Regulation of endometrial blood flow in ovariectomized rats: assessment of the role of nitric oxide

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Regulation of endometrial blood flow in ovariectomized rats: assessment of the role of nitric oxide. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2009–H2017, 1997.—The purpose of this study was to evaluate the role of nitric oxide (NO) in the maintenance of basal endometrial blood flow of ovariectomized rats and in the increase of endometrial blood flow after administration of estradiol 17β (E2b). Endometrial blood flow was repeatedly measured with the H2 gas clearance technique in ovariectomized rats. Nω-nitro-L-arginine methyl ester (L-NAME) dose dependently reduced basal endometrial blood flow and increased mean arterial blood pressure and endometrial vascular resistance. E2b (1 µg/kg iv) increased endometrial blood flow and reduced endometrial vascular resistance, which peaked by 2 h after the injection. The vasoconstrictive activity of L-NAME (an inhibitor for NO synthesis) was compared with that of phenylephrine (PE, an α-receptor agonist acting through an NO-independent mechanism). Doses of L-NAME (1 and 3 mg/kg iv) were matched with those of PE (3.2 and 6.4 mg·kg−1·h−1 iv), as they induced an approximately equivalent percent increase in basal endometrial vascular resistance. The percent increases of endometrial vascular resistance in E2b-treated animals by the two agents in matched doses were also of a similar magnitude. When animals were first treated with L-NAME or PE, E2b lost the ability to reduce endometrial vascular resistance. Enzyme activity and gene expression of NO synthase in the rat uterine tissue were also examined after E2b treatment, and no significant changes were observed. These data raise doubts about the role of NO in the regulation of endometrial blood flow after acute administration of E2b and suggest that other mechanisms may be involved.

hydrogen gas clearance; estradiol 17β

ADMINISTRATION OF estradiol 17β (E2b) to ovariectomized mice increases uterine blood flow (10, 11, 20, 24). This increase in uterine blood flow occurs after a delay of 30–60 min and peaks ~2 h after E2b administration (11, 24). Although the time course and the magnitude of this E2b-induced increase in uterine blood flow have been thoroughly described, the exact mechanism by which this occurs is not known. Various vasoactive substances, such as prostanoids and vasoactive polypeptides, have been implicated as the mediators involved in this phenomenon (4, 5, 11). However, the increase in uterine blood flow has not been successfully antagonized with antagonists to the suggested mediators.

The increase in uterine blood flow by E2b is a localized response, inasmuch as E2b injection into one uterine artery of the ewe produced an increase in uterine blood flow only to the ipsilateral horn (11, 18). Pretreatment with cycloheximide, an inhibitor of protein synthesis, prevented this E2b-induced increase in uterine blood flow (11), indicating that the production of an enzyme or a polypeptide is a necessary intermediate for this E2b response. More recently, another vasodilator, nitric oxide (NO), has been implicated as a possible mediator for this E2b response (8, 23, 27). Whereas chronic exposure of tissues to E2b increases NO synthesis, as demonstrated by pharmacological (8) and biochemical and molecular biology techniques (27), the mechanism that underlies the acute effects of E2b on vascular tone is controversial. Some investigators reported that the acute vasorelaxation by E2b is an endothelium-independent process (13, 28), whereas other investigators indicated that NO is the mediator (23).

The only evidence that suggests a role of NO in mediating the acute effects of E2b on the uterine vascular bed comes from one group of investigators using the sheep model (23). In support of their hypothesis, these investigators demonstrated that administration of Nω-nitro-L-arginine methyl ester (L-NAME) of NO synthase (NOS), was able to reduce, in a dose-dependent manner, the E2b-induced increase in uterine blood flow. However, the inhibition of NO synthesis leads to vasoconstriction in all vascular beds, inasmuch as NO is responsible for maintenance of basal vascular tone (17, 25). It is therefore possible that the results observed by Van Buren et al. (23) are due to a physiological antagonism in which the increase of uterine blood flow by one substance (i.e., E2b) is offset by the decrease in uterine blood flow by another substance (i.e., L-NAME), the net effect depending on the potencies of the substances involved. In the present study, we reassessed whether NO is the mediator for the acute effects of E2b on the uterine vascular bed by using pharmacological, biochemical, and molecular biology techniques.

MATERIALS AND METHODS

Animal Preparation

The experimental procedures have been previously described in detail (30). Briefly, virgin female Sprague-Dawley rats (200–225 g; Harlan, Indianapolis, IN) were housed under conditions of controlled temperature and light cycle and were provided free access to food pellets and water. Under pentobarbital sodium anesthesia (40 mg/kg ip), the animals were ovariectomized bilaterally 3–5 days before the experiment.

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To measure endometrial blood flow using the H₂ gas clearance technique, the animals were anesthetized with urethane (1.25 g/kg ip). A femoral vein was cannulated for saline and drug administration, and a carotid artery was cannulated to monitor the arterial blood pressure on a Gilson recorder via a pressure transducer (model P23Db, Statham). The body temperature of the animals was monitored with a rectal probe and was maintained at 36.5–37.0°C under an incandescent lamp.

For assessments of NOS activity as well as for endothelial NOS (eNOS) and inducible NOS (iNOS) gene expression in the uterus, an external jugular vein was cannulated for drug delivery, and the uterine horns were removed from the animals under anesthesia with urethane (1.25 g/kg ip). The uterine horns were washed with sterile phosphate-buffered saline, frozen quickly in liquid nitrogen, and stored in a freezer until they were homogenized.

H₂ Gas Clearance Technique

The H₂ gas clearance technique (1) was used to measure endometrial blood flow of ovariectomized rats. Details of this technique have been described previously (30). Briefly, the trachea was intubated with PE-240 tubing to maintain a patent airway and to facilitate the delivery of 2% H₂ during blood flow measurement. After a midline laparotomy, an Ag-AgCl reference electrode was placed in the abdominal cavity. A platinum electrode (125 µm diameter, A-M System, Everett, WA) was then inserted into the endometrium of the right uterine horn. Care was taken to avoid apparent blood vessels and to minimize damage to the tissue (30). The uterine horn was then covered with a thin piece of saline-moist gauze, and the area over the incision was covered with a moist gauze, and the area over the incision was covered with a piece of Parafilm to prevent evaporation. To measure endometrial blood flow, the electrodes were connected to a polarographic and amplifying unit (Val Tech Electronics, Sherman Oaks, CA) and the electrode current during the inhalation of 2% H₂ and subsequent removal of H₂ was traced on the Gilson recorder. Before the electrode connection, the output voltage of the polarographic unit was adjusted to match the electrode voltage between the platinum electrode and the reference electrode. This was done to set the electrode current to zero (or close to zero) in the absence of H₂ and to ensure a more reliable recording (30). Through an ADALAB analog-to-digital converter, the signals were sent to a computer. During the 15 min of H₂ inhalation, the current tracing gradually rose and reached a plateau as the tissue was saturated with H₂. When H₂ was discontinued for 15 min, a desaturation curve was generated because of the washout of H₂ from the tissue by blood flow. The endometrial blood flow was then calculated by analyzing the H₂ desaturation curve using a monoexponential curve-fitting program (12).

NOS Assay

The arginine-to-citrulline conversion assay (2) was used to measure NOS activity in uterine homogenate. A 20% homogenate of uterine horn was prepared in 50 mM triethanolamine (TEA)-HCl, pH 7.4, containing 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 µM pepstatin A, and 2 µM leupeptin at 4°C. The homogenate was centrifuged at 20,000 g for 60 min at 4°C, and the supernatant was used to assay NOS activity. NOS activity was determined by measuring the formation of [³H]citrulline from [³H]arginine (2). Enzymatic reactions were conducted at 37°C for 10 min in 50 mM TEA-HCl, pH 7.4, containing 50 µM l-arginine (77 Ci/mmol, with ~20,000 cpm of l-[2,3,4,5-³H]arginine-HCl), 100 µM NADPH, 10 µM tetrahydrobiopterin, 10 µM flavin adenine dinucleotide, 10 µM flavin mononucleotide, 2 mM CaCl₂, 50 mM l-valine, 1 µg of calmodulin, and 0.2–0.4 mg of supernatant protein in a final incubation volume of 200 µl. Ca²⁺-independent NOS activity was measured in the absence of Ca²⁺ and calmodulin and in the presence of 2 mM EDTA and 2 mM EGTA. Ca²⁺-dependent NOS activity was obtained by subtracting the Ca²⁺-independent NOS activity from the total NOS activity. The L-[2,3,4,5-³H]arginine-HCl was purified by anionic exchange chromatography on columns of Dowex AG 1-X8, OH⁻ form (prepared from the acetate form), 100–200 mesh, to remove traces of contaminating [³H]citrulline. Enzymatic reactions were terminated by addition of 2 ml of ice-cold buffer (20 mM sodium acetate, pH 5.5, containing 1 mM l-citrulline, 2 mM EDTA, and 0.2 mM EGTA), and samples were loaded onto columns (1 cm diameter) containing 1 ml of Dowex AG 50W-X8, Na⁺ form (prepared from H⁺ form), that had been preequilibrated with stop buffer for chromatography. After the 2 ml of eluate were collected in a test tube, each column was washed again with 2 ml of water and collected in the same test tube. Aquasol-2 (12 ml) was added to one-half (2 ml) of the final eluate, and the samples were counted in a liquid scintillation spectrometer (model LS 3801, Beckman).

Reverse Transcriptase Polymerase Chain Reaction

The reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to assess the eNOS and iNOS gene expression in rat uterus. Tri-Reagents (Molecular Research Center, Cincinnati, OH) were used to homogenize rat uterine horns and to extract the total RNA. Reverse transcription was performed by using 5 µg of total RNA sample, 50 U of Moloney murine leukemia virus RT, and 100 pmol of oligo(dT) and running the reaction at 42°C for 30 min. The resulting cDNA samples were ePCR amplified using the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) in 100 µl of reaction mixture. The final reaction mixture was treated by heat denaturation at 94°C for 5 min and PCR amplification for 40 cycles, each consisting of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. This was followed by a final extension at 72°C for 5 min. The amplification procedure for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) consisted of 30 cycles each of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, 1 µg of total RNA sample.

The following primers were used (Custom Primers, GIBCO-BRL, Gaithersburg, MD): eNOS, 5'-GTGATGGCCGAAGCCTGTGAAG-3' (sense) and 5'-CCGAGCGGAACACAGCAGAC-3' (antisense) (19); iNOS, 5'-CATGGCTTGCCCCTGGAAGTTTCT-3' (sense) and 5'-CCCTGCTAGTGTCCTCAGCAGATC-3' (antisense; gene bank accession M84327) (29); GAPDH, 5'-GTTGAG-GTGGTTGAAGCGGATTTG-3' (sense) and 5'-CAGAGCTCTCTT-GAGTGGCATG-3' (antisense) (22). One hundred nanograms of sense and antisense primers were used in each PCR in a final volume of 100 µl. The amplified DNA fragments obtained were of expected base-pair (bp) size: eNOS, 422 bp; iNOS, 747 bp; GAPDH, 558 bp. Each final PCR product sample (30 µl) was loaded on a 1.5% agarose gel, electrophoresed, and visualized by ethidium bromide staining under ultraviolet light. The identities of the products were confirmed by Southern blot hybridization with internal oligonucleotides that were ³²P labeled using T4-polynucleotide kinase from respective cDNA sequences. We cloned the PCR products and sequenced them to confirm the identities of the amplified fragments. The eNOS and iNOS cDNA fragments were then excised from the agarose gel and eluted into a small vial. These fragments were subsequently cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI) and sequenced using a Taq dye-terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 377 DNA sequencer (Perkin Elmer, Norwalk, CT).
products into PCR 2.1 vector using a TA cloning kit (Invitrogen). Their identity as iNOS, eNOS, and GAPDH was confirmed by DNA sequencing (data not shown). The quantity of RNA for RT-PCR and the number of cycles were initially determined to ensure that saturation did not occur.

Chemicals and Solutions

The stock solution of E2β was made by dissolving the powder (Sigma Chemical) in 100% ethanol to a concentration of 250 µg/ml and stored in a freezer. Immediately before intravenous injection, 10 µl of this stock were mixed with 990 µl of 0.9% NaCl. L-NAME (Sigma Chemical) was dissolved in 0.9% NaCl and stored in a freezer before use. The phenylephrine (PE) solution for intravenous infusion was made by dissolving the PE powder (Sigma Chemical) in 0.9% NaCl to a concentration of 1 mg/ml and stored in a refrigerator before use. Lipopolysaccharide (LPS; Sigma Chemical) was dissolved in 0.9% NaCl to a concentration of 1 mg/ml before intravenous injection.

TEA-HCl, l-arginine-HCl free base, l-citrulline, peptatin, leupeptin, dithiothreitol, sodium acetate, EDTA, EGTA, NADPH, flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin, CaCl2, and calmodulin were obtained from Sigma Chemical. L-[2,3,4,5-3H]arginine-HCl was obtained from Amersham. Dowex AG 1-X8 and Dowex AG 50W-X8 were from Bio-Rad Laboratories (Richmond, CA).

Experimental Design

After the surgical procedure, a stabilization period of 90–120 min was allowed before the measurement of endometrial blood flow by the H2 gas clearance technique (30). At times indicated, E2β and L-NAME were administered intravenously in a volume of 0.1 ml over a 1-min period, and PE was given as a constant intravenous infusion. Control endometrial blood flow was measured before administration of these agents. Endometrial blood flow in response to E2β was measured at 2 h after E2β administration, and the endometrial blood flow in response to L-NAME or PE was measured 15–30 min after the injection or the start of infusion. Approximate values of endometrial vascular resistance were obtained by dividing the mean arterial pressure (MAP) by endometrial blood flow. Uterine venous pressure was not included in the calculation because of the technical difficulties in measuring it in rats.

For NOS assays and RT-PCR experiments, E2β or LPS was injected intravenously in a volume of 0.1 ml over a 1-min period, and the uterus was removed and frozen in liquid nitrogen at 30 min or 2 h after the injection. In a separate group of animals, neither E2β nor LPS was given before the uterus was removed. These animals were in the time 0 group and were used as the negative control for baseline comparison.

Experimental Protocols

Study I: Cumulative dose response of L-NAME on MAP, endometrial blood flow, and endometrial vascular resistance. After the control endometrial blood flow measurement, cumulative doses of L-NAME were given at 30-min intervals to evaluate the role of NO in the regulation of basal endometrial blood flow and to obtain dose-response effects on MAP, endometrial blood flow, and endometrial vascular resistance.

Study II: Reversal of the E2β response by L-NAME and PE. Doses of L-NAME (1 and 3 mg/kg) and PE (3 and 6.4 mg·kg⁻¹·h⁻¹) that induced comparable percent increase in endometrial vascular resistance in the absence of E2β were given 2 h after the administration of E2β to test and compare the abilities of L-NAME and PE to reverse the E2β-induced changes in endometrial blood flow and endometrial vascular resistance.

Study III: Blockade of the E2β response by L-NAME and PE. L-NAME injection (1 and 3 mg/kg) or PE infusion (3.2 and 6.4 mg·kg⁻¹·h⁻¹) was given or started immediately before E2β injection to compare the effectiveness of L-NAME and PE in blocking E2β-induced changes in endometrial blood flow and endometrial vascular resistance. In preliminary experiments we observed that L-NAME (1 and 3 mg/kg) increased uterine vascular resistance; this increase was maintained for at least 3 h after its administration.

Study IV: Assessment of NOS activity and eNOS and iNOS gene expression. E2β, vehicle, or LPS was administered to the animals to test their effects on uterine NO activity as well as on eNOS and iNOS gene expression. LPS was utilized as the positive control, inasmuch as other investigators (26) demonstrated an increase in iNOS protein and gene expression after its administration. The NOS assay was also performed in the presence of L-NAME to confirm the specificity of the assay.

Fig. 1. Response of mean arterial pressure (MAP), endometrial blood flow (EBF), and endometrial vascular resistance (EVR) to cumulative intravenous injection of Nω-nitro-L-arginine methyl ester (L-NAME). L-NAME was given at 30-min intervals as 1, 2, 7, and 20 mg/kg (cumulative doses of 1, 3, 10, and 30 mg/kg). Values are means ± SE from 6 animals. *Significantly different from control; †significantly different from 1 mg/kg L-NAME group; ‡significantly different from 3 mg/kg L-NAME group (P < 0.05).
Data Analysis

Values are means ± SE. MAP, endometrial blood flow, and endometrial vascular resistance values were compared using repeated-measures analysis of variance. Values of NOS activity were compared using one-way analysis of variance. Pairwise post hoc comparisons between means were made using Tukey's (least significant difference) criterion. Differences were considered to be significant at \( P < 0.05 \).

RESULTS

Study I: Cumulative Dose Response of L-NAME on MAP, Endometrial Blood Flow, and Endometrial Vascular Resistance

Administration of L-NAME dose dependently produced increases in MAP and endometrial vascular resistance and a decrease in endometrial blood flow (Fig. 1). The increases in MAP and endometrial vascular resistance and the decrease in endometrial blood flow were near maximum with 3 mg/kg of L-NAME.

Study II: Reversal of the E\(_2\) Response by L-NAME and PE

The E\(_2\)-induced increase in endometrial blood flow and decrease in endometrial vascular resistance were reversed after administration of L-NAME (Fig. 2) and PE (Fig. 3). The relative changes in endometrial vascular resistance induced by L-NAME (Fig. 4A) and PE (Fig. 4B) were also compared under baseline as well as E\(_2\)-treated conditions. Doses of L-NAME and PE are...
considered matched, since the relative changes of endometrial vascular resistance are similar between the control groups in Fig. 4. E2β-treated animals were pretreated with 1 µg/kg of E2β 2 h before treatment with L-NAME or PE. No significant difference in relative endometrial vascular resistance values was found between L-NAME and PE treatments at matched doses or between the baseline and the E2β-treated groups.

Fig. 4. Relative changes of EVR induced by L-NAME or PE under control or E2β-treated conditions. Data are from a total of 30 animals. EVR values after L-NAME administration or during PE infusion are shown as relative changes normalized to values under control or E2β-treated conditions and plotted as means ± SE. In A, data for control group are from experiments shown in Fig. 1, and data for E2β-treated group are from experiments shown in Fig. 2. In B, control group was studied under control conditions, PE was infused at 3.2 or 6.4 mg·kg⁻¹·h⁻¹ for 30 min, and data for E2β-treated group are from experiments shown in Fig. 3.

Fig. 5. Blockade of E2β response by L-NAME. L-NAME [1 mg/kg (A, n = 7) or 3 mg/kg (B, n = 8)] was given to animals before injection of 1 µg/kg E2β. MAP, EBF, and EVR were measured at times indicated and are plotted as means ± SE. *Significantly different from control (P < 0.05).
Study III: Blockade of the E<sub>2</sub>β Response by L-NAME and PE

A decrease in endometrial vascular resistance induced by E<sub>2</sub>β was observed in animals pretreated with 1 mg/kg of L-NAME (Fig. 5A), but not in animals pretreated with 3 mg/kg of L-NAME (Fig. 5B). The E<sub>2</sub>β-induced decrease in endometrial vascular resistance was not observed with infusion of PE at 3.2 mg·kg<sup>-1</sup>·h<sup>-1</sup> iv (Fig. 6).

Study IV: Assessment of NOS Activity and eNOS and iNOS Gene Expression

There was greater Ca<sup>2+</sup>-dependent NOS activity than Ca<sup>2+</sup>-independent NOS activity in rat uterus (Fig. 7). E<sub>2</sub>β injection did not change the Ca<sup>2+</sup>-dependent or the Ca<sup>2+</sup>-independent NOS activities (Fig. 7). By contrast, Ca<sup>2+</sup>-independent NOS activity was increased 2 h after LPS injection (Fig. 8). This increase of Ca<sup>2+</sup>-independent NOS activity was accompanied by an increase in gene expression for iNOS (Fig. 9, top), but not for eNOS (Fig. 9, middle). There was no change in iNOS (Fig. 9, top) or eNOS (Fig. 9, middle) gene expression after E<sub>2</sub>β administration. Low NOS activities in the presence of L-NAME (Figs. 7 and 8) confirmed the specificity of the assay. Comparable GAPDH gene expressions from all samples (Fig. 9, bottom) indicate that equal amounts of RNA were used for each RT-PCR set.

DISCUSSION

The identification of endothelium-derived relaxing factor as NO (9, 15) stimulated numerous investigators to elucidate whether the mechanism by which E<sub>2</sub>β modulates vascular tone is by release of NO (7, 8, 23, 27). The precursor for NO synthesis is L-arginine (14, 21), and various analogs of arginine act as competitive inhibitors of NOS (17, 25). These analogs have been utilized to assess the physiological role of NO in vitro and in vivo (3, 6, 8, 17, 23). Two forms of NOS have been identified: constitutive NOS (cNOS) and iNOS. cNOS is Ca<sup>2+</sup>-calmodulin dependent, whereas iNOS is Ca<sup>2+</sup>-calmodulin independent. eNOS, which mediates endothelium-dependent vascular relaxation through the release of NO, is a type of cNOS.
The relationship between E2β and NOS is controversial. Gisclard and colleagues (7) obtained a significant increase in acetylcholine-induced relaxation of the femoral artery after chronic E2β replacement in castrated rabbits. In these studies, rabbits were injected daily with 35 µg/kg im E2β, which increased the circulating E2β concentration to levels seen during estrus. We (8) also previously demonstrated that the basal release of NO from the rabbit thoracic aorta was higher in females than in males, and ovariectomy abolished this difference. However, some investigators demonstrated that the acute effects of E2β in modulating vascular tone of isolated vascular rings are NO independent (13, 28). The primary objective of this study therefore was to assess whether the E2β-induced acute increase in endometrial blood flow is mediated by NO. Endometrial, rather than myometrial, blood flow was estimated, as our previous study indicated that acute administration of E2β caused more profound changes in the blood flow to the endometrium than to the myometrium (30).

In study I, administration of L-NAME to ovariectomized animals raised the MAP and increased endometrial vascular resistance in a dose-dependent manner (Fig. 1). This indicated that NO is involved in the regulation of the basal tone of the uterine vascular bed, even in the absence of E2β, and that a basal release of NO keeps this vascular bed in a partially dilated state similar to that suggested for other vascular beds (17). Doses of 1 and 3 mg/kg of L-NAME were selected for our studies, as MAP and endometrial vascular resistance were significantly higher than basal values after these doses, and the responses reached near-maximal values after administration of 3 mg/kg of L-NAME. In the present study we also observed that when PE was infused at 3.2 and 6.4 mg·kg⁻¹·h⁻¹, the relative increases (normalized to control values) in endometrial vascular resistance under control conditions were comparable to those produced by 1 and 3 mg/kg of L-NAME (Fig. 4). Therefore, these doses of PE and L-NAME were approximately matched in their abilities to raise endometrial vascular resistance.

The decrease in endometrial vascular resistance after E2β administration is apparent by 60 min, and the maximal effect occurs within 2 h (30). In study II, at 2 h after E2β injection, administration of L-NAME (Fig. 2) and PE (Fig. 3) produced graded increases in endometrial vascular resistance, and the relative increases in endometrial vascular resistance were comparable between the two vasoconstrictors (Fig. 4). The magnitudes of these relative changes in endometrial vascular resistance were not different from those produced by these agents in the absence of E2β treatment (Fig. 4). If E2β-induced increase in NO synthesis was responsible for the decrease in endometrial vascular resistance, we would expect a much greater increase in endometrial vascular resistance after L-NAME administration in E2β-treated than in control animals; we would also

![Fig. 8. Effect of vehicle and lipopolysaccharide (LPS) on Ca²⁺-independent NOS activity of rat uterus. At 30 min or 2 h before removal of uterus, animals were injected intravenously with vehicle for E2β (0.1 ml of 1% ethanol) or 1 mg/kg LPS. Assay was also performed in presence of L-NAME in LPS-treated animals to confirm specificity of assay. Values are means ± SE from 5 animals in each group. *Significantly different from control at time 0 (P < 0.05).](http://ajpheart.physiology.org/)

![Fig. 9. Time course of gene expression of inducible NOS (iNOS), endothelial NOS (eNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in rat uterine horn. Lane 1, negative control, where reverse transcriptase polymerase chain reaction was done in absence of RNA. Lanes 2–4, 3–5, and 6–8 represent time course after administration of a single dose of vehicle, E2β, and LPS, respectively. In each treatment group, 1st point represents time 0 (lanes 2, 5, and 8), 2nd point represents 30 min (lanes 3, 6, and 9), and 3rd point represents 2 h (lanes 4, 7, and 10) after administration of each agent. Lane M, φX174/HaeIII molecular weight markers. Arrows, position of reverse transcriptase polymerase chain reaction products for iNOS, eNOS, and GAPDH. Data are from a single experiment that is representative of 3 separate experiments.](http://ajpheart.physiology.org/)
expect a greater increase in endometrial vascular resistance by L-NAME than by PE in E$_2$$\beta$-treated animals, inasmuch as PE causes vasoconstriction through an NO-independent mechanism. However, our data are not consistent with this notion and, therefore, raise doubt that the increase in endometrial blood flow after acute E$_2$$\beta$ treatment is mediated by an increased synthesis of NO. Our interpretation therefore differs from that of other investigators (23). This might be due to the fact that these investigators did not examine the effects of L-NAME on basal uterine vascular tone and did not use PE as a control vasoconstrictor. The dose-dependent reduction in E$_2$$\beta$-elevated uterine blood flow by L-NAME in their study could be simply due to the blockade of NO synthesis that was already present to the same extent under the baseline conditions.

In a study that first demonstrated that NO synthesis could actually be induced in vascular tissue (16), active tone was first induced in isolated aortic rings with PE, then LPS was added to the Krebs solution in the tissue bath containing the vascular ring. LPS was able to reduce the vascular tone in these PE-preconstricted vascular rings, whereas this effect of LPS was not observed in the presence of an inhibitor of NO synthesis. These findings indicated that the LPS-induced decrease in vascular tone was mediated by NO. In study III we utilized a similar protocol to further evaluate whether acute administration of E$_2$$\beta$ can increase NO synthesis. As in the isolated aortic ring study (16), the endometrial vascular resistance was initially increased from basal values by infusion of PE at 3.2 mg·kg$^{-1}$·h$^{-1}$. Then, E$_2$$\beta$ was administered (Fig. 6). A decrease in endometrial vascular resistance was not observed for up to 3 h after E$_2$$\beta$ administration, as would be expected if E$_2$$\beta$ were to increase NO synthesis. On the other hand, a slight decrease in endometrial vascular resistance was observed after E$_2$$\beta$ administration only after the endometrial vascular resistance was increased from basal values by 1 mg/kg, but not by 3 mg/kg, of L-NAME (Fig. 5). These observations provide further evidence that the E$_2$$\beta$-induced increase in endometrial blood flow is not mediated by an increase in NO synthesis.

In study IV, administration of E$_2$$\beta$ to the animals did not increase NOS enzyme activity, nor did it enhance NOS gene expression in the uterus when an increase of endometrial blood flow was expected. On the other hand, LPS, which we used as our positive control, increased the Ca$^{2+}$-calmodulin-independent NOS enzyme activity and iNOS gene expression in the uterus. These data further suggest that an E$_2$$\beta$-induced increase in endometrial blood flow is unlikely to be mediated by an increase in NO synthesis. It is possible that the acute administration of E$_2$$\beta$ may be different, in the ability of modulating NOS activity, from chronic E$_2$$\beta$ replacement, where an increase in the release of NO (8) and an increase in eNOS gene expression are observed (27). The difference between our studies and those of other investigators (23) may also be due to differences in experimental designs or animal models. In our studies we assessed the E$_2$$\beta$-induced increase in endometrial blood flow in ovariectomized rats, whereas other investigators studied the effects of E$_2$$\beta$ on total uterine blood flow in ovariectomized sheep (23).

In conclusion, although the acute effects of E$_2$$\beta$ in decreasing endometrial vascular resistance may be mediated by increased protein synthesis, it seems unlikely that NOS is increased. Other possible mechanisms need to be considered.

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