

# Increased Nitric Oxide Synthase Activity and Expression in the Human Uterine Artery During Pregnancy

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**Abstract**—Evidence exists that NO plays a role in the vasodilation that occurs during pregnancy. The purpose of the present study was to determine whether the role of NO is associated with an increase in the activity and protein expression of NO synthase (NOS) in the human uterine artery. Uterine arteries were obtained from pregnant patients (P arteries) and nonpregnant patients (NP arteries). NOS activity was estimated with the L-[<sup>3</sup>H]-arginine-to-L-[<sup>3</sup>H]-citrulline conversion method and on the basis of changes in tissue levels of cGMP. Western immunoblotting and immunohistochemistry were used to assess NOS protein expression. Ca<sup>2+</sup>-dependent NOS activity was 8 times greater ( $P < 0.01$ ) in P than in NP arteries. Although most of this pregnancy-induced increase in NOS activity was Ca<sup>2+</sup> dependent (64%), a considerable portion was Ca<sup>2+</sup> independent. Expressions of endothelial NOS (eNOS) and neuronal NOS, but not inducible NOS, were demonstrated in P and NP arteries. The eNOS was located in the endothelium and stained with a qualitative order of P arteries > NP arteries (follicular) > NP arteries (luteal). The neuronal NOS was located in the adventitia of P and NP arteries. Basal NO-dependent and bradykinin-stimulated levels of cGMP were higher ( $P < 0.05$ ) in P than in NP arteries. These results indicate that an upregulation of eNOS protein expression could account for the increased NO synthesis/release in the human uterine artery during pregnancy. (*Circ Res.* 2000;87:406-411.)

**Key Words:** cyclic GMP ■ bradykinin ■ immunohistochemistry ■ N<sup>ω</sup>-nitro-L-arginine methyl ester  
■ Ca<sup>2+</sup> ■ nitric oxide synthase

Normal pregnancy is associated with an increase in uterine blood flow and a decrease in uterine vascular resistance.<sup>1-4</sup> The low resistance is attributed to a loss of smooth muscle in myometrial resistance vessels (spiral arteries and terminations of radial arteries) as well as to dilation of the larger uterine arteries.<sup>5</sup> The dilation of the uterine arteries could be due to an increased role of endogenous vasodilators.

Considerable evidence indicates that NO plays a role in pregnancy-induced uterine vasodilation. We have previously reported that acetylcholine is more potent and efficacious in producing dilation of isolated uterine arteries from pregnant than from nonpregnant patients.<sup>6,7</sup> The acetylcholine-induced relaxation was blocked by NO synthase (NOS) inhibitors and thus is apparently mediated by NO.<sup>6,7</sup> Furthermore, pregnancy-induced increases in basal NO production have been found in the uterine vasculature of rats,<sup>8,9</sup> guinea pigs,<sup>10,11</sup> and sheep.<sup>3,12,13</sup>

NO is produced by NOS, of which 3 isoforms have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).<sup>14</sup> The NOS isoforms share a common overall catalytic scheme for the oxidation of L-arginine to NO and L-citrulline but can be divided into 2 functional classes based on the dependence of Ca<sup>2+</sup> for

activity.<sup>14</sup> The constitutive forms, eNOS and nNOS, require Ca<sup>2+</sup> for activity, but the inducible isoform, iNOS, has a Ca<sup>2+</sup>-independent activity. Ca<sup>2+</sup>-independent activity for eNOS also has been reported.<sup>15-18</sup>

In the present study, we tested the hypothesis that the greater NO-mediated relaxation of the human uterine artery during pregnancy is associated with increases in the activity and protein expression of the constitutive eNOS. The specific objectives of the study were to determine (1) whether NOS activity is greater in uterine arteries from pregnant patients than in arteries from nonpregnant patients, (2) the extent to which the NOS activity is Ca<sup>2+</sup> dependent, (3) whether the changes in NOS activity are associated with an increase in eNOS protein expression, (4) whether the enhanced NOS protein expression is located in the endothelium, the nerves, or the vascular smooth muscle cells, and (5) whether the changes in NOS activity and protein expression observed in pregnancy are associated with elevated levels of cGMP (ie, the secondary mediator for NO actions).

## Materials and Methods

### Procurement of Arteries

Specimens of the ascending branch of the uterine artery were obtained from multiparous patients who were undergoing hysterectomy.

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tomy for various medical reasons. The use of uterine arteries obtained from patients after hysterectomy was approved by the institutional review boards for The University of Texas Medical Branch (Galveston, Tex) and Baylor College of Medicine (Houston, Tex), and the procedures followed were in accordance with institutional guidelines. Uterine arteries were obtained from 15 pregnant patients and 30 nonpregnant patients (the arteries are referred to as P and NP arteries, respectively). The mean ages of the pregnant and nonpregnant patients were  $32.3 \pm 1.5$  and  $39.6 \pm 1.2$  years, respectively. The mean arterial blood pressure of the pregnant patients ( $83.1 \pm 3.2$  mm Hg) was significantly ( $P < 0.05$ ) lower than that of the nonpregnant patients ( $91.6 \pm 3.5$  mm Hg). None of the pregnant patients were diagnosed with hypertension or preeclampsia. The pregnant patients were near-term gestation ( $37.2 \pm 0.6$  weeks). Reasons for the cesarean/hysterectomy included fibroids, repeat cesarean section, abnormal placentation (placenta accreta, placenta previa), and breech position. The patients delivered healthy babies with no apparent growth retardation. The nonpregnant patients were in different phases of the menstrual cycle. The phase (follicular or luteal) of the cycle was determined by the date of the last menstrual period. Unfortunately, many of the patients' charts did not contain such information. Reasons for the hysterectomy included fibroids, menorrhagia, dysmenorrhea, pelvic pain, leiomyomata, severe pelvic adhesions, and enlarged uterus. According to the patients' charts, most of the pregnant and nonpregnant patients were not on medications. None of the patients included in the present study were on estrogen or progesterone therapy. A few patients had taken ibuprofen or aspirin. The patients received diazepam or midazolam, morphine sulfate, and atropine, scopolamine, or glycopyrrolate for preanesthetic medication. The pregnant patients also received an antacid and ranitidine for aspiration prophylaxis. Anesthesia, which was similar in both pregnant and nonpregnant patients, was induced with sodium thiopental and maintained with nitrous oxide/oxygen and either isoflurane or fentanyl.

### Tissue Dissection

Sections of the uterus that contained the ascending branch of the uterine artery were placed in cold ( $2^{\circ}\text{C}$ ) Krebs-bicarbonate solution equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  immediately after hysterectomy and before being taken to the laboratory, where the arteries were dissected from the surrounding tissue. In preliminary studies with the NP arteries, there was no loss of NOS activity during the first 180 minutes after hysterectomy if the tissues were kept in cold ( $2^{\circ}\text{C}$ ) Krebs' solution.

### NOS Activity

Evaluation of the NOS activity in human uterine arteries was made by measuring [ $^3\text{H}$ ]-arginine conversion to [ $^3\text{H}$ ]-citrulline. The method for NOS activity was a modified method of Magness et al<sup>3</sup> and Conrad et al.<sup>8</sup> Within 90 minutes of surgical removal of the uterus, the arteries were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until NOS activity was measured. The tissues were stored at this temperature for no more than 2 weeks. The tissues were homogenized with an Ultra-Turrax T25 homogenizer (IKA Works) in a 1:10 dilution of ice-cold homogenizing buffer, which contains (in mmol/L) sucrose 50, HEPES 25, DL-dithiothreitol 1, leupeptin 10, pepstatin A 10, chymostatin 10, antipain 10, soybean trypsin inhibitor 10, and phenylmethylsulfonyl fluoride 100.

The samples were centrifuged at 1000g for 15 minutes at  $4^{\circ}\text{C}$ . Dowex (50W-X8 200-400 mesh; Bio-Rad) cation exchange resin (200 mg) was added to 2 mL of the homogenate to eliminate endogenous L-arginine. After centrifugation, the supernatant (100  $\mu\text{L}$ ) was added to incubation buffer (100  $\mu\text{L}$ ), which contained 20 mmol/L flavin adenine dinucleotide, 4  $\mu\text{mol/L}$  tetrahydrobiopterin, 20 U/mL calmodulin, 40  $\mu\text{mol/L}$  L-arginine, 0.625 mCi/L L-[ $^3\text{H}$ ]-arginine (Amersham), 1 mmol/L NADPH, 50 mmol/L sucrose, 25 mmol/L HEPES, and 1 mmol/L  $\text{CaCl}_2$ . To determine the degree of  $\text{Ca}^{2+}$  dependence of this NOS activity,  $\text{Ca}^{2+}$  was omitted and 2 mmol/L EGTA was added to chelate any remaining  $\text{Ca}^{2+}$ . After a 30-minute incubation period at  $25^{\circ}\text{C}$ , 1.3 mL of "stop buffer" (80 mmol/L HEPES, 8 mmol/L EDTA) was added. The [ $^3\text{H}$ ]-

citrulline produced by the enzyme reaction was separated from [ $^3\text{H}$ ]-arginine by the addition of 1 mL of Dowex (50W-X8 200-400 mesh) and water mixture (1:1) and subsequently centrifuged at 200g for 15 minutes. Supernatant (0.5 mL) was removed, mixed with 4.5 mL of scintillation fluid, and counted on a Beckman LS 3801 scintillation counter. The values of samples heated at  $65^{\circ}\text{C}$  for 30 minutes to inactivate enzymes were subtracted from total counts to reflect apparent NOS activity. The NOS inhibitors *N*<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME) and nitroarginine were added to aliquots of homogenate to inhibit the conversion of L-arginine to L-citrulline. The amount of arginine metabolites reduced by the NOS inhibitors reflects the NOS activity (NO and citrulline production). The protein concentration was determined according to the Bradford method. The NOS activity was expressed in picomoles of arginine metabolites produced per milligram of protein per minute.

### Western Analysis

The method of Dong et al<sup>19</sup> was used for the analysis. Human uterine arteries ( $\approx 100$  mg) were homogenized in 50 mmol Tris/L buffer (pH 7.4) containing 0.1 mmol EGTA/L, 0.14 mL  $\beta$ -mercaptoethanol/mL, 100 mmol phenylmethylsulfonyl fluoride/L, and 0.2 mg trypsin inhibitor/mL and centrifuged. The pellet (membranous fraction) was resuspended in buffer. The proteins in all subcellular fractions were measured with the BCA kit (Pierce). Positive control proteins were obtained from the membrane fractions of human umbilical endothelial cells (eNOS) and the cytosolic fractions of rat cerebellum (nNOS) and cytokine-stimulated RAW 264.7 cells (iNOS). The latter 2 positive controls were cross-reactive with human tissue (Transduction Laboratories). In each blot, 1 lane was loaded with protein from an appropriate positive control.

Equal amounts of protein were size fractionated on 7.5% (wt/vol) SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blots were then incubated with specific primary antibodies (monoclonal; Transduction Laboratories) for 1 hour at room temperature. The final dilutions were 1:1000 for nNOS, 1:1000 for iNOS, and 1:500 for eNOS (vol/vol) in the blocking buffer. After the blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody, the membranes were washed and the enhanced chemiluminescence reagent (ECL kit; Amersham) was added and incubated for 1 minute at room temperature. The blots were exposed to autoradiographic film, and the intensity of specific immunoreaction bands was quantified through densitometric scanning. The data are expressed as densitometric units. Both the elimination of the primary antibody and the use of non-NOS-related monoclonal antibody indicated the specificity of the NOS protein bands at appropriate molecular sizes (data not shown).

### Immunohistochemistry

Uterine arterial segments were fixed in 10% formalin in phosphate buffer, embedded in paraffin, and cross-sectioned at 6  $\mu\text{m}$ . Sections from pregnant and nonpregnant patients were immunostained at the same time and under similar conditions so a direct comparison could be made among the groups. After deparaffinization, sections were rehydrated in graded ethanol and then incubated in 3%  $\text{H}_2\text{O}_2$  in absolute methanol for 10 minutes to quench endogenous peroxidase activity. To eliminate nonspecific background, sections were incubated in a nonimmune serum (normal goat serum). The localization of eNOS, nNOS, and iNOS was attempted by using isoform-specific antibodies (Alexis Biochemicals) and indirect immunoperoxidase detection via the Labeled-[strept] Avidin-Biotin method (Histostain SP-Broad Spectrum/AEC Kit; Zymed). Sections were incubated with eNOS (bovine monoclonal antibody, 1:250), nNOS (rat monoclonal antibody, 1:1000), or iNOS (rat macrophage monoclonal antibody, 1:500) for 1 hour in a moist chamber at room temperature. All of the antibodies were cross-reactive with the respective human NOS. Optimal concentrations of the primary antibodies were determined based on preliminary studies. For a negative control, the primary antibody was replaced with normal mouse or rabbit IgG (Vector). Sections were then rinsed for 15 minutes in 10 mmol/L PBS, incubated for 10 minutes at room temperature with a biotinylated

secondary antibody (Histostain SP Kit), washed in PBS, and incubated for 10 minutes with a streptavidin–horseradish peroxidase complex. Sections were washed in PBS and incubated in substrate (hydrogen peroxide–chromogen [amino-ethylcarbazol] mixture). Sections were counterstained with hematoxylin and mounted. The sections were examined by 4 investigators who were blinded to the identity of the tissue. The presence of a brownish-red stain was qualitatively assessed with a scale of 0 to 4 (no staining to heavy staining) at 3 locations: endothelium, media, and adventitia.

### Basal Levels of cGMP Production

Duplicate uterine arterial segments (10 to 30 mg) from pregnant and nonpregnant patients were incubated for 60 minutes in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> in Krebs' buffer at 37°C. Endothelium was removed from some of the segments before incubation. Segments were changed to tubes containing Krebs' solution (for basal levels) or Krebs' solution with L-NAME (0.1 mmol/L) and incubated for 30 minutes or Krebs' solution with bradykinin (1 μmol/L) added for the final 10 minutes. The studies were not conducted in the presence of a phosphodiesterase inhibitor such as 3-isobutyl-1-methylxanthine because of the interest in determining the levels of cGMP that existed during various experimental conditions in earlier studies on pregnancy-induced changes in constrictor functions of the uterine artery.<sup>6,7</sup> At the end of the incubation period, segments were quickly frozen in liquid nitrogen, immersed in ice-cold 1% trichloroacetic acid, homogenized, and centrifuged. The supernatant was frozen (−70°C) until assayed for cGMP with a radioimmunoassay kit (cGMP [<sup>125</sup>I] assay system with Amerlex-M magnetic separation; Amersham International). The results are reported in femtomoles of cGMP per milligram protein.

### Endothelium Removal

To evaluate the role of endothelium on NO-mediated cGMP production in the vascular smooth muscle, the endothelium was removed from randomly selected arterial rings by gently rubbing the endothelial layer with tungsten wire (0.38 mm) for 1 minute. Control arterial rings from the same patient were studied in parallel. Confirmation of endothelium removal by this method has been reported with histological and physiological studies on human uterine arteries.<sup>6</sup>

### Statistical Analysis

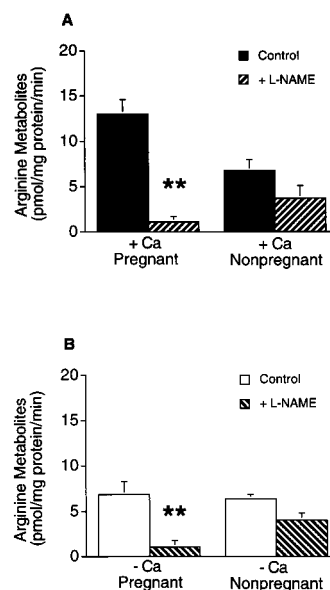
Data were analyzed using the Mann-Whitney test or Student's *t* test, as appropriate. Data presented are mean ± SEM. Results were considered significant at the *P* < 0.05 level.

## Results

### NOS Activity

The rate of formation of arginine metabolites in the presence and absence of Ca<sup>2+</sup> was determined in uterine arteries from pregnant patients (P arteries) and from nonpregnant patients (NP arteries). In the presence of Ca<sup>2+</sup>, the total arginine metabolite formation was significantly greater (*P* < 0.003) in P arteries than in NP arteries. In Ca<sup>2+</sup>-free incubation medium containing EGTA, the rate of formation of arginine metabolites was not significantly different between P and NP arteries. The difference between activity in the presence and absence of Ca<sup>2+</sup>, reflecting Ca<sup>2+</sup>-dependent enzyme activity, was 8.6 ± 0.8 pmol · mg protein<sup>−1</sup> · min<sup>−1</sup> (*P* < 0.01) in the P arteries and 1.7 ± 0.9 pmol · mg protein<sup>−1</sup> · min<sup>−1</sup> (*P* = NS) in the NP arteries. Thus, the rate of Ca<sup>2+</sup>-dependent formation of arginine metabolites was much greater (*P* < 0.003) in the P arteries than in the NP arteries.

L-NAME (0.4 mmol/L), in the presence of Ca<sup>2+</sup> (Figure 1A), decreased the production of total arginine metabolites by 91.9 ± 8.6% (*n* = 7) in paired P arteries and by 45.8 ± 6.7%

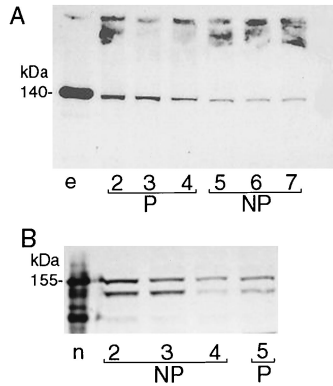


**Figure 1.** Rate of total arginine metabolite formation in the absence and presence of the NOS inhibitor L-NAME (0.4 mmol/L) in uterine arteries from pregnant patients and nonpregnant patients. A, In the presence of Ca<sup>2+</sup> (+Ca), the difference between the total arginine metabolite formation in the absence and presence of L-NAME represents total NOS activity. Vertical bars represent SEM of metabolites in 7 paired P arteries and 7 paired NP arteries. \*\*Significantly greater NOS activity in the P arteries than in the NP arteries (*P* < 0.01). B, In a Ca<sup>2+</sup>-free medium (with EGTA), the difference between the arginine metabolite formation in the absence and presence of L-NAME represents Ca<sup>2+</sup>-independent NOS activity. Vertical bars represent SEM of arginine metabolites in the absence and presence of L-NAME in 7 paired P arteries and 7 paired NP arteries. \*\*Significantly greater Ca<sup>2+</sup>-independent NOS activity in the P arteries than in the NP arteries (*P* < 0.01).

(*n* = 7) in paired NP arteries (*P* < 0.01). Higher concentrations (1 and 4 mmol/L) of L-NAME did not produce any greater inhibition of arginine metabolite formation. Another NOS inhibitor, nitroarginine (0.4 mmol/L), inhibited the total arginine metabolite formation to a similar extent as that of L-NAME in P arteries (*n* = 3) and NP arteries (*n* = 3). The total NOS activity expressed as the difference between the amount of arginine metabolites formed in the absence and presence of L-NAME was 11.7 ± 1.8 (*n* = 7) and 3.1 ± 0.9 (*n* = 7) pmol · mg protein<sup>−1</sup> · min<sup>−1</sup> (*P* < 0.01) in P and NP arteries, respectively. Thus, the total NOS activity was 3 times greater in the P arteries than in the NP arteries.

In Ca<sup>2+</sup>-free medium L-NAME (0.4 mmol/L) decreased the formation of arginine metabolites by 84.0 ± 6.9% (*n* = 7) in paired P arteries and by 36.3 ± 5.0% (*n* = 7) in paired NP arteries (Figure 1B). The NOS activity in a Ca<sup>2+</sup>-free incubation medium, expressed as the difference between the amount of arginine metabolites formed in the absence and presence of L-NAME, was 5.9 ± 0.5 (*n* = 7) and 2.4 ± 0.6 (*n* = 7) pmol · mg protein<sup>−1</sup> · min<sup>−1</sup> (*P* < 0.01), respectively, in P and NP arteries (Figure 1B). The Ca<sup>2+</sup>-independent NOS activity was significantly greater (*P* < 0.01) in the P arteries than in the NP arteries. Furthermore, the Ca<sup>2+</sup>-dependent NOS activity, expressed as the difference between the NOS activity in the presence and absence of Ca<sup>2+</sup>, was significantly greater (*P* < 0.01) in the P arteries than in the NP arteries.





**Figure 2.** Examples of Western immunoblots of NOS protein expression in uterine arteries from pregnant (P) and nonpregnant (NP) patients. Each lane number represents a different patient. A, Expression of eNOS. The positive control was human umbilical vein endothelial cells (e) (30  $\mu$ g). The monoclonal antibody against eNOS reacted with a protein band at 140 kDa. B, Expression of nNOS. The positive control was rat cerebellum (n) (40  $\mu$ g). The monoclonal antibody against nNOS reacted with the protein band at 155 kDa.

**NOS Expression**

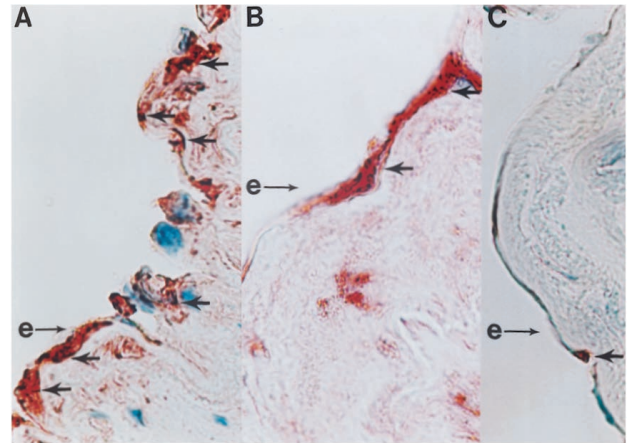
Figure 2A depicts representative Western immunoblots of eNOS protein expression in uterine arteries from 3 pregnant and 3 nonpregnant patients. A single band at 140 kDa was detectable with both P and NP arteries and was similar in size to the band with human umbilical vein endothelial cell protein standard. The expression of eNOS protein was consistently greater in the P than in the NP arteries. Densitometry of the bands shows that the eNOS expression was 1.5 times greater ( $P < 0.05$ ) in the P arteries ( $1.3 \pm 0.2$  densitometric units,  $n = 6$ ) than in the NP arteries ( $0.8 \pm 0.1$  densitometric unit,  $n = 7$ ).

In early experiments, the expression of nNOS was demonstrated in 3 of 5 P arteries and in 3 of 6 NP arteries. In subsequent studies, when care was taken to prevent damage to the adventitia during dissection, nNOS was demonstrated in all of the uterine arteries (1 P artery and 3 NP arteries, Figure 2B). Of the arteries that demonstrated nNOS expression, there was no apparent difference in the relative units of nNOS expression in the P arteries ( $1.2 \pm 0.4$  densitometric units,  $n = 4$ ) and NP arteries ( $1.9 \pm 0.7$  densitometric,  $n = 6$ ).

No iNOS expression was detected in either the P or NP arteries. It should be noted that the human uterine artery appears to have the capacity for iNOS induction, because iNOS expression could be demonstrated when NP arteries ( $n = 3$ ) were exposed to 100 U interleukin-1B (results not shown).

**NOS Localization**

Sections of 5 P arteries and 7 NP arteries were examined for isoform specific staining of NOS. Of the NP arteries, 4 patients were in the follicular phase and 3 patients were in the luteal phase of the menstrual cycle. In the P arteries, there was consistently heavy staining of eNOS in the endothelium (Figure 3A). In the NP arteries, the staining varied from moderate in those from patients in the follicular phase (Figure 3B) to slight in those from patients in the luteal phase (Figure 3C). Thus, qualitatively, the staining of eNOS appeared to be



**Figure 3.** Examples of immunohistochemical localization of eNOS in endothelium of uterine arteries from a pregnant patient (A), a nonpregnant (follicular) patient (B), and a nonpregnant (luteal) patient (C). IgG served as a negative control for each section (not shown). Note that the section of the uterine artery from a patient in the follicular phase of the menstrual cycle appeared to have greater immunostaining than the section from a patient in the luteal phase. Arrows point to examples of positive staining for eNOS in the endothelium (e). Magnification  $\times 1000$ .

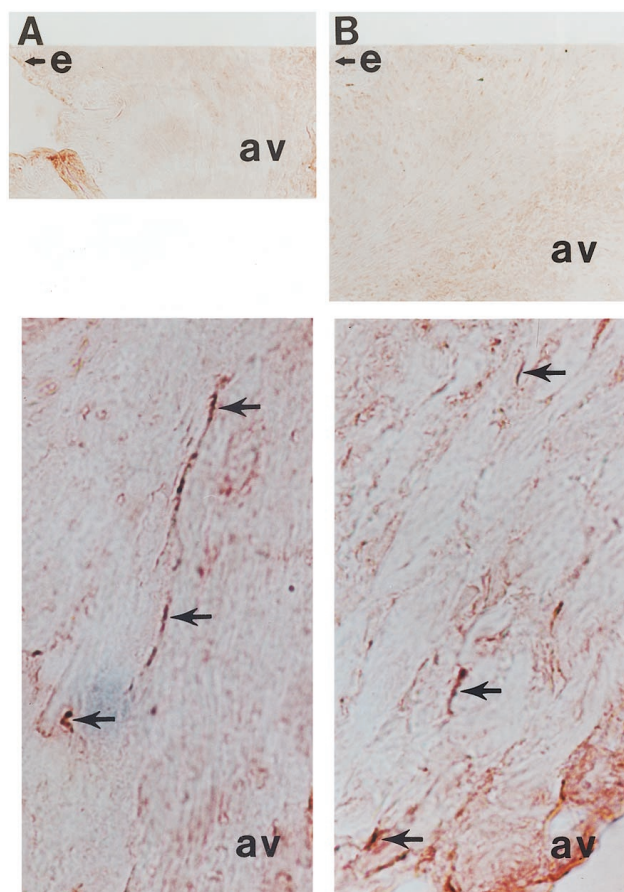
in the order of P arteries > NP (follicular) arteries > NP (luteal) arteries. There was slight but detectable staining for nNOS in the adventitia (Figure 4, top and bottom), but not the endothelium or smooth muscle (Figure 4, top), of all of the arteries examined. In contrast to the heavy staining for eNOS, the staining of nNOS appeared to be similar in the P arteries (Figure 4A) and NP arteries (Figure 4B). None of the arteries exhibited any detectable staining of iNOS in the vascular smooth muscle or the endothelium. Also, there was no positive staining in the negative control sections exposed to mouse or rabbit IgG instead of the primary antibodies (results not shown).

**cGMP Levels**

Basal levels of cGMP in uterine arteries with endothelium were 1.7 times higher in the P arteries than in the NP arteries (Table). L-NAME (0.1 mmol/L) reduced the basal content of cGMP by  $70.7 \pm 3.0\%$  in the P arteries ( $n = 7$ ) and by  $22.7 \pm 5.2\%$  in the NP arteries ( $n = 7$ ) ( $P < 0.01$ ). After removal of the endothelium, the basal content of cGMP was reduced by  $43.2 \pm 5.0\%$  in the P arteries ( $n = 5$ ) ( $P < 0.01$ ) and by  $27 \pm 6.9\%$  in the NP arteries ( $n = 6$ ) ( $P < 0.05$ ) (Table). Bradykinin (1  $\mu$ mol/L), a receptor-dependent activator of eNOS, increased the levels of cGMP by  $118.5 \pm 16.5\%$  in the P arteries ( $n = 5$ ) and by  $68.0 \pm 8.3\%$  in the NP arteries ( $n = 6$ ) ( $P < 0.05$ ) (Table). To determine whether the incubation of the human uterine artery resulted in an induction of iNOS, experiments with the NP arteries ( $n = 8$ ) were conducted in the absence and presence of dexamethasone (10  $\mu$ mol/L), an inhibitor of induction of iNOS. The results showed that the presence of dexamethasone did not alter the basal cGMP levels (data not shown).

**Discussion**

The results of the present study show that in the human uterine artery, there is a pregnancy-associated increase in



**Figure 4.** Examples of immunohistochemical localization of nNOS in the adventitia of uterine arteries from a pregnant patient (A) and a nonpregnant patient (B). Top, Cross sections of uterine artery at a magnification of  $\times 100$ . The endothelium (e) is indicated by arrow. Bottom, Adventitia (av) at a magnification of  $\times 1000$ . Arrows point to examples of positive staining for nNOS. IgG served as a negative control for each section (not shown).

$\text{Ca}^{2+}$ -dependent NOS activity, eNOS protein expression, and NO-dependent cGMP levels. These findings are in agreement with the results of our functional studies that show that in the human uterine artery, pregnancy causes an increase in the NO-dependent relaxation responses to acetylcholine or periaxillary nerve stimulation.<sup>6,7</sup> Furthermore, the results are consistent with the findings that pregnancy increases  $\text{Ca}^{2+}$ -dependent NOS activity and eNOS protein expression in the uterine artery of sheep<sup>13</sup> and in  $\text{Ca}^{2+}$ -dependent NOS activity in the uterine artery of the guinea pig.<sup>11</sup>

The following results indicate that eNOS accounts for most of the pregnancy-associated increase in NO production. First, the  $\text{Ca}^{2+}$ -dependent NOS activity, indicating involvement of the constitutive isoforms of NOS (eNOS, nNOS), was  $\approx 8$  times greater in the P arteries than in the NP arteries. Second, the protein expression of eNOS was greater in the P arteries than in the NP arteries (Figure 2A). Third, immunohistochemical staining with eNOS-specific antibody revealed a qualitatively greater staining in the endothelium of the P arteries than of the NP arteries (Figure 3). Fourth, bradykinin, known to increase cGMP levels by releasing NO from the endothelium, produced a much greater increase in cGMP

#### Effects of L-NAME and Endothelium Removal on Basal Levels of cGMP (fmol/mg protein) and of Bradykinin Stimulation of cGMP in Uterine Arteries From Pregnant and Nonpregnant Patients

Condition	Pregnant Control Subjects	Nonpregnant Patients
Control	45.4 $\pm$ 9.4 (7)*	26.4 $\pm$ 3.2 (7)
L-NAME	13.3 $\pm$ 4.7†	20.4 $\pm$ 2.4
Control	42.7 $\pm$ 9.7 (5)*	22.2 $\pm$ 3.3 (6)
- Endothelium	24.3 $\pm$ 3.3‡	15.7 $\pm$ 2.4§
+ Bradykinin	93.3 $\pm$ 5.4	37.3 $\pm$ 3.1¶

Values represent mean $\pm$ SEM. Numbers in parentheses represent number of patients. Control values represent basal levels of cGMP.

\* $P < 0.05$ , statistically significant difference between pregnant and nonpregnant controls.

† $P < 0.01$ , significant difference between control and L-NAME-treated paired P arteries.

‡ $P < 0.01$ , significant difference between control and paired P arteries without endothelium.

§ $P < 0.05$ , significant difference between control and paired NP arteries without endothelium.

|| $P < 0.05$ , significant difference between control (unstimulated) and bradykinin-stimulated paired P arteries.

¶ $P < 0.05$ , significant difference between control (unstimulated) and bradykinin-stimulated paired NP arteries.

levels in the P arteries than in the NP arteries. Thus, it can be reasonably concluded that the pregnancy-enhanced NOS activity in uterine arteries reflects mainly an increased eNOS activity.

Both the P and NP arteries exhibited staining for nNOS. The staining appeared to be located in nerve fibers mainly in the adventitia (Figure 4). The presence of nNOS in the neuronal innervation is consistent with our previously reported findings that the response of the human uterine artery to periaxillary nerve stimulation contains a NO-mediated vasodilator component.<sup>6</sup> Similarly, Morris<sup>20</sup> reported that the uterine arteries of guinea pigs exhibited a vasoconstrictor and a vasodilator response to nerve stimulation. The vasodilator response was partially blocked by a NOS inhibitor. On the other hand, Magness et al<sup>13</sup> did not find nNOS staining in the ovine uterine artery.

Western analysis demonstrated nNOS expression in the P and NP arteries. That nNOS protein expression was not greater in P arteries suggests that the increase in the  $\text{Ca}^{2+}$ -dependent NOS activity (Figure 1A) was due to eNOS, not nNOS. The protein expression is in accord with the findings that the human uterine arteries consistently exhibited both positive immunostaining for nNOS (present study) and NO-mediated neurogenic vasodilatation.<sup>6</sup>

The iNOS isoform of NOS in the P and NP arteries was not detectable by Western analysis or immunohistological staining with a monoclonal antibody for iNOS that is cross-reactive with human tissue. Magness et al<sup>13</sup> concluded that there also was little or no iNOS activity in the ovine uterine artery. Their conclusion was based in part on the finding that the NOS activity in the ovine uterine artery was largely  $\text{Ca}^{2+}$ -dependent. Interestingly, in the present study we found ample  $\text{Ca}^{2+}$ -independent NOS activity, especially in the P arteries (Figure 1B). However, the fact that there is  $\text{Ca}^{2+}$ -independent

NOS activity despite the lack of any detectable iNOS suggests the existence of a  $\text{Ca}^{2+}$ -independent mechanism for activation of the constitutive isoforms of NOS in the human uterine artery. In support of this suggestion, there is evidence that shows a  $\text{Ca}^{2+}$ -independent component in the signal transduction pathway for activation of eNOS.<sup>15–18</sup>

In the present experiments, we expressed the NOS activity as the difference between the amount of arginine metabolites produced in the absence and presence of maximal inhibitory concentrations of L-NAME. Surprisingly, the amount of other arginine metabolites (eg, ornithine, arginosuccinate) that could possibly be produced in the presence of the NOS inhibitors was consistently and significantly greater in the NP arteries than in the P arteries (Figure 1). We have no explanation for this finding.

As noted in Materials and Methods, many of the uterine arteries were obtained without having available a clear indication of the phase of the menstrual cycle at the time of surgery. However, such information was available for the arteries used in the staining experiments that qualitatively demonstrated greater staining for eNOS in the arteries obtained from patients in the follicular phase than from those in the luteal phase. Although these results are qualitative, they are included in light of the controversy regarding whether NOS is modulated by estrogen in normally cycling women. These results are consistent with previous studies that show an increased responsiveness to acetylcholine in uterine arteries removed from patients with high plasma levels of estrogen during the follicular phase of the menstrual cycle.<sup>21</sup> Also, we previously reported that the acetylcholine-induced NO/cGMP-dependent vasodilatation of the human uterine artery was greater in the follicular phase than in the luteal phase.<sup>22</sup> These results are in agreement with a growing body of evidence that indicates a role for estrogen in the upregulation of NOS activity in the uterine vasculature.<sup>4,23,24</sup>

In summary, the present study demonstrates that pregnancy is associated with increased eNOS levels in the human uterine artery. In both the P and NP arteries, eNOS was primarily located in the endothelium and nNOS was detected mainly in the adventitia. No detectable iNOS was found in either the P or NP arteries. The  $\text{Ca}^{2+}$ -dependent NOS activity was 8 times greater in the P arteries than in the NP arteries. Although most of this pregnancy-induced increase in activity was  $\text{Ca}^{2+}$  dependent, a significant portion was clearly independent of  $\text{Ca}^{2+}$ . The NO-mediated basal and bradykinin-stimulated levels of cGMP were markedly greater in the P than the NP arteries. These results, together with our previously reported functional studies that show that pregnancy causes increased NO-dependent relaxation responses to acetylcholine or periarterial nerve stimulation, constitute strong evidence for a prominent role of NO in the maintenance of human uterine vasodilation during pregnancy.

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