Antiproliferative Effects of Inducible Nitric Oxide Synthase Inhibition on Decidualization in Pseudopregnant Rats (44266)

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Abstract. **The aim of this study was to assess the involvement in decidual proliferation of nitric oxide (NO), a regulator of many cellular processes, that is synthesized from L-arginine by NO synthase. The investigation was conducted on pseudopregnant (PG) rats in which the decidual cell reaction, the basis for the decidualization process, was surgically induced by uterine trauma on PG Day 4. Groups of animals (n = 5) were pretreated with either 2 doses/day of N^G-nitro-L-arginine methyl ester (L-NAME) that inhibits NO synthase, or twice daily doses of L-NAME plus L-arginine combined. Drug application times coincided with 3 hr after lights on or 3 hr before lights off. The two treatment regimens (PG Days 1–4 or 5–8) respectively preceded or followed decidual induction. Animals were sacrificed at mid-light on PG Day 9, the day of maximal growth response to the deciduogenic stimulus. Parallel, time-dependent increases in both NO synthase activity and decidual growth occurred mainly in the endometrium. L-NAME produced reductions in endometrial and myometrial growth that were reversed by the combined L-NAME plus L-arginine treatments. These inhibitory effects by L-NAME were caused by only the pretraumal (PG Days 1–4) administration. Hormonally, circulating progesterone levels were similarly affected by this early treatment and may also contribute to the reduced decidual sensitivity. In contrast, serum estradiol, along with the zinc metalloenzymes, alkaline phosphatase and the matrix metalloproteinases—prominent decidualization biomarkers—were all unaffected by either the pre- or post-decidual induction dosings. The study demonstrates that inducible NO synthase/endogenous NO may physiologically participate in uterine metabolism during the decidual cell reaction. Moreover, by virtue of L-NAME inhibition of the decidual response, it appears that NO synthase/NO may influence decidual growth either by directly increasing uterine sensitivity to the deciduogenic stimulus or by indirectly affecting endometrial vascularity and subsequent availability of decidual metabolites.** [P.S.E.B.M. 1998, Vol 218]

The decidual cell reaction (DCR), a feature of rats, mice, and other mammals, is manifested under normal circumstances as a response to the implanting blastocyst. The response can also be artifically induced on Day 4 of pseudopregnancy (PG) in rats (1). In either case, it is characterized by uterine hypertrophy, hyperplasia, and differentiation of the uterine stromal cells (2). When induced (i.e., by surgical uterine trauma in rats) pseudopregnancy is prolonged into decidualization. So essentially the process mimics gestation but without fetoplacental formation. However, a maternal placenta or deciduoma develops and attains maximal growth in rats and mice, ranging from (PG) Day 9 for the antimesometrial cells to Days 11–13 for the mesometrial cells (3). Physiologically, the reaction depends on early estrogen/progesterone priming followed by obligatory progesterone support (4, 5).

Nitric oxide (NO), a recently acclaimed neurotransmitter, is synthesized by NO synthase from L-arginine (6, 7). The enzyme exists in two isoforms: one is constitutive cal-

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cium/calmodulin-dependent, and the other is inducible calcium-independent. Hormonally, an L-arginine-NO system was shown to be localized in the rat uterus where L-arginine responses were increased by progesterone (8). Additionally, NO was able to function as a neuroactive agent in stimulating hypothalamic gonadotropin releasing hormone secretion and in affecting reproductive function (9, 10). Other studies on rats demonstrated that the biosynthesis of calcium-independent NO synthase was increased during pregnancy (11, 12), and that estrogen was able to regulate both NO synthase isozymes (13). NO was also affected by androgens in male rats (14). Functionally, it was revealed that chronic inhibition of NO synthase retarded intrauterine growth in pregnant rats (15). In a recent study on immature rats, it was suggested that an L-arginine-NO system might contribute to estrogen-induced uterine edema and growth (16). However, despite these studies, the mechanisms responsible for NO synthase expression during pregnancy remain unresolved. Consequently, with both estrogen and progesterone required at restricted times in the regulation of decidual formation, the aim of this study was to examine the role of NO in deciduoma formation, and associative hormonal and biochemical mechanisms. The involvement of NO in deciduoma development was determined by utilizing N^G-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue that inhibits NO synthase. Secondly, the study was aimed at clarifying whether the inhibition of NO during the DCR would be more or less effective during the estrogen/progesterone primed, pre-decidual induction phase (Days 1–4), than during the post-traumal period (Days 5–8) when progesterone action was preeminent.

Materials and Methods

Animals and Treatments. Female Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 200–240 g, were housed under controlled temperature (20° ± 2°C) and photoperiod (12L:12D, lights on at 0700–1900 hr) and had free access to food and water. Estrous cycles were monitored daily (0900–1000 hr) by vaginal lavages. Only animals displaying two consecutive 4- or 5-day estrous cycles were used in the experiments. PG was initiated by mechanically stimulating the uterine cervix with a blunt glass rod during proestrus and estrus (1000–1100 hr). Day 1 PG was designated as the first day following cervical stimulation when leukocytes infiltrated the vaginal smears. DCR, the basis for decidualization, was artifically induced *via* laparotomy, under light ether anesthesia, by bilateral scratch trauma. The procedure was conducted on the antimesometrial portion of the uterine epithelium with a blunt surgical needle on Day 4 PG at 1000–1200 hr (17). All procedures involving the animals were approved by the Institutional Animal Care and Use Committee.

Experiments. The first experiment was designed to determine the expression of NO synthase in the endometrium and myometrium, and the relationship between NO synthase activity and decidual growth. Rats (5/group) were periodically sacrificed at mid-light (1300 hr) on PG Days 3, 6, 9, 12, and 15.

Next we set out to evaluate possible biochemical and hormonal involvement in NO synthase/NO action and the decidual process by employing treatments with L-NAME alone, or L-NAME plus L-arginine combined. Growth parameters in the endometrium and myometrium were measured at sampling time (1300 hr on PG Day 9) in rats (5/group). Each group was treated twice per day during the pre-decidualized induction period of Days 1–4 (Experiment 2), and similarly during the post-traumal period Days 5–8 (Experiment 3).

Dosing Procedures. The injection doses of twice daily L-NAME (20 mg/kg ip) and L-arginine (600 mg/kg sc) were previously described (16). The animals were injected 3 hr after lights on and 3 hr before lights off. The vehicle was 2 ml/kg saline. The animals were sacrificed under light ether anesthesia by decapitation; trunk blood was collected and uteri removed. Endometrial tissue was mechanically removed in cold saline by scraping away with lancets from the underlying myometrium.

Hormones. After preparation of pooled endometrial and myometrial tissues by homogenization and centrifugation, protein concentration was determined by the method of Lowry *et al.* (18) using bovine serum albumin as a standard: DNA content was quantified by the diphenylamine method of Burton (19). Serum progresterone and estradiol levels were measured by radioimmunoassays after a method adopted from Albrecht *et al.* (20). The sera or standards were mixed with suitably diluted antisera (Sigma Chemical Co., St. Louis, MO) and 3000 cpm of labeled tracer [^{3}H]estradiol or [^{3}H]progesterone (NEM Life Sciences Products, Boston, MA) in 0.1 *M* Tris-HCl buffer (pH 7.4), containing 0.1% BSA. Bound and free tracer were separated by the dextran-coated charcoal suspension method. A Logit-log dose regression equation was used for calculating the levels of estradiol and progesterone.

Enzyme Assays. NO synthase activity was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (21). The tissue (endometrial and myometrial separately) was homogenized in 50 m*M* Tris buffer (pH 7.4) containing 0.1 m*M* EDTA, 1 m*M* DTT, trypsin inhibitor (0.2 mg/ml) and 10 m*M* phenylmethanesulfonyl and then centrifuged at 1000*g* for 15 min. 100 μl of the supernatant were incubated for 30 min at 37°C with 100 μl of the assay buffer in a final volume of 50 m*M* Tris-HCl buffer (pH 7.4), 20 μ*M* FAD, 4 μ*M* tetrahydrobiopterin, calmodulin (5 U/ml), 1 μ*M* arginine, 2 m*M* EGTA, 1 m*M* NADPH and [^{3}H]arginine (1 μCi/ml). After incubation, the assay was terminated with 0.8 ml of ice-cold stop solution (80 m*M* Hepes, 8 m*M* EDTA, pH 5.2). The samples were immediately passed through 2 ml Dowex-50W cation exchange columns, preequilibrated with stop solution to bind and remove the positively charged arginine. After column pas-

sage, the columns were eluted with 4 ml distilled H_2O. The radioactivity of [^{3}H]citrulline was quantified by liquid scintillation, and the NO synthase activity was expressed as the fmole of citrulline formed/milligram protein/min. The matrix metalloproteinases (MMP) a group of gelatinase enzymes, were detected by means of substrate zymography (22), employing SDS-PAGE containing 0.1% gelatin as the substrate for gelatinolysis. Molecular masses of the MMP enzymes were determined by comparison with SDS-PAGE markers (Bio-RAD, Hercules, CA). MMP activity was quantified by scanning densitometry with Molecular Analyst software (Bio-Rad, Hercules, CA). Alkaline Phosphatase (ALP) activity was calculated by a colorimetric method incorporating liberated p-nitrophenol and adopted from Pollard *et al.* (23).

Western Blot Analysis. Endometrial tissue was homogenized in 50 m*M* Tris buffer (pH 7.4) containing 0.1 m*M* EDTA, 1 m*M* DTT, trypsin inhibitor (0.2 mg/ml) and 10 m*M* phenylmethanesulfonyl and then centrifuged at 1000*g* for 15 min. The supernatant was centrifuged at 100,000*g* for 30 min. The supernatant (cytosolic fraction) was then decanted from the pellet, and the pellet (membranous fraction) was solubilized by electrophoresis sample buffer. The extracted proteins, resolved on 9% SDS-PAGE for 90 min at 4°C were transferred onto polyvinylidene difluoride membranes, and blots were then incubated for 1 hr with a blocking buffer (1% BSA in 20 m*M* Tris-buffered saline, pH 7.5, with 0.05% Tween 20). Proteins were probed successively with diluted anti-inducible NO synthase monoclonal antibody (Transduction Laboratories, Lexington, KY), washed in 20 m*M* Tris-HCl buffer, pH 7.5 containing 150 m*M* NaCl and 0.05% Tween 20, and then with diluted alkaline phosphatase-conjugated anti-mouse IgG antibody (Promega Inc., Madison, WI), and finally washed with the buffered solution. The reactive bands on the blots were visualized by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate.

Statistical Analysis. Results are expressed as means ± SEM. Statistical significance was determined by analysis of variance for multiple comparisons with a statistic program, PROSTAT (Salt Lake City, UT). Comparisons between control and experimental means were done with the Student's *t* test. *P* values smaller than 0.05 were considered significant.

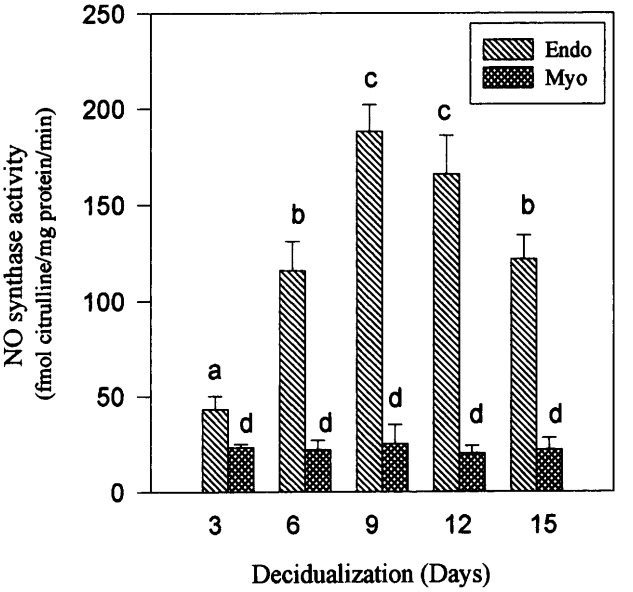

Figure 1. Time-course expression of inducible NO synthase activity. Shown here are the results (mean ± SEM) of five pooled individual tissues (10 used on PG Day 3), each calculated from three independent replicates. Values without the same letter are significantly different ($P < 0.01$).

Results

The data in Figure 1 indicate a time-related parabolic profile in the activities of inducible NO synthase in the endometrium during decidualization. There was a dramatic, progressive increase from Day 3 through Day 6 to peak levels at Day 9 (from 43 ± 7 to 188 ± 14 fmol citrulline/mg protein/min), and declining thereafter from PG Days 12–15. Moreover, all post-decidual induction increments in NO synthase (from Days 6–15) were particularly robust, ranging from approximately 2- to 3-fold increases over the pretraumal level at PG Day 3. However, a nonparallel relationship existed between NO synthase and decidual growth in the myometrium where the values were basal and invariable.

The results in Table I demonstrate that the inhibitory

Table I. Endometrial Growth and Hormonal Levels after Pretreatment with L-NAME or L-NAME + L-Arginine

Treatment	Endometrium g/uterus	Protein mg/endo	DNA mg/endo	Serum P ng/ml	Serum E_2 pg/ml	ALP activity[a] mIU/mg prot
D_{1-4} Control	1.464 ± 0.090	204 ± 13	3.25 ± 0.20	130 ± 11	15.0 ± 2.3	15.5 ± 1.2
D_{1-4}+ NAME	0.750 ± 0.145[c]	104 ± 20[c]	1.60 ± 0.31[c]	99 ± 6[b]	13.8 ± 5.0	15.8 ± 1.1
D_{1-4} + NAME + Arg	1.419 ± 0.050	222 ± 24	3.08 ± 0.11	112 ± 11	15.4 ± 3.7	16.1 ± 1.4
D_{5-8} Control	1.424 ± 0.069	201 ± 10	3.13 ± 0.16	126 ± 13	14.6 ± 3.6	16.0 ± 1.9
D_{5-8} + NAME	1.443 ± 0.090	198 ± 12	3.22 ± 0.49	126 ± 17	16.6 ± 8.6	18.6 ± 4.5
D_{5-8} + NAME + Arg	1.476 ± 0.098	204 ± 14	3.32 ± 0.22	124 ± 11	16.5 ± 3.2	19.4 ± 4.5

Note. Values represent the means ± SEM ($n = 5$). Animals were pretreated twice per day with L-NAME (20 mg/kg, ip) for 4 days (PG Days 1–4 or 5–8) or similarly with L-NAME (20 mg/kg, ip) + L-arginine (600 mg/kg, sc). End points were all measured on PG Days 9.
[a] Results for alkaline phosphatase (ALP) represent three independent replicates/point from five pooled animals. [b]$P < 0.05$, [c]$P < 0.01$.

effect of inducible NO synthase was pronounced after pretreatment with L-NAME during PG Days 1–4, and was ineffective following Days 5–8 exposure. Reductions in growth indices (weight, protein, and DNA) were all significantly decreased ($P < 0.01$) by early L-NAME administration. Hormonally, L-NAME also produced a significant reduction ($P < 0.05$) in the serum progesterone concentration following pretraumal application (dropping from Days 1–4 saline control values of 130 ± 11 ng/ml in intact animals, to 99 ± 6 ng/mg). Progesterone titers for ovariectomized animals that were saline-injected during Days 1–4 were very low at 24 ± 5 ng/ml ($n = 3$) on Day 9. Serum estradiol titers for these animals under similar experimental conditions were unchanged from values on intact controls (15.0 ± 2.3 pg/ml, $n = 5$) to L-NAME-treated animals (13.8 ± 5.0 pg/ml, $n = 5$). Serum estradiol concentration for vehicle-injected (Days 1–4) ovariectomized animals was also very basal at 6.0 ± 1.5 pg/ml ($n = 3$) on Day 9. These ovariectomized values are not shown in Table I. The combined treatment of L-NAME plus L-arginine lessened or reversed these reductions. From Table II, these growth endpoints in the myometrium were significantly affected ($P < 0.01$) by Days 1–4 treatment.

Post-traumal treatments (Days 5–8) with L-NAME, or L-NAME plus L-arginine, were ineffective on endometrial and myometrial growth and serum progesterone levels (Table I). In addition, circulating estradiol levels were low and unaffected by L-NAME treatments. Days 5–8 saline injected ovariectomized control values ($n = 3$) of 27 ± 4 ng/ml progesterone and 6.8 ± 1.4 pg/ml estradiol (not shown in Table I) were both extremely low when compared with the intact and L-NAME-treated animal (Table I). Enzymatically, ALP activities were unchanged by the early and late treatments (Table I). Endometrial extracts examined on Day 9 yielded a NO synthase protein band at 130 kDa, corresponding to the mouse macrophage inducible NO synthase control. This was confirmed with mouse macrophage antibody by Western blot analysis (Fig. 2, top panel-A). Results from zymography indicate the presence of two protein bands (MMP 2 at 72 kDa and MMP 9 at 92 kDa) for these enzymes in both uterine compartments (Fig. 2, lower panel-B). The relative activities for the MMP remained essentially unaltered in both the endometrium and myometrium by both dosings, irrespective of the treatment period.

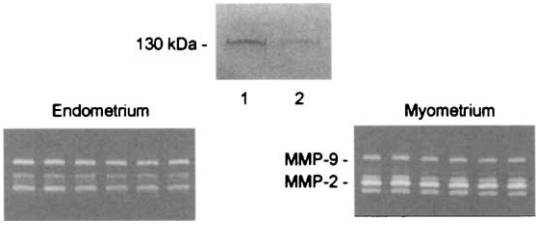

Figure 2. (A) Western blot analysis of inducible NO synthase expression in the endometrium on PG Day 9. Lane A1, mouse macrophage; Lane A2, PG Day 9 endometrium. Protein on polyvinylidene difluoride membranes were probed successively with monoclonal antibody. (B) A representative zymogram of the intensity profiles of decidualized endometrial and myometrial matrix metalloproteinases after treatment with L-NAME or L-NAME + L-arginine on PG Days 1–4 or 5–8. Lane B1, PG Days 1–4 control; Lane B2, PG Days 1–4 plus L-NAME; Lane B3, PG Days 1–4 plus L-NAME and arginine; Lane B4, PG Days 5–8 control; Lane B5, PG Days 5–8 plus L-NAME; Lane B6, PG Days 5–8 plus L-NAME and arginine.

Discussion

The study demonstrates that features of maximal decidual proliferation in the endometrium and myometrium (increases in wet weight, in DNA for hyperplasia and protein for hypertrophy, along with enhanced progesterone for maintenance of this growth) were all adversely affected by the inhibition of inducible NO synthase. This action occurred during the pre-decidual induction period when estrogen/progesterone priming was prominent. By contrast, post-traumal NO synthase inhibition was ineffective on peak decidual development during PG Day 9. This apparent dependence of NO synthase on the pre-traumal hormonal status, and the subsequent interaction between NO synthase and the progesterone-dominated decidual growth, suggest an involvement of inducible uterine NO synthase/localized NO with these steroid hormones. Specifically, the results strongly suggest that L-NAME inhibition of the pre-decidual ovarian secretion of progesterone may also be responsible for the suboptimal decidual sensitivity and subsequent reduced uterine proliferation. In this context, based on RU 486 association with decreased NO synthase protein during preterm labor, it was hypothesized that inducible NO synthase could be partially regulated by progesterone (12), and under a different circumstance by sex steroids in general (13). The results indicate that estradiol concentrations, normally insignificant during the high point in decidual growth, were unaffected by both pre- and post-traumal inhibitory treatments of NO synthase. Additionally, two important decidualization biomarkers (i.e., alkaline phosphatase of uncertain function (24), and the matrix metalloproteinases engaged in degradation and remodeling of the extracellular matrix during decidualization (25)) were unchanged by the two pretreatments, in both uterine compartments.

The data provide significant evidence for the involvement of NO in the decidual cell reaction in rats. The findings establish parallel time-dependency between enhanced levels of inducible calcium-independent NO synthase, here verified and previously identified (12) by a 130-kDa protein band, and progression of decidualized proliferation to its maximal antimesometrial level on PG Day 9. This temporal link suggests that NO may physiologically participate in the decidualization process. So what may be the specific significance of this relationship that exists only in the endometrium? Firstly, comparable studies on the pregnant rat uterus provided evidence of substantial increases in inducible NO synthase protein (12) and in NO synthase activity (11, 13). Then, a recent study by Rao *et al.* (16), demon-

Table II. Effects of L-NAME or L-NAME + L-Arginine on Myometrial Growth

Treatment	Myometrium g/uterus	Protein mg/myo	DNA mg/myo	ALP activity[a] mIU/mg prot
D_{1-4} Control	1.416 ± 0.086	170 ± 10	3.22 ± 0.20	10.8 ± 0.7
D_{1-4}+ NAME	0.959 ± 0.044[b]	104 ± 5[b]	2.22 ± 0.10[b]	11.0 ± 1.6
D_{1-4} + NAME + Arg	1.524 ± 0.078	168 ± 9	3.35 ± 0.17	11.2 ± 1.4
D_{5-8} Control	1.383 ± 0.100	166 ± 12	3.12 ± 0.22	10.9 ± 1.0
D_{5-8} + NAME	1.350 ± 0.081	157 ± 4	2.93 ± 0.08	11.3 ± 1.2
D_{5-8} + NAME + Arg	1.461 ± 0.099	172 ± 12	3.28 ± 0.21	11.2 ± 1.0

Note. Presented are the means ± SEM ($n = 5$). Animals were pretreated twice per day with L-NAME (20 mg/kg, ip) for 4 days (PG Days 1–4 or 5–8) or similarly with L-NAME (20 mg/kg, ip) + L-arginine (600 mg/kg, sc). End points were all measured on PG Days 9.
[a] Results for alkaline phosphatase (ALP) represent three independent replicates/point from five pooled animals. [b] $P < 0.01$.

strated that the blocking action of L-NAME on estradiol-induced uterine growth in immature rats, can effectively be prevented by simultaneous pretreatment with L-arginine. Functionally, NO primarily affects either blood flow dynamics (11, 26, 27) or smooth muscle contractility (8, 28). From a vascular standpoint, characteristic local hyperemia and increased capillary permeability in the decidualized endometrium can assist in providing access to the anabolic substrates (i.e., water, protein, DNA, glycogen) that are required for the hyperplasia and hypertrophy accompanying the decidual response. The vaso-regulatory influences of NO include estradiol-induced increases in uterine blood flow (26), stimulation of uterine vasodilatation (11), and even increases in testicular blood flow in rats (27). Hence when these viewpoints are considered together, it appears that the enhanced endometrial expression of NO synthase activity during the decidualization process may be closely linked to the increased endometrial vasculature. On the other hand, the absence of fetuses during decidualization minimized or may have even precluded myometrial contractility as a factor. Consequently, the basal unaltered levels in myometrial NO synthase activity at this time could be equated with the essential quiescence that distinguishes decidual establishment.

In summary, the present study indicates two significant aspects of NO involvement in decidual metabolism: 1) a prevalent, time-dependent increase in endometrial NO synthase activity (PG Days 1–9); and 2) the predominance of NO synthase inhibition during the pre-decidual induction period (PG Days 1–4) in the endometrium and myometrium. Because of these two events, it is concluded that the antiproliferative effects of inhibited inducible NO synthase/endogenous NO on maximal decidual responses (PG Day 9) encompassing growth and associative progresterone support, may occur directly by way of reduced uterine sensitivity to the deciduogenic stimulus, surgically applied on PG Day 4, or indirectly through impaired endometrial vascularity. The latter action could result in reductions in the metabolites that are available for optimal decidual cell reaction. Furthermore, the study provides some insight into the mechanisms of action of the inducible NO synthase/NO system during deciduoma formation. These include: hormonally, its reliance on the early pre-decidual estrogen/progesterone milieu and later postinductional mediation by progesterone; biochemically, the utilization of DNA and protein as effector molecules; and enzymatically, the apparent noninvolvement of characteristic zinc metalloenzymes.

1. DeFeo VJ. Temporal aspect of uterine sensitivity in the pseudopregnant or pregnant rat. Endocrinology **72:**305–316, 1963.
2. Finn CA, Pope M, Milligan SR. Control of uterine stromal mitosis in relation to uterine sensitivity and decidualization in mice. J Reprod Fertil **103:**153–158, 1995.
3. DeFeo VJ. Decidualization. In: Wynn RM, Ed. Cellular Biology of the Uterus. New York: Appleton-Century-Crofts/Meredith, pp 191–290, 1967.
4. Yochim JM, DeFeo VJ. Hormonal control of the onset, magnitude, and duration of uterine sensitivity in the rat by steroid hormones of the ovary. Endocrinology **72:**317–326, 1963.
5. Finn CA. The implantation reaction. In: Wynn RM, Ed. Biology of the Uterus. New York: Plenum Press, pp 245–308, 1977.
6. Palmer RMJ, Rees DD, Ashton DS, Moncada S. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem Biophys Res Commun **153:** 1251–1256, 1988.
7. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev **43:**109–142, 1991.
8. Yallampalli C, Izumi H, Byum-Smith M, Garfield RE. An L-arginine–nitric oxide–cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. Am J Obstet Gynecol **170:**175–185, 1993.
9. Moretto M, Lopez FJ, Negro-Vilar A. Nitric oxide regulates luteinizing hormone-releasing hormone secretion. Endocrinology **133:**2399–2402, 1993.
10. Mani SK, Allen JM, Rettori V, McCann SM, O'Malley BW, Clark JH. Nitric oxide mediates sexual behavior in female rats. Proc Natl Acad Sci USA **91:**6468–6472, 1994.
11. Conrad KP, Joffe GM, Kruszyna H, Kruszyna R, Rochelle LG, Smith RP, Chavez JE, Mosher MD. Identification of increased nitric oxide biosynthesis during pregnancy in rats. FASEB J **7:**566–571, 1993.
12. Dong Y-L, Gangula PRR, Yallampalli C. Nitric oxide synthase isoforms in the rat uterus: Differential regulation during pregnancy and labour. J Reprod Fertil **107:**249–254, 1996.
13. Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthase by sex hormones. Proc Natl Acad Sci USA **91:**5212–5216, 1994.
14. Chamness SL, Ricker DD, Crone JK, Dembeck CL, Maguire MP, Burnett AL, Chang TSK. The effect of androgen on nitric oxide synthase in the male reproductive tract of the rat. Fertil Steril **63:**1101–1107, 1995.
15. Molnar M, Suto T, Toth T, Hertelendy F. Prolonged blockade of nitric oxide synthesis in gravid rats produces sustained hypertension, pro-

teinuria, thrombocytopenia, and intrauterine growth retardation. Am J Obstet Gynecol **170:**1456–1466, 1994.
16. Rao VSN, Chaves MC, Ribeiro RA. Nitric oxide synthase inhibition and the uterotropic response to oestrogen in immature rats. J Reprod Fertil **105:**303–306, 1995.
17. Brann DW, Mahesh VB. Robe of corticosteroids in female reproduction. FASEB J **5:**2691–2698, 1991.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193:**265–275, 1951.
19. Burton K. A Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J **62:**315–323, 1955.
20. Albrecht ED, Haskins AL, Pepe GJ. The influence of fetectomy at midgestation upon the serum concentration of progesterone, estrone, and estradiol in baboons. Endocrinology **107:**726–731, 1980.
21. Bush P, Gonzales N, Ignarro L. Biosynthesis of nitric oxide and citrulline from L-arginine by constitutive nitric oxide synthase present in rabbit corpus cavernosum. Biochem Biophys Res Commun **186:**680–685, 1992.
22. Curry TE Jr., Mann JS, Huang MH, Keeble SC. Gelatinase and proteoglycanase activity during the periovulatory period in the rat. Biol Reprod **46:**256–264, 1992.
23. Pollard JW, Jahon M, Butterworth PJ. Characterization and expression of uterine and placental alkaline phosphatases in the mouse. J Reprod Fertil **89:**735–742, 1990.
24. Kennedy TG, Ross HE. Effect of prostaglandin E_2 on the rate of decidualization in rats. Prostaglandins **46:**243–250, 1993.
25. Nothnick WB, Dylan RE, Leco KJ, Curry TE Jr. Expression and activity of ovarian tissue inhibitors of metalloproteinases during pseudopregnancy in the rat. Biol Reprod **53:**681–691, 1995.
26. Van Buren GA, Yang D, Clark KE. Estrogen-induced uterine vasodilation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. Am J Obstet Gynecol **167:**828–833, 1992.
27. Lissbrant E, Lofmark U, Collin O, Bergh A. Is nitric oxide involved in the regulation of the rat testicular vasculature? Biol Reprod **56:**1221–1227, 1997.
28. Jackson WF, Busse R. Elevated guanosine 3′-5′-cyclic monophosphate mediates the depression of nitro-vasodilator reactivity in endothelium-intact blood vessels. Arch Pharm **344:**345–350, 1991.