-1} , 0.1% (v/v) Tween-20, pH 7.5) for 1 h at room temperature. The blots were then incubated with specific primary antibodies for 1 h at room temperature. All the primary antibodies were monoclonal, obtained from Transduction Laboratories (Lexington, KY) and were used at different final dilutions (NOS I: 1:1000; NOS II: 1:1000; NOS III: 1:500 (v/v)) in the blocking buffer. These antibodies were raised using the following NOS proteins as immunogens: NOS I, a 22.3 kDa protein fragment corresponding to amino acids 1095–1289 of human NOS I; NOS II, a 21 kDa protein fragment corresponding to residues 961–1144 of mouse NOS II; NOS III, a 20.4 kDa protein fragment corresponding to amino acids 1030–1209 of human NOS III. The blots were washed three times for 30 min each with wash buffer (10 mmol Tris l^{-1} , 100 mmol NaCl l^{-1} , 0.1% (v/v) Tween-20, pH 7.5) and then incubated with horseradish peroxidase-conjugated goat-antimouse immunoglobulin antibody (Transduction Laboratories) diluted in 5% (w/v) non-fat milk in wash buffer. The membranes were washed with wash

buffer three times for 30 min and the enhanced chemiluminescence reagent, ECL kit (Amersham, Arlington Heights, IL) was added and incubated for 1 min at room temperature. The blots were exposed to autoradiographic film and the intensity of specific immunoreactive bands was quantified using densitometric scanning. Densitometric units of specific protein bands are expressed relative to the values from nonpregnant animals (North *et al.*, 1994). Both the elimination of primary antibodies and the use of a non-NOS related monoclonal antibody indicated the specificity of the NOS protein bands at appropriate molecular sizes (data not shown). In each blot at least one lane was loaded with protein from an appropriate positive control.

Nitric oxide synthase activity

NOS activity in the uterine homogenate was determined by monitoring the formation of [³H]-citrulline from [³H]-arginine as described by Bush *et al.* (1992). Briefly, NOS reaction was performed in duplicate for 45 min at 37°C in 200 μl buffer containing 2.5 mg ml^{-1} protein, 20 μmol FAD, 4 μmol tetrahydrobiopterin l^{-1} , 50 in calmodulin, 1 μmol L-arginine l^{-1} , 10 nmol [³H]-arginine and with or without 1 mmol NADPH l^{-1} and with 1.25 mmol CaCl₂ l^{-1} or with 1 mmol EGTA l^{-1} . All reactions were stopped by dilution with ice-cold Hepes buffer (80 mmol Hepes l^{-1} , 8 mmol EDTA l^{-1} , pH 5.2). [³H]-citrulline product was separated from [³H]-arginine by DOWEX (AG 50W-8) cation exchange (Brown *et al.*, 1992). With this method DOWEX retained $96 \pm 2\%$ of [³H]-arginine. [³H]-arginine was incubated without protein and the resulting counts were subtracted from [³H]-citrulline product counts of each replicate tube. Since NOS activity is NADPH dependent (Marletta, 1993), the activity in the absence of NADPH was subtracted from total activity and the results are expressed as NADPH-dependent activity. NOS activity is expressed as fmol L-citrulline $\text{mg}^{-1} \text{min}^{-1}$.

Statistical analysis

One-way ANOVA or Student's *t* test was used to evaluate differences between various treatments and the differences were assumed significant at $P < 0.05$.

Results

NOS I

The NOS I antibody reacted with 155 kDa protein from the cytosolic fraction of the rat cerebellum corresponding to NOS I (Fig. 1). The cytosolic fraction from the uterus of nonpregnant rats showed a specific band of 155 kDa that corresponds to NOS I. Densitometric analysis of this band from six animals shows that the NOS I was minimal during pregnancy, delivery and postpartum (Table 1). The NOS I is present in the uterus of not only cyclic nonpregnant rats but also noncyclic prepubertal rats (Fig. 2). There were no apparent changes in NOS I content of pregnant uterus during delivery induced by antiprogesterone, RU486.

Fig. 1. Western blotting using a monoclonal antibody against nitric oxide synthase I from two representative nonpregnant rats at dioestrus stage (NP), and pregnant rats on day 18 of gestation, during delivery, or on day 1 postpartum (PP). B: rat cerebellum. The antibody reacted with a protein band at 155 kDa. Note that this 155 kDa band is present only in the nonpregnant rat uterus.

Fig. 2. Western immunoblotting for nitric oxide synthase I in various tissues; rat cerebellum (B), uterus of rats on day 18 of gestation with or without administration of antiprogesterone, RU486, and of prepubertal rats, E: human endothelial cells; M: mouse macrophages.

Fig. 3. Western blotting using a monoclonal antibody against mouse macrophage-nitric oxide synthase (NOS II) in the uterus from two representative nonpregnant rats during dioestrus (NP), and pregnant rats on day 18 of gestation, during delivery, and on day 1 postpartum (PP). M: cytosolic fraction from cytokine stimulated mouse macrophages. The antibody reacted with two protein bands at 125 and 130 kDa. Note that these protein bands are detectable only during pregnancy on day 18 of gestation.

NOS II

The monoclonal antibody to NOS II reacted with the appropriate band corresponding to the 130 kDa protein from cytokine stimulated murine macrophage cell line, RAW264.7, but not with that of cerebellum or endothelial cells, indicating the specificity of this antibody to detect NOS II (Figs 3 and 4). In the uterine specimens obtained from days 16–22 of gestation, this antibody reacted with two major protein bands, one with a similar size (130 kDa) to that from the macrophage cell line and the other slightly smaller in size (125 kDa). Many earlier studies indicated that the size of the NOS II isoform varied between 120 kDa and 135 kDa. Although the reacted

Fig. 4. Western immunoblotting for nitric oxide synthase II of proteins from cytokine-stimulated mouse macrophage cells (M), uterus from rats on day 18 of gestation with or without administration of antiprogesterone, RU486, and uterus from prepubertal rats. Note that this antibody did not react with proteins from either rat cerebellum (B) or human endothelial cells (E).

protein bands in the uterus range from 125 kDa to 130 kDa, we analysed only the 130 kDa band corresponding to the size of the positive control, for comparisons among different groups. The NOS II protein in the uterus is detectable predominantly during pregnancy (days 16–22 of gestation), and this was 518–726% relative to the amount of protein in the uterus from nonpregnant rats at dioestrus (100%) and it declined markedly during delivery and day 1 postpartum (Table 1). NOS II was undetectable in the uterus on day 18 of gestation during RU486-induced preterm delivery (Fig. 4) and also undetectable in the uterus from prepubertal rats. These data indicate that the amount of NOS II is minimal in the nonpregnant state, is upregulated during pregnancy and is downregulated during term and preterm delivery.

NOS III

The antibody against NOS III isoenzyme reacted with a specific band at 140 kDa from the membrane fraction of human endothelial cells, but not with that of cerebellum or macrophages, indicating the specificity of this antibody (Figs 5 and 6). A band at 140 kDa corresponding to the size of NOS III protein was detectable in the uterus from nonpregnant rats and rats at all stages of gestation examined (Fig. 5); however, there were no significant changes in the amount of NOS III among different groups (Table 1). Furthermore, NOS III content of the uterus did not change during preterm delivery induced by RU486 (Fig. 6). In addition, the NOS III protein was present in the uterus of prepubertal rats (Fig. 6). These data indicate that NOS III protein content in the uterus is not regulated at gestation.

Nitric oxide synthase activity in rat uterus during pregnancy and labour

The NADPH-dependent conversion of L-arginine to L-citrulline was detectable in rat uterine tissues during pregnancy and delivery. The NADPH-sensitive NOS activity which is dependent upon Ca^{2+} was similar in rats during pregnancy and delivery (term and RU486-induced) (Table 2). However, the Ca^{2+} -independent NOS activity in the uterus

Table 1. Relative changes in the protein concentration of nitric oxide synthase (NOS) isoforms in rat uterus, measured as densitometric units of specific bands on western blots

NOS isoform	Non-pregnant	Pregnant						RU486 induced preterm labour
		Day 16	Day 18	Day 20	Day 22	Delivery	Postpartum day 1	
NOS I	100	10.3 ± 1.5*	11.0 ± 1.7*	10.2 ± 1.5*	10.0 ± 1.4*	9.5 ± 2.3*	11.0 ± 1.6*	9.5 ± 2.2*
NOS II	100	517.8 ± 18.0*	726.3 ± 29.2*	682.0 ± 18.2*	561.0 ± 25.0*	88.7 ± 9.9	96.8 ± 8.4	93.5 ± 8.1
NOS III	100	90.8 ± 10.9	96.0 ± 9.5	92.3 ± 10.2	89.3 ± 10.4	91.3 ± 14.4	89.7 ± 10.8	97.0 ± 8.4

Data are expressed as percentage change from nonpregnant animals. Values are means ± SEM for six animals per group.
**P* < 0.01 versus nonpregnant animals by ANOVA.

Fig. 5. Western blotting, using a monoclonal antibody against endothelial cell-nitric oxide synthase (NOS III), of proteins from the uterus of two representative nonpregnant rats during dioestrus (NP), and pregnant rats on day 18 of gestation, during delivery, or on day 1 postpartum (PP). E: human endothelial cells. The antibody reacted with a protein band at 140 kDa.

Fig. 6. Western immunoblotting for nitric oxide synthase III of proteins from human endothelial cells (E), uterus from rats on day 18 of gestation with or without administration of antiprogesterone, RU486, and uterus from prepubertal rats. Note that this antibody did not react with proteins from either rat cerebellum (B) or cytokine stimulated mouse macrophages (M).

was significantly (*P* < 0.05) lower in animals during delivery at term or preterm (induced by RU486), than it was during pregnancy (on day 18 of gestation) (Table 2).

Discussion

Nitric oxide production in the rat uterus is upregulated during pregnancy and downregulated during delivery and it is postulated that increased endogenous NO production during pregnancy may play a role in maintaining uterine quiescence during gestation (Yallampalli *et al.*, 1994a, b). In the present study, we

Table 2. Nitric oxide synthase activity (fmol citrulline mg⁻¹ protein min⁻¹) in uteri of rats during pregnancy and labour and antiprogesterone-treated rat uterus

Day of pregnancy	Calcium dependence	Calcium independence
Pregnant, day 18	80 ± 13	170 ± 30
Spontaneous labour	100 ± 30	20 ± 8*
RU486-induced labour	120 ± 20	70 ± 30*

Enzyme activity was measured using the [³H]arginine to [³H]citrulline conversion assay. The decrease in calcium-independent NOS activity during labour, both spontaneous and RU486-induced, was significant (**P* < 0.05) compared with that during pregnancy (*n* = 4).

examined isoform(s) of NOS in the rat uterus that may contribute to the changes in NO production at different stages of gestation. The protein for NOS II was abundant in the uterus during pregnancy, on days 16–22 of gestation, and was undetectable during spontaneous labour at term, RU486-induced labour at preterm, and in nonpregnant, adult or prepubertal rats. The changes in the NOS II content associated with pregnancy and delivery parallel the changes in Ca²⁺-independent NOS activity and suggest that NOS II may be the major contributing enzyme for variation in NO production during pregnancy and delivery. However, the content of NOS III in the uterus was unchanged during the different stages of gestation examined. NOS I was undetectable during pregnancy although it was present in nonpregnant rats. These studies indicate that increased NOS II protein is responsible, at least in part, for the increased NO production during pregnancy and therefore for uterine quiescence during pregnancy, and a decrease in this isoform is associated with the decrease in NO activity during delivery.

The major finding is that NOS II protein in the rat uterus is extremely low during nonpregnant stages and is substantially increased during pregnancy and is decreased during both spontaneous delivery at term and RU486-induced preterm delivery. This is consistent with changes in Ca²⁺-independent NOS activity in these animals. Natuzzi *et al.* (1993) reported that calcium-independent NOS activity (a well known characteristic of NOS II) in the rat uterus was maximum during pregnancy and is decreased substantially during spontaneous

delivery and postpartum. In addition, using rabbit decidual tissues, Sladek *et al.* (1993) showed that the NOS activity, which has the characteristics of an inducible isoform of the enzyme, is substantially lower at the end of gestation. These studies, together with the present study, provide evidence that the uterine NOS II protein is increased during pregnancy and this increased protein may be responsible for increased NOS activity and thus for uterine quiescence. Although RU486-induced preterm labour has been established for many years, our data demonstrated for the first time that RU486-induced preterm labour was associated with substantially decreased NOS II protein, indicating that the expression of NOS II may be, at least partially, regulated by progesterone.

NOS III isoform may also contribute to the total NOS activity in the uterus, since NOS III protein was readily detectable in the rat uterus at various stages examined. However, there were no significant changes either in NOS III protein content or in Ca^{2+} -dependent activity in the rat uterus associated with pregnancy and delivery, indicating that this isoform may not be primarily responsible for the changes in total NOS activity. This conclusion is further supported by the studies of Natuzzi *et al.* (1993) and Sladek *et al.* (1993), in which there were no significant pregnancy-associated changes in Ca^{2+} -dependent NOS activity. Therefore, we propose that the relative contribution of the NOS III isoform to the pregnancy associated changes in total NOS activity in the uterus is marginal.

In contrast to NOS II and NOS III, NOS I protein was undetectable in the rat uterus during pregnancy and, therefore, the relative contribution of NOS I for total NOS activity during pregnancy is also negligible. It is interesting to note that NOS I protein was detectable in the uterus of prepubertal rats and in adult rats during the nonpregnant stage. This observation is further supported by a recent report that uteri from non-pregnant rats contain nerve-associated NOS activity (Shew *et al.*, 1993). The decline in NOS I during pregnancy could be attributed to the degeneration of nerves in the uterus during this period (Stjernquist and Sjöberg, 1994). The cellular location of different isoforms of NOS in the uterus is uncertain at present, and requires immunohistochemical studies.

The monoclonal antibodies to NOS I, II, and III reacted with proteins with molecular weights specific to the corresponding isoforms. The antibodies to both NOS II and III did not crossreact with other isoforms. However, NOS I antibody reacted with a protein from macrophages at 130 kDa, in addition to NOS I (protein at 155 kDa obtained from rat cerebellum). On the basis of the molecular size of the protein reacted with a specific antibody and crossreactivity, the data on changes in the content of different NOS isoforms, NOS I, II and III in the uterus during pregnancy and labour, are valid.

The mechanisms responsible for an increase during pregnancy and a decrease during labour in the NOS II protein in the uterus are unclear at present. It is unclear from the present study exactly when during pregnancy these changes in NOS II actually occur in the uterus. It is also not clear whether the changes in NOS II protein are modulated by female sex steroid hormones, although studies indicate that antiprogesterins reduce NOS II protein in the rat uterus. It has been reported that NOS activity in several maternal tissues increases early in pregnancy

(Weiner *et al.*, 1994) and that several types of uterine cells, myometrial mast cells and endometrial epithelial cells, produce NOS II, the expression of this enzyme appearing to be modulated by ovarian steroid hormones (Huang *et al.*, 1995). Cytokines induce NOS II protein in a variety of tissues and several studies have reported changes in a variety of cytokines associated with pregnancy (Hunt, 1989). Notwithstanding the type and the timing of the stimulus for the increase and decline in NOS II in the uterus, the changes in this isoform and thus NO production in this tissue may play a significant role in the maintenance of uterine quiescence during pregnancy and initiation of labour.

In summary, we demonstrated that all the three isoforms of NOS: NOS I, NOS II and NOS III are detectable in the rat uterus by western immunoblotting. The NOS II protein and Ca^{2+} -independent NOS activity is increased during pregnancy and decreased during labour (both at term and preterm) without changes in NOS III content, while NOS I protein was undetectable during gestation. Taken together with our previous observations (Yallampalli *et al.*, 1993, 1994a, b), present studies suggest that NOS II could play a significant role in the generation of NO in the uterus and thus in the maintenance of uterine quiescence during pregnancy.

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