Endothelial vasodilator production by uterine and systemic arteries. VII. Estrogen and progesterone effects on eNOS

HEIDI L. RUPNOW,1 TERRANCE M. PHERNETTON,1 CYNTHIA E. SHAW,1 MARY L. MODRICK,1 IAN M. BIRD,1 AND RONALD R. MAGNESS1,2

Departments of 1Obstetrics and Gynecology, Perinatal Research Laboratories, and 2Animal Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53715

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Rupnow, Heidi L., Terrance M. Phernetton, Cynthia E. Shaw, Mary L. Modrick, Ian M. Bird, and Ronald R. Magness. Endothelial vasodilator production by uterine and systemic arteries. VII. Estrogen and progesterone effects on eNOS. Am J Physiol Heart Circ Physiol 280: H1699–H1705, 2001.—Uterine blood flow (UBF) and uterine artery endothelial nitric oxide synthase (eNOS) expression are greatest during the follicular vs. luteal phase. 17β-Estradiol (Eβ) increases UBF and elevates eNOS in ovine uterine but not systemic arteries; progesterone (P4) effects on Eβ changes of eNOS remain unclear. Nonpregnant ovariectomized sheep received either vehicle (n = 10), P4 (0.9 g Controlled Internal Drug Release vaginal implants; n = 13), Eβ (5 μg/kg bolus + 6 μg·kg−1·day−1; n = 10), or P4 + Eβ (n = 12). Reproductive (uterine/mammary) and nonreproductive (omental/renal) artery endothelial proteins were procured on day 10, and eNOS was measured by Western analysis. P4 and Eβ alone and in combination increased (P < 0.05) eNOS expression in uterine artery endothelium (vehicle = 100 ± 16%, P4 = 251 ± 59%, Eβ = 566 ± 147%, P4 + Eβ = 772 ± 211% of vehicle). Neither omental, renal, nor mammary artery eNOS was altered, demonstrating the local nature of steroid-induced maintenance of uterine arterial eNOS. In the myometrial microvasculature, eNOS was increased slightly (P = 0.06) with Eβ and significantly with P4 + Eβ. Systemic NOx was increased with P4 and P4 + Eβ, but not Eβ, suggesting differential regulation of eNOS expression and activity, since P4 increased eNOS in uterine artery endothelium while Eβ and the combination further increased eNOS protein.

during the follicular phase of the estrous cycle, when the estrogen-to-progesterone (P4) ratio is increased, uterine blood flow (UBF) is also elevated. UBF increases to its maximum just before ovulation, coinciding with the maximum estrogen-to-P4 ratio. During the luteal phase, when plasma P4 concentrations are high and estrogen is low, UBF returns to basal levels (7). Prolonged systemic estrogen administration to ovariectomized ewes acutely and dramatically elevates reproductive tissue blood flows (e.g., uterine and mammary blood flows) within 120 min; however, UBF falls on days 1–3 and remains slightly elevated above basal levels through 10 days of treatment (13–16). Although total nonreproductive tissue blood flows are elevated by acute and prolonged administration of estrogen, certain nonreproductive systemic vascular beds, i.e., omental and renal, do not exhibit altered perfusion. Previous studies have demonstrated that the acute estrogen-induced increases in UBF are mediated in part by increased expression of endothelial nitric oxide synthase (eNOS) and elevated nitric oxide (NO) production (23, 27, 28). Clearly, NO production can be altered by changes in eNOS expression and activation. This expression of eNOS and its regulation by both exogenous and endogenous estrogen is localized primarily to the endothelium rather than the vascular smooth muscle (VSM) of uterine arteries (17a, 27). After estrogen treatment of ovariectomized ewes, eNOS expression is progressively and substantially elevated in uterine, but not mammary, omental or renal artery endothelium (27). It is unknown, however, if the estrogen-induced rise in mammary blood flow (13) is also regulated by elevations in NO production as was reported for UBF (23, 27). P4 treatment alone does not stimulate UBF; however, it can partially attenuate the acute (≈120 min) nongenomic, estrogen-induced increase in UBF in ovariectomized sheep (5, 9, 14, 22). In contrast, during prolonged estrogen infusion, concomitant P4 treatment does not alter the estrogen-mediated elevation in UBF (1, 14). Anderson et al. (1) observed that caruncular blood flow was increased with the addition of P4 compared with 17β-estradiol (Eβ) alone while P4 decreased the percentage of the total UBF to the cervix and myometrium and the endometrial blood flow is not affected by treatment. It is unknown if exogenous P4 treatment alone or with estrogen coadministration alters eNOS protein expression in reproductive or nonreproductive arteries.

In the present study, we hypothesized that prolonged treatment with P4 alone would have little or no effect on eNOS protein expression in the uterine or systemic artery endothelium and, when given in combination with estrogen, would partially attenuate the

Address for reprint requests and other correspondence: R. R. Magness, Dept. of Obstetrics and Gynecology, Univ. of Wisconsin, Perinatal Research Laboratories, 7E Meriter Hospital, 202 S. Park St., Madison, WI 53715 (E-mail: rmagness@facstaff.wisc.edu).

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estrogen-induced increase in eNOS. The specific objectives of this study were to determine 1) the effects of P₄ alone and in combination with estrogen on eNOS expression in the reproductive (uterine and mammary) vs. nonreproductive (renal and omental) endothelial-isolated proteins; 2) whether P₄ ± estrogen treatment would affect changes in eNOS levels in the VSM of any of the artery types studied; 3) whether P₄ ± estrogen would increase eNOS protein expression in the microvessels of the endometrium, caruncles, or myometrium; and 4) whether systemic plasma NO levels were increased during P₄ and/or estrogen treatment in correlation with eNOS protein expression.

MATERIALS AND METHODS

Prolonged estrogen and P₄ treatment. Mixed Western breed ewes (n=45) were ovarioectomized via a midventral laparotomy as previously described (13, 15, 16), and, after at least 10 days, steroid replacement therapy was administered. For the 17β-estradiol-treated ewes (E₂; n=10), an indwell- ing 19-gauge polyvinyl catheter was placed in the right ven- tricle via the jugular vein. Animals then received a 5 μg/kg bolus of E₂β (Sigma; St. Louis, MO) mixed in 3 ml (~10–11% ethanol) of saline; the catheter was flushed with 5 ml of saline followed immediately by 6 μg·kg⁻¹·day⁻¹ continuous infusion in 9.5% ethanol isotonic saline (0.0123 ml/min) for 10 days. E₂β dose and timed tissue collection were based on eNOS expression and hemodynamic responses as well as blood levels of E₂β achieved in our previous studies (15, 16, 27). For administration of P₄ (n=13), three controlled internal drug release (CIDR) im- plants with 9 g P₄ (Carter Holt Harvey) were placed in the vagina of ovarioctomized ewes for 10 days. For combined treatment (P₄ + E₂β; n=12), both E₂β infusion and CIDR P₄ implants were compared as described above. Control ewes (vehic- le; n=10) received vehicle (ethanol in saline) infusion and/or blank CIDR implants. With this protocol, P₄ levels in systemic circulation were elevated in P₄ and P₄ + E₂β- treated ewes above luteal-phase ewes and control (time 0; control = 0.06 ± 0.04 ng/ml; luteal = 2.44 ± 0.35 ng/ml; P₄ = 3.53 ± 0.27 ng/ml; P₄ + E₂β = 3.16 ± 0.69 ng/ml). P₄ levels during E₂β treatment were 0.20 ± 0.16 ng/ml and were not different from control values (0.18 ± 0.10 ng/ml). These observed P₄ levels are slightly lower than those observed by Scudamore et al. (26) with the use of three CIDR implants. E₂β levels during E₂β and combination treatments were elevated above controls but were very variable because of interassay variability (data not shown). The ewes were euthanized with pentobarbital sodium, which was given intra- venously (≈50 mg/kg). Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin-Madison Research Animal Care Committees of both the Medical School and the College of Agriculture and Life Sciences and followed the recommended American Veterinary Medicine Association guidelines for euthanasia of laboratory farm animals.

Isolation and preparation of vessels. We used the previously described rapid isolation procedure to obtain endothelial-derived proteins that are devoid of VSM contamination (17). Briefly, uterine, mammary (reproductive), omental, and renal (nonreproductive) arteries were excised, placed in PBS (8 mM sodium phosphate, 2 mM potassium phosphate, and 0.15 M NaCl, pH = 7.4; Sigma), dissected free of connective tissue, and rinsed free of blood. Portions of each artery type were opened longitudinally, and the endothelium/tunica in- tima was gently scraped (3–6 times) from the artery and placed in lysis buffer (50 mM Tris, 0.15 M NaCl, and 10 mM EDTA, pH = 7.4, plus the addition of 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 M phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml apro tin; all from Sigma) by using a curved-end spatula, as previously described (17, 27). The remaining “scraped” vessel was rubbed with a wet cotton swab, and any remaining adventitia was extensively removed before the denuded artery (VSM) was placed in lysis buffer. The endothelial-isolated proteins and denuded arter- ies (VSM) were snap-frozen in liquid nitrogen immediately on collection and were stored at −20°C. Additional intact artery segments were collected for immunohistochemistry and fixed in 4% formaldehyde in sodium cacodylate buffer (0.1 M, pH = 7.4; EM Science; Fort Washington, PA) for 24 h and then stored at 4°C in sodium cacodylate buffer containing 0.01% sodium azide until dehydration and placement in paraffin blocks.

Isolation of uterine microvessels. Uterine tissue was ob- tained from the above-treated ewes at the time of death. Caruncles, endometrium, and myometrium were removed from the uterus. Microvessels (=200–500 μm for endome- trium, 300–500 μm for caruncles, and 500–800 μm for myo- metrium) were identified, and the surrounding tissue was gently teased away. The microvessels were placed in lysis buffer and snap-frozen.

Preparation of tissues and Western analysis. VSM from uterine, mammary, renal, and omental arteries and the uter- ine microvessels were homogenized in lysis buffer and then sonicated. Endothelial-isolated proteins from uterine, mammary, renal, and omental arteries were also sonicated. After centrifugation (250 g for 10 min) to remove particulate mat- ter from vessel preparations, the protein concentrations were determined using a modified Lowry assay procedure (Bio- Rad; Hercules, CA). Proteins (30 μg for VSM and endometrial microvessels, 10 μg for endothelial-isolated proteins, 20 μg for caruncles and myometrial microvessels) were resolved on precast 7.5% polyacrylamide gels (15 well; Bio-Rad) with 0.1% SDS at 100 V for 1.5 h at room temperature before transfer to Immobilon P membranes at 100 V for 2 h. Mem- branes were blocked with Tris buffer (20 mM Tris base, 5% skim milk for VSM and endometrial microvessels, 10% for endothelial-isolated proteins, 20% for caruncles and myometrial microvessels) and then stored at −20°C. The primary eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY; N30020, 1:750 dilution), and a positive control, human umbilical endothelial cell lysate was included on each blot. The secondary antiserum was a sheep anti-mouse Fab₂ se- rum (1:2,000; Amersham; Piscataway, NJ). Primary antibody was dissolved in Tris buffer containing 1% BSA and Tween 20, and the secondary antibody was dissolved in Tris buffer with Tween 20 and 0.5% skim milk. Primary antibody incubations were for 2 h at room temperature, and secondary antibody incubations were for 1 h with one 15-min and three 5-min washes with Tris buffer and Tween 20 after each antibody incubation. The membrane was probed as described by Amersham using the enhanced chemiluminescence kit and exposure to Hyperfilm for 10 min. The relative level of eNOS in each sample was determined using a Bio-Rad scan- ning transmission densitometer model 670 coupled with Bio- Rad Molecular Analyst software (version 1.5, build 468). Data are expressed as percentage of vehicle calculated from the mean absorbance units noted on the same Western blot.

Immunohistochemical analysis of arteries. Immunohisto- chemical analysis for eNOS was performed with the use of a Vectastain ABC Elite kit (Vector Laboratories; Burlington,
CA) and a mouse monoclonal antibody from Transduction Laboratories, as previously described (17, 27).

Blood NOx analysis. Blood samples obtained from each ewe immediately before death were spun, and plasma was stored at \(-20^\circ C\). At the time of NO analysis, a 500-\(\mu\)l blood sample was added to 1 ml chilled 100% ethanol, vortexed, and centrifuged (12,000 \(g\)) for 5 min. NO analysis on the supernatant was measured using a Seivers Instruments model 280 Nitric Oxide Analyzer (Boulder, CO) that measures NOx based on a gas-phase chemiluminescence reaction between NO and ozone. Briefly, samples were injected in the purge vessel where nitrates and nitrites in the sample react with \(V_3Cl_3\) to produce NO. The NO gas then flowed into the nitric oxide analyzer, where it reacted with ozone to produce nitrite, which could be quantified by luminescence. The area under the peak was calculated, and a value for the amount of NO in the sample was determined against a standard curve (17a, 29, 34).

**Statistical analysis.** Differences in treatment groups (vehicle vs. \(P_4\), \(P_4 + E_2\beta\), and \(P_4 + E_2\beta\)) for each vessel and plasma were analyzed using Student’s \(t\)-tests and one-way ANOVA.

**RESULTS**

Protein expression for eNOS in endothelial-isolated proteins and VSM of uterine and systemic arteries. Elevations in eNOS protein expression with steroid treatment were specific to the endothelium of the uterine vasculature since this was the only vascular bed studied where significant changes in eNOS were observed. Expression of eNOS protein was increased \((P < 0.05)\) in uterine artery endothelium under prolonged \(P_4\), \(E_2\beta\), and \(P_4 + E_2\beta\) treatments \((251 \pm 59, 566 \pm 147, \text{and } 772 \pm 211\% \text{ of vehicle, respectively})\) when compared with vehicle \((100 \pm 16\%); Fig. 1). Furthermore, protein expression with \(E_2\beta\) treatment alone and the combination of steroid hormones was significantly elevated over \(P_4\) treatment alone. There were no significant differences observed in eNOS protein expression in the endothelium with any hormone treatment in the mammary artery, the other reproductive artery studied \((Fig. 2)\). Neither systemic nonreproductive artery \((omentum or renal)\) showed any increase in eNOS protein expression \((Fig. 2)\). The level of eNOS protein was undetectable by our methods in the VSM of uterine, renal, omental, and mammary arteries, consistent with our previous data \((17, 27; \text{data not shown})\).

Immunohistochemistry of intact uterine and systemic arteries for eNOS followed similar qualitative trends to the results seen by Western blot analysis \((data not shown)\) as was previously shown \((27)\). Localization of eNOS was primarily in the endothelium; however, some patchy staining was noted in the VSM of all artery types studied using immunohistochemical staining similar to what was previously observed, but this was not consistent \((27)\).

**eNOS protein expression in the uterine microvasculature.** Uterine microvessels obtained from the endometrium, myometrium, and caruncles of steroid-treated ewes were analyzed for eNOS levels by Western blot analysis. No significant differences in eNOS expression were observed in the caruncular or endometrial microvessels \((Fig. 3)\). In contrast, significant increases in eNOS were observed only in the myometrial microvessels with \(P_4 + E_2\beta\) treatments, whereas \(E_2\beta\) showed a slight elevation \((P = 0.06;\)
Blood NOx levels. NOx levels in plasma of the ovariec-tomized nonpregnant sheep averaged 4.42 ± 1.07 μM. P4 treatment alone increased (P < 0.05) NO levels over estrogen but in comparison with vehicle did not reach significance (Fig. 4; P = 0.10; vehicle = 100 ± 26%, P4 = 203 ± 54%, E2β = 282 ± 79%, P4 + E2β = 235 ± 57% of vehicle).

**DISCUSSION**

Data from the current study confirm and extend our previous data and are the first to demonstrate that eNOS protein expression is elevated in the uterine artery endothelium with the administration of P4 as well as estrogen alone and that, when given in combination, there is an increase similar to estrogen alone. Treatment with estrogen or both P4 plus estrogen increased eNOS protein expression in the myometrial microvessels. Furthermore, treatment with P4 increased the amount of NO in the systemic circulation, and estrogen had little effect on this P4-induced increase. Thus changes in eNOS protein expression in the uterine artery endothelium alone are insufficient to account for changes in NOx.

UBF is also elevated during prolonged administration of estrogen (13, 14, 16). Estrogen appears to mediate this elevation in UBF through eNOS, since NOS inhibition reduces the estrogen-induced increase in UBF (17, 23, 27). The current study confirmed the prolonged estrogen-induced increases in uterine artery eNOS protein expression first observed by us (27) and confirmed by others (25) and was designed to determine the relevance, if any, that P4 had on this effect.

P4 alone has little vasodilatory effect on the uterine vasculature (1, 5, 9, 14, 22). However, in this study, we showed that P4 administered alone for 10 days induced eNOS protein expression in uterine artery endothelium. The lack of an increase in blood flow to parallel an increase in the enzyme producing a potent vasodilator may indicate a failure to activate the protein. There is a paucity of studies on the in vivo effects of P4 treatments on vascular function in vitro. In vitro BHT-920 treatment, which induces vascular relaxation via endothelial α2-adrenergic receptors, caused maximum relaxation in canine coronary arteries from animals treated with estrogen. In contrast, when an in vivo combination of P4 and estrogen was given, the estrogen-associated BHT-920-induced coronary artery relaxation was no longer achieved (18). Goetz et al. (8) showed that estrogen increases translocation of eNOS in bovine aortic endothelial cells while P4 alone had no effect on cellular distribution and so, by implication, activation of eNOS.

Estrogen induces a rapid acute increase in UBF with 120 min of infusion that remains elevated, although at a more modest level, above control through 10 days of infusion (7, 9, 13–16). We have previously shown that estrogen treatment alone increases eNOS protein expression in uterine artery endothelium with maximum expression seen at 8–10 days of treatment (27). In fetal pulmonary artery endothelial cell cultures, eNOS protein and mRNA expression are stimulated within 48 h at physiological doses of estrogen (11). Estrogen treatment also increases nNOS in uterine artery VSM (25), prolonged estrogen-induced increases in uterine artery eNOS protein expression first observed by us (27) and confirmed by others (25) and was designed to determine the relevance, if any, that P4 had on this effect.
which may account for some portion of the vasodilatory effects of estrogen.

During prolonged combined P4 and estrogen treatment, UBF is intermediate between that seen with the two hormones alone (5, 9, 14, 22). However, we found that treatment with the combination of the two hormones for 10 days showed elevations in eNOS protein expression that were greater than P4 alone but similar to estrogen alone. This elevation of eNOS was nearly as high as the increases seen during pregnancy, a time of increase in both estrogen and P4 (17, 17a). Although the combination treatment with estrogen and P4 may partially mimic the rises in uterine artery endothelial-derived eNOS expression (Fig. 1), it did not increase UBF to the very high levels observed in late-gestation sheep (12), presumably again through a failure to achieve activation of eNOS. Furthermore, during pregnancy, other factors, such as the growth factors vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF), from the placenta may be necessary for activation as we have previously seen these growth factors involved in the activation of NO (4, 12).

In the myometrial microvessels of the uterus, we observed that eNOS protein expression was significantly upregulated by the combination of estrogen plus P4 and tended to be increased by estrogen alone. We did not, however, observe significant steroid-induced changes in eNOS levels in endometrial or caruncular microvessels. To our knowledge, this is the first comparison of microvascular resistance vessel changes in eNOS expression within the uterus. Anderson et al. (1) showed increases in blood flow to the caruncles during P4 treatment compared with estrogen alone and increased flow to the myometrium and cervix during estrogen treatment. They also observed no hormone effect on blood flow to the endometrium and intermediate flows with estrogen and P4 treatment together (1). Our recent data (14) show increases in blood flow to all regions of the uterus with estrogen and combination treatment. In the current studies, however, we only observed increases in eNOS expression in the myometrial microvessels (Fig. 3), consistent with a lack of concomitant activation of eNOS.

The other reproductive vascular bed studied in the current experiment (mammary artery) showed no eNOS protein elevations during any hormone treatment. However, we have observed elevations in blood flow to the mammary gland during prolonged estrogen, P4, and combination treatment (14). In mammary arteries obtained from men undergoing bypass surgery, using a ring suspension method, Nechmad et al. (20) showed that E2β induced marked vasorelaxation in rings with endothelium compared with those without. Furthermore, they showed that this vasorelaxation could be completely inhibited with the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) and the estrogen receptor antagonist tamoxifen and greatly enhanced with the calcium ionophore A23187. This indicates that the mammary artery in this system is dilating due to NOS enzyme activation rather than elevations in total protein levels, as we do not see any increase in eNOS protein expression in the mammary artery endothelium with our in vivo treatment regimen.

Systemic vascular beds (omentum and renal) in the sheep do not show elevations in blood flow during physiological states such as pregnancy and the follicular phase of the ovarian cycle or with exogenous ovarian steroid administration (7, 12, 14, 15). Furthermore, it has been shown that eNOS protein expression is not regulated during the ovarian cycle or pregnancy in the renal artery used as a systemic bed (17, 27). Our results confirm that eNOS expression is not regulated in either of these particular nonreproductive vascular endothelia by estrogen and further add that P4 does not alter eNOS levels in these tissues. Veille et al. (28) showed that, in estrogen-treated ewes, uterine arteries had increased NOS expression, as measured by calcium-driven arginine-to-citrulline conversion (NOS activity) compared with vehicle controls and was completely blocked with the NOS inhibitor L-NAME. In contrast, renal arteries from the same estrogen-treated ewes showed no such increase in NOS expression (28). It is noteworthy that these studies were not performed in the presence of P4.

NO was elevated in the systemic blood with P4 and P4 plus estrogen. There are several mechanisms that could explain why NO is higher in P4-treated and not estrogen-treated ewes. One possibility is that P4 is increasing NO production by stimulating other isoforms of NOS in tissues other than the vascular beds that we investigated. Yallampalli et al. (30) showed decreased production of NO in the uterus with estrogen- and estrogen plus P4-treated rats compared with P4 alone. Recently, it has been shown that estrogen given to late pregnant rats decreased NO production and decreased inducible NOS (iNOS) mRNA and protein expression in a dose-dependent manner (31). It has been hypothesized that iNOS may generate larger amounts of NO compared with that of eNOS (19). It is noteworthy that we and others could not detect iNOS protein expression in uterine arteries from sheep (27) or humans (21) as well as ovine uterine tissue (33). Therefore, it is possible that P4 may be affecting iNOS in other organs/tissues not investigated herein, thus increasing NO in the systemic circulation. However, in human, postmenopausal women under estrogen replacement therapy showed elevations in circulating NO levels above baseline and placebo controls within 1 mo of administration, which remained elevated through 6 mo of study (6). Another estrogen replacement study showed elevations in plasma NO, but these studies were also in the presence of some P4 (2, 24). It is therefore possible that P4 may be causing some of the increase in systemic NO. Bezooijen et al. (3) showed that, in ovariectomized rats, oral administration of ethinyl estradiol significantly increased plasma NO while E2β was not able to increase NO when given orally but did so when administered subcutaneously. These data indicate that route of administration and/or type of estrogen may play a role in the observed ste-
Estradiol-mediated elevations of plasma NO. Furthermore, in this study they showed that P₄ given alone and in combination with ethinyl estradiol increased plasma NO; thus, as was shown in our current study, P₄ may influence circulating levels of NO.

To achieve increases in NOₓ measured in the systemic circulation, eNOS expression and eNOS activation may be necessary. We (14, 17a) and others (32, 34) have observed increased systemic NO and uterine artery eNOS levels in late-gestation sheep compared with nonpregnant controls when estrogen and P₄ are both elevated and blood flow is maximum. In perfused uterine arteries from pregnant ewes, NO is increased over uterine arteries from nonpregnant ewes at basal levels and with stimulation using both ATP and the calcium ionophore A23187 (29). eNOS protein expression is also increased in these perfused uterine arteries (29). Nelson et al. (21) recently reported that uterine arteries from pregnant women produced greater basal and bradykinin- and ionomycin-induced NO-mediated cGMP production. Further work in our laboratory with a uterine artery endothelial cell culture model has shown that cell signaling mechanisms are different between the pregnant and nonpregnant cells independent of calcium elevation (4). For example, extracellular signal-regulated kinase 2 phosphorylation was only achieved in pregnant vs. nonpregnant cells with ANG II, ATP, bFGF, and VEGF stimulation. Therefore, it is possible that activation of eNOS protein may require the presence of other factors, as seen in the pregnant state. Furthermore, it has been shown in fetal pulmonary artery endothelium that estrogen acutely stimulates NOS activity but that this activation is independent of changes in eNOS protein expression, suggesting activation at the cell signaling level (10, 11).

In conclusion, both estrogen and P₄ alone and in combination increase eNOS protein expression in uterine but not systemic artery endothelium. These increases in eNOS protein expression only partially account for the elevations in UBF seen during prolonged estradiol and P₄ and during physiological states such as the follicular phase of the ovarian cycle. Therefore, future work discerning the mechanisms of activation of eNOS and/or signal transduction is necessary to understand the physiological effects of ovarian steroids on UBF.

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