Expression of iNOS mRNA and Inhibitory Effect of NO on Uterine Contractile Activity in Rats Are Determined by Local Rather Than Systemic Factors of Pregnancy

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Abstract. Our purpose was to investigate whether the local or systemic factors of pregnancy are associated with inducible nitric oxide synthase (iNOS) mRNA expression and to determine the inhibitory effects of pharmacological agents that increase cGMP levels in rat myometrium. iNOS mRNA expression was determined in uterine tissues from nonpregnant rats and on day 17 of gestation in the pregnant and non-pregnant uterine horns by RT-PCR. In addition, uterine rings from the pregnant and non-pregnant uterine horns were placed in Krebs-Henseleit solution for isometric recordings of spontaneous contractions. Concentration-inhibition relationships to diethylamine/nitric oxide complex, 8-bromo-cGMP, and the selective phosphodiesterase V inhibitor were obtained. Compared to nonpregnant rats, expression of iNOS mRNA in myometrium increased during pregnancy, which was maximal on day 17, followed by a decrease on day 21 of gestation. Expression of iNOS mRNA at day 17 of gestation was greater in pregnant uterine horns than in nonpregnant ones. Maximal inhibition of phosphodiesterase V and increasing cGMP induced similar inhibition of spontaneous contractions in nonpregnant and pregnant uterine horns, while NO induced less inhibition in the former. The results suggest that the local pregnancy factor is needed for signal transduction from NO to soluble guanylate cyclase at a time when maximal expression of iNOS mRNA is evident.

Keywords: iNOS mRNA, nitric oxide, contractility, pregnancy, uterus

Introduction

Nitric oxide (NO), a unique and ubiquitous biologic messenger, plays a major role in physiological and pathological processes such as smooth muscle relaxation, neurotransmission, host defense, and inflammation (1). The NO-soluble guanylate cyclase (sGC)-cyclic guanosine 3',5'-monophosphate (cGMP) pathway is believed to regulate uterine contractile activity, maintaining relative myometrial quiescence throughout gestation, while decreased production of NO, as well as sensitivity to NO, close to term may promote labor and delivery (2 – 4). NO-induced activation of sGC increased intracellular levels of cGMP, thus mediating smooth muscle relaxation (5). NO is synthesized from L-arginine via three distinct isoforms of NO synthases (NOS) (6). Expression of inducible NOS (iNOS) mRNA, the main isoform present in the uterus, is increased during pregnancy and decreased during labor (7, 8).

Myometrium in pregnancy is under the influence of both systemic factors such as circulating neurohumoral factors, and local factors such as locally released neurohumoral and local physical factors like myometrial stretching due to increasing uterine volume, and the placental and myometrial hormones and autacoids. Effects of systemic and local factors can be studied separately in animals with double uteri, where one of the uterine horns may be left sterile. Isolated observations have indicated deviations in the behavior of the pregnant and nonpregnant uterine horns (9 – 11).
It is not known how pregnancy affects the expression of iNOS mRNA and contractility and responsiveness to the agents affecting the sGC-cGMP pathway in non-pregnant and pregnant uterine horns.

The aim of this study was to investigate whether gestation by itself (systemic factors) or the pregnancy in one uterine horn (local factors) with the absence of the pregnancy in the other horn affects iNOS mRNA expression and responsiveness to agonists of the sGC-cGMP pathway in rat uterine tissues.

**Materials and Methods**

**Animals**

Nonpregnant (NP) and pregnant Wistar rats on day 10 (P10), 14 (P14), 17 (P17), and 21 (P21) of gestation (term delivery at day 22) were utilized in this study. Successful mating was confirmed by analyzing the restriction sites or by direct sequencing using an automated DNA sequencer. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR products were cut out, purified, and used as a template for the PCR analysis.

A pair of sense and antisense primers specific for rat iNOS was designed based on published cDNA sequences. For rat iNOS (12), the nucleotide sequence of each primer and the location in cDNA are as follows: CTGTCACCGAGATCAATGCA (sense, 1232 – 1251) and CATGAGCAAAGGCACAGAAC (antisense, 1909 – 1890). A calmodulin cDNA subclone was used as a control in all RT-PCR. The predicted length of PCR products for rat iNOS is 658 bp. The polymerase chain reaction was carried out in a 10-μL solution containing the following: 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 125 μmol of each deoxy-nucleotide triphosphate, 0.5 μmol primer mix, and 25 U/μL Taq DNA polymerase. The PCR conditions were 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 72°C, and 1 cycle at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR products were confirmed by analyzing the restriction sites or by direct sequencing using an automated DNA sequencer.

**Measurement of uterine contractility in vitro**

Uterine rings were positioned between rigid stainless steel wire (250 μm) stirrups and placed in an 10-mL organ chambers containing Krebs-Henseleit solution, bubbled with 5% CO₂ in air, 37°C, pH approximately 7.40, of the following composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 0.026 mM EDTA, and 11.5 mM glucose. The passive tension was gradually increased to the optimal level of 2 g during the equilibration period of more than 90 min.

The spontaneous contractions of the uterine rings registered with isometric force transducers (TB-611T; Nihon Kohden, Tokyo) were recorded and stored by an on-line computer and analyzed by means of Chart (Bio Research, Nagoya) software as previously described (13). Uterine ring spontaneous contractile activity was analyzed as integral contractile activity for 10 min before and after each concentration of the agents, and the induced changes were expressed as a percentage of the basal integral activity. Concentration-inhibition relationships to NO donor diethylamine/nitric oxide complex (DEA/NO), membrane permeable cGMP, 8-bromo-cGMP (8-br-cGMP), and selective phosphodiesterase V inhibitor UK-114542 were assessed. The changes occurred in parallel-run control rings were subtracted from the changes induced by the agents.

**Drugs used**

DEA/NO was obtained from Research Biochemicals.
International (Natick, MA, USA), 8-br-cGMP was from Sigma (St. Louis, MO, USA), UK-114542 was obtained from Pfizer, Inc. (London, UK). Stock solutions of the agents were made using deionized water, aliquoted, and kept frozen at −20°C until used. The desired concentration of the agents were made with deionized water at the day of experiment and were kept on ice. The agents were applied to the organ chamber in the volumes of 10 – 23 μL to obtain one half log increments in the concentration-inhibition relationships studies.

Data analyses

Images were entered into a computer and analyzed by NIH Image 1.62 software program (Research Services Branch, Bethesda, MD, USA) (available at: http://rsb.info.nih.gov/nih-image/). The results were normalized using the amount of calmodulin PCR products obtained in a parallel PCR experiment. Data are presented as means ± S.E.M. The Dunnett-ANOVA test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

All values of uterine contractile activity are presented as means ± S.E.M. The pD$_2$ values (−log M of concentration of DEA/NO, 8-br-cGMP, and UK-114542 causing 50% inhibition of uterine ring contractile activity) were calculated and compared by means of the one-way ANOVA test. A probability of 0.05 or less was considered statistically significant.

Results

iNOS mRNA expression

The changes in expression of iNOS mRNA (RT-PCR products) in uterine tissues during progression of gestation (P10, P14, P17, and P21) and the expression of iNOS mRNA in uterine tissues from non-pregnant rats are demonstrated in Fig. 1a, and quantitative data based on densitometric scanning are shown in Fig. 1b. Calmodulin (CaM) mRNA, used as an internal control in these experiments, migrated as a unique 1.4-kilobase (kb) band in rat uterine tissues as have been described for other tissues (14). In our experiment, CaM mRNA expression was constant during the course of pregnancy (NP: 100%, P10: 121 ± 12%, P14: 124 ± 14%, P17: 97 ± 10%, P21: 119 ± 13%; $n = 4$), as observed in rat (15) and mouse (16) uterus. Expression of iNOS mRNA was progressively and significantly greater on day 10, 14, and 17 of gestation than those in uterine tissues from non-pregnant rats ($P < 0.05$) and returned dramatically on day 21 of gestation to values not statistically different from those in the tissues from non-pregnant rats.

Expression of iNOS mRNA in nonpregnant uterine horns on day 17 of pregnancy did not significantly differ in tissues from nonpregnant rats. However, the expression of iNOS mRNA in pregnant uterine horns was greater than those in uterine tissues from both non-pregnant animals and the nonpregnant uterine horns (Fig. 2).

Contractility studies in vitro

DEA/NO, UK-114542, and 8-br-cGMP concentration-dependently inhibited spontaneous contractile activity in the rings from the nonpregnant and pregnant uterine horns (Fig. 3). The maximal inhibitions induced by all three agents in pregnant uterine horns were

![Image](Fig 1.png)

Fig. 1. Representative reverse transcription-polymerase chain reaction (RT-PCR) products of iNOS mRNA in uterine tissues from nonpregnant (NP) and pregnant rats on day 10 (P10), 14 (P14), 17 (P17), and 21 (P21) of gestation. a: RT-PCR products of calmodulin (CaM) and iNOS mRNA in uterine tissues. b: Quantitative data based on densitometric scanning of iNOS mRNA. Each value represents the mean ± S.E.M. ($n = 4$). *$P < 0.05$, **$P < 0.01$ vs NP; †$P < 0.05$ vs P10.
similar. DEA/NO-induced maximal inhibition, but not pD_{2}, was significantly less in the rings from the non-pregnant versus pregnant uterine horns (Fig. 3, Table 1). Sensitivity to UK-114542 was higher in pregnant versus nonpregnant uterine horns with similar maximal effects (Fig. 3, Table 1). There was no difference in either sensitivity or maximal inhibition of uterine contractions induced by 8-br-cGMP in rings from pregnant versus nonpregnant horn (Fig. 3, Table 1).

Discussion

The data presented demonstrate that: 1) Expression of iNOS mRNA in rat myometrium increases with progression of gestation and decreases at term; 2) Expression of iNOS mRNA on day 17 of gestation is greater in pregnant versus non-pregnant uterine horn; 3) Inhibitor of phosphodiesterase V and agent mimicking increased level of cGMP induced similar inhibition of nonpregnant and pregnant uterine horn contractions. NO induced, similar to the above agents, inhibition of contractions in pregnant horn rings being less effective in rings from nonpregnant horns.

![Fig. 2](representative reverse transcription-polymerase chain reaction (RT-PCR) products of calmodulin (CaM) and iNOS mRNA in nonpregnant rat uterine horns, non-ligated (Lig (-)) and ligated (Lig (+)), and in pregnant uterine horns (PrUth) and nonpregnant uterine horns (nonPrUth) on day 17 of gestation. NP, non-pregnant; P17, day 17 of pregnancy.)

![Fig. 3](Concentration-inhibition relationships of (DEA/NO), UK-114542, and 8-br-cGMP in pregnant uterine horn rings (PrUth) and non pregnant uterine horn rings (nonPrUth) on day 17 of gestation. Abscissa, concentration of the agent (−log, M); Ordinate, percent of basal integral contractile activity. Each point represents the mean ± S.E.M. (n = 6–8).)

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<tr>
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<th>nonPrUth</th>
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<td><strong>pD\textsubscript{2}</strong></td>
<td><strong>Max</strong></td>
<td><strong>pD\textsubscript{2}</strong></td>
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<tr>
<td>DEA/NO</td>
<td>5.01 ± 0.16</td>
<td>34.9 ± 3.5</td>
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<tr>
<td>8-br-cGMP</td>
<td>5.41 ± 0.10</td>
<td>64.0 ± 4.8</td>
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<tr>
<td>UK-114542</td>
<td>4.60 ± 0.07</td>
<td>61.4 ± 7.9</td>
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Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01 vs nonPrUth, n = 6 – 8.
NO is produced by three NOS isoforms: endothelial NOS, iNOS, and neuronal NOS, which convert the amino acid L-arginine into L-citrulline and NO (6). Three types of NOS PCR products were detected in the myometrium, with the iNOS PCR product being the most prominent (7, 8). It is widely accepted that the increased production of NO by the pregnant myometrium serves to maintain uterine relative quiescence and the decrease of NO production close to term may be one of the trigger signals to start delivery activity (7, 8). The up-regulated expression of iNOS was demonstrated in myometrial smooth muscle of pregnant rats (7, 8), rabbits (17), guinea pigs (14), and humans (18), although the role of NO in human myometrium was questioned (19). In our experiments, expression of iNOS mRNA reached a maximum level on day 17 of gestation and was decreased on day 21 before the delivery, which confirms the previous data (7). Taken together, these studies support the hypothesis that endogenously produced NO via iNOS maintains myometrial relative quiescence during pregnancy.

The greater expression of iNOS mRNA in pregnant uterine horn than in non-pregnant uterine horn at day 17 of gestation may result from the presence of some local factors, namely, the factors from feto-placental units, since both uterine horns are under similar influences of circulating hormonal factors. These experiments do not specify the nature of the local factors, although it may possible be the placental factors or obvious an anatomical difference between the pregnant and non-pregnant uterine horns. The fact that the difference in iNOS mRNA expression between the two horns is prominent on day 17 of gestation suggests that the local placential hormonal factors may be of major importance. Mechanical stretching may not be the reason, since the iNOS mRNA expression decreases toward term, although pregnant uterus continue to increase in size from day 17 till term. In our experiments, expression of mRNA of adenylyl cyclase (AC) 2, but not AC4, AC6, and AC9, in pregnant uterine horn was significantly greater than in non-pregnant uterine horn on day 17 of gestation (9). Thus, gene expressions of AC4, AC6, and AC9 may be regulated by a systemic factors, whereas AC2 gene expression seems to be under control of local factors.

It has been demonstrated that there is greater mechanical activity and sensitivity to oxytocin in pregnant versus non-pregnant rabbit uterine horns that persisted for about one week after delivery, suggesting that the anatomical difference was of major importance, since the hormonal levels should have equalized by that time (10). Skinned myometrial fibers from pregnant horn demonstrated greater sensitivity to Ca\(^{2+}\) versus non-pregnant horn fibers on days 20 – 21 of gestation, suggesting the role local mechanical (distention) factors (11). Increased expression of oxytocin receptor mRNA, but not prostaglandin F\(_{2\alpha}\) receptor mRNA, during labor occurred in pregnant, but not in non-pregnant, uterine horn (20, 21) and the requirement of both hormonal and mechanical factors was postulated (20).

Inhibitory effect on uterine contractile activity of NO is accentuated during pregnancy and decreased toward term (2, 4, 6, 16). DEA/NO spontaneously releasing NO with an estimated half-life of about 2.1 min in solution with pH 7.4 at 37°C (22) relaxed vascular smooth muscle (8) and inhibited spontaneous contractions of myometrium during pregnancy, but not at term (2, 4, 23). The greater inhibitory effect of DEA/NO on spontaneous uterine contractions in pregnant uterine horn is associated with greater expression of iNOS mRNA in this experiments.

UK-114542, a potent reversible inhibitor of the cGMP-specific phosphodiesterase, phosphodiesterase V, an important modulator of smooth muscle relaxation (24), is similar to sildenafil that inhibited uterine contraction induced by oxytocin, acetylcholine, and prostaglandin E\(_2\) in non-pregnant rat uterine tissues (25). Similar to sildenafil, UK-363664 inhibited oxytocin-evoked contractions of preterm rat myometrium and this effect decreased at term (26), the effect being demonstrated by the selective cGMP phosphodiesterase inhibitor zaprinast (27). Sensitivity to UK-114542 was significantly higher in the uterine rings from pregnant versus non-pregnant horn with no difference in maximal effects in our study. In vascular smooth muscle, the relaxation induced by sildenafil, similar to UK-114542, at 10\(^{-7}\) M or less were suggested to be attributable to their inhibitory effects on phosphodiesterase V in isolated tissues (28). Sildenafil at 10\(^{-7}\) M or more caused further relaxation in the isolated rat aorta. However, it is suggested that this relaxation was independent of the inhibition of phosphodiesterase V (28). On the other hand, the less sensitivity of uterine versus vascular smooth muscle to NO and differential selectivity of NO for inducing relaxation between vascular and non-vascular smooth muscle suggests the differential pharmacological properties of NO (2, 29). Therefore, in the uterine tissue, it has yet not been determined if this relaxation by UK-114542 at high concentrations is dependent or independent of the inhibition of selective phosphodiesterase V. The membrane-permeable analog of cGMP, 8-br-cGMP, inhibited contractile activity of rings from both pregnant and non-pregnant uterine horns with no differences in either pD\(_2\)’s or maximal response, which is consistent with published data (21). Thus, inhibitory effects of UK-114542 and 8-br-cGMP...
on uterine spontaneous contractions does not depend on iNOS mRNA expression/pregnancy state, while inhibition induced by NO depends on it.

In conclusion, we confirm the existence of the NO-sGC-cGMP pathway in rat uterine smooth muscle. The pathway is activated at pregnancy; it is activated in pregnant, but not in nonpregnant horn. Some local (placental) pregnancy factor is necessary for signal transduction during activation of soluble guanylate cyclase by NO.

References