Vasorelaxant action of 17β-estradiol in rat uterine arteries: role of nitric oxide synthases and estrogen receptors

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Submitted 26 June 2007; accepted in final form 15 October 2007

Scott PA, Tremblay A, Brochu M, St-Louis J. Vasorelaxant action of 17β-estradiol in rat uterine arteries: role of nitric oxide synthases and estrogen receptors. Am J Physiol Heart Circ Physiol 293: H3713–H3719, 2007. First published October 19, 2007; doi:10.1152/ajpheart.00736.2007.—The uterine vasculature plays an important role during pregnancy by providing adequate perfusion of the maternal-fetal interface. To this end, substantial remodeling of the uterine vasculature occurs with consequent changes in responsiveness to contractile agents. The purpose of our study was to characterize the vasorelaxant effects of estrogens on vascular smooth muscles of the rat uterine artery during pregnancy and to evaluate the involvement of estrogen receptors (ESR) and nitric oxide synthases (NOS). To do so, we measured NOS expression in the whole uterine and mesenteric circulatory bed by Western blotting. Vasorelaxant effects of 17β-estradiol (17β-E2) were assessed on endothelium-denuded uterine arteries with wire myographs in the absence and presence of pharmacological modulators [nitro-L-arginine methyl ester (L-NAME), ICI-182780, tamoxifen]. All experiments were performed on arteries from nonpregnant (NP) and late pregnant (P) rats. In the uterine vasculature of the latter group, NOS3 (endothelial NOS) expression was increased, while NOS1 (neuronal NOS) was reduced compared with NP of the latter group, NOS3 (endothelial NOS) expression was increased, while NOS1 (neuronal NOS) was reduced compared with NP rats. Expression of the NOS2 (inducible NOS) isoform was undetectable in the two groups. Both 17β-E2 and 17α-E2 induced uterine artery relaxation, but the latter evoked lower responses. Endothelium-denuded arteries from NP rats showed larger relaxation with 17β-E2 than P rats. This larger relaxation disappeared in the presence of 17β-estradiol (E2) range between 0.1 and 0.4 ng/ml during the normal menstrual cycle (1). After conception, the E2 serum level increases progressively until it reaches 6–30 ng/ml at term (21). In nonpregnant rats, E2 ranged from 3 to 19 pg/ml during the estrous cycle, rising to 30–57 pg/ml on the last (22nd) day of gestation (15, 20). E2 has been shown to augment blood flow in the uterine vasculature (22, 32). Given the high exposure of the utero-placental bed to the direct and massive secretion of estrogens during gestation, these hormones should play a significant role in the uterine arterial circulation during pregnancy. Estrogen may act via two different pathways: genomic and nongenomic (2). The simplified classical view of the genomic pathway needs estrogen to bind estrogen receptor (ESR1) (formerly ERα) or ESR2 (formerly ERβ), favoring dimerization. The dimer formed can regulate the transcription of target genes that have estrogen response element sequences in their promoter region. On the other hand, estrogen has been reported to induce some of its effects in a short period of time that could not involve new protein synthesis. These rapid actions of estrogen, referred to as nongenomic actions, could arise from endothelial cell activation or occur directly on vascular smooth muscle (VSM) cells (VSMC). Many studies have disclosed that arterial relaxation mediated by estrogen could result from endothelial cell activation, which releases nitric oxide (NO) (7) and leads to cGMP pathway activation in VSMC that could activate calcium-activated potassium channels (33). However, this pathway is probably not the main route through which E2 elicits vasorelaxation, because it has been demonstrated several times that E2 can relax endothelium-denuded arteries (9, 18). With the use of pharmacological levels of E2, vascular relaxation in the absence of endothelium was correlated with decreased Ca2+ influx (17, 40), increased K+ efflux (41, 44), and intracellular cAMP elevation (25) in VSMC. However, it has been discerned in the porcine coronary artery that E2 relaxation of VSM requires protein kinase G activation by augmenting cAMP and then possibly opening large-conductance Ca2+-activated K+ channels (BKCa) (18). A more recent study suggested that E2-induced relaxation involves NO activation of BKCa in human coronary smooth muscle cells after stimulation of neuronal nitric oxide synthase (nNOS, NOS1) (16).

In addition, seven-transmembrane G protein-coupled receptor 30 (GPR30) has been reported to trigger rapid cellular signaling when activated by estrogen (14). Binding to this receptor was found to be highly specific to 17β-E2, with 17α-E2 having much less affinity, similar to that of these estrogens; pregnancy

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compounds for ESR (29). GPR30 has been localized in the cytoplasm (13) and probably in the endoplasmic reticulum (29), possibly with a small GPR30 pool at the plasma membrane (12). It has also been shown that GPR30, expressed intracellularly, is capable of initiating cellular signaling, and insufficient GPR30 is expressed on the cell surface to initiate signaling in response to impermeable ligands [E2:bovine serum albumin (BSA)] (30).

NOS are responsible for the production of NO, a volatile gas with well-known vasorelaxant properties on vascular tissues. NOS exist in three isoforms: NOS3 [endothelial NOS (eNOS)], mainly present in the vascular endothelium; NOS1 (nNOS), present in neurons and innervated tissues, such as smooth muscles; and NOS2 [inducible NOS (iNOS)], inducible and expressed ubiquitously. Several studies have reported that NOS3 expression is increased in the uterine vasculature during estrogen treatment, the follicular phase, and pregnancy, suggesting that endothelially generated NO is involved in the vasorelaxant actions of E2 (23, 27, 28). However, little is known about changes in NOS2 and NOS1 expression in uterine arteries during pregnancy. NOS1 expression has been shown to be completely abolished in the VSMC of human umbilical cords after preeclamptic pregnancy (36).

Since increased NOS3 expression in the uterine arterial endothelium during pregnancy is well-documented, what are the direct effects of E2 on uterine arterial smooth muscle (in the absence of endothelium)? How are these effects modulated during pregnancy in regard to the significant remodeling that occurs in arterial walls during this condition? Our study was designed to characterize the acute effect of estrogens on VSM of uterine arteries, determine how it is affected by pregnancy, and establish whether ESR and NO participates in the acute relaxant action of E2. We hypothesized that the acute action of 17β-E2 on endothelium-denuded uterine arteries is mediated by a pathway independent from ESR but involving some participation of nonendothelial NOS.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Charles River Canada, St-Constant, QC, Canada; total of 120) weighing 225–250 g were mated with fertile males. The morning on which spermatozoa were observed in vaginal smears was considered day 1 of pregnancy. Virgin rats of the same age (±14–15 wk of age) served as controls without consideration of the stage of the estrous cycle. All animals had ad libitum access to food (Teklad global 18% protein rodent diet, Harlan Teklad; Montreal, QC, Canada) and tap water. They were killed by decapitation on day 22 of pregnancy (parturition on day 23). Their main uterine arteries in the middle portion along the uterine horn were cleaned of fat and connective tissues, and rings (1.5–2.0 mm) were prepared and mounted in wired myographs as described previously (39), with some modifications. For instance, the endothelium was removed by passing Triton X-100 (0.03% vol/vol) in the artery before its mounting on myograph supports similarly to the method of Connor and Feniuk (11). The passive tension applied on the uterine arteries was equivalent to 60 mmHg (Lpas), which is the passive circumference of the arterial segment would have had at this transmural pressure.

After the arterial segments were set up in myographs, they were stretched three times to minimize subsequent hysteresis and allowed to equilibrate (10 min) in the absence of passive tension. The segments were stretched in steps of ∼50 μm in diameter until ~2–3 mN/mm (1 mN = 102 mg) of wall tension was reached. The vessels were held at each length for 2–3 min, and wall tension was recorded. The data were fitted to the exponential curve \( y = Ae^{tx} \) to evaluate \( L_{sp} \), as previously described (39). After equilibration at this passive length for 30 min, reactivity of the arterial segments was evaluated by challenge with 1 μmol/l phenylephrine (Phe). At plateau response the absence of a functional endothelium was verified by adding 10 μmol/l carbachol, and only arteries that did not respond were used. The arterial segments were washed three times and, 45 min later, the vessels were restimulated with 1 μmol/l Phe in the absence or presence of nitro-l-arginine methyl ester (l-NNAME, 100 μmol/l) or ICI-182780 (20 μmol/l) added 15 min before Phe. When the plateau response was reached, cumulative concentrations of estrogens (17α-E2 or 17β-E2) were added to generate concentration-relaxation curves. We also performed these experiments with 17β-E2/BSA conjugates, followed by another relaxation to 17β-E2.

Statistical analysis. For Western blotting, each value was standardized to β-actin from the same blot. Differences between pregnant (P) and nonpregnant (NP) groups were determined by Student’s t-test. In the myotropic experiments, responses to estrogens were expressed in percentage of relaxation from the increased tone induced by Phe (1 μmol/l). Concentration-relaxation curves were analyzed by nonlinear regression with Prism software (GraphPad Software, San Diego, CA): \( Y = E_{max}/1 + 10^{logEC_{50} - X_{slope}} \), where \( X \) is the logarithm of estrogen.
concentration and $Y$ is the response obtained (% relaxation). Maximum response ($E_{\text{max}}$) and the concentration of agonist producing 50% of $E_{\text{max}}$ ($EC_{50}$) were compared between groups by F-test (Prism).

**Drugs and chemicals.** All salts used in these experiments were of analytical grade and obtained from Fisher Scientific (Montreal, QC, Canada) and Bio-Rad. Phe (phenylephrine hydrochloride), carbachol (carbamylcholine chloride), $17\alpha$-E2, and $17\beta$-E2 were purchased from Sigma (St. Louis, MO). ICI-182780 was procured from Tocris Cookson (Ellisville, MO). 1,3,5(10)-Estratrien-3,17$\beta$-diol-6-one 6-carboxymethyloxime:BSA and 1,3,5(10)-estratrien-3,17$\beta$ diol 17-hemisuccinate:BSA were purchased from Steraloids (Newport, RI). Stock solutions of ICI-182780, $17\alpha$-E2, and $17\beta$-E2 were prepared in DMSO, and $17\beta$-estradiol:BSA conjugates were prepared in water.

**RESULTS**

Uterine artery diameter, under passive tension equivalent to 60 mmHg transmural pressure, increased twofold at the end of pregnancy compared with NP rats (NP 290 ± 5 μm, P 560 ± 7 μm; $P < 0.001$, $n = 60$ each). As documented previously (39), Phe (1 μmol/l) induced a much larger response (almost double) in arteries of P compared with NP rats (Fig. 1, A and B, insets), and this response was not affected by preincubation with L-NAME. $17\beta$-E2 evoked concentration-dependent relaxation of the uterine arteries in both groups of animals. However, relative (%) relaxation by $17\beta$-E2 in endothelium-denuded uterine arteries was greater in NP compared with P rats ($E_{\text{max}}$, 118 ± 3% for NP vs. 106 ± 4% for P; $P < 0.05$, $n = 10$ each) (Fig. 1). This larger relaxation observed in the arteries of NP rats was blocked when their tissues were preincubated with L-NAME ($E_{\text{max}}$ from 118 ± 3% to 100 ± 6%; $P < 0.01$, $n = 10$ each; Fig. 1A). The stereoisomer $17\alpha$-E2, documented to have a reduced affinity for ESR (19), induced relaxation of the uterine arteries in both groups, but of significantly reduced magnitude compared with $17\beta$-E2. For instance, maximum relative relaxation with $17\alpha$-E2 in the uterine arteries of NP rats was 76 ± 10% (Fig. 2B), which was similar for the arteries of P animals. However, sensitivity to both agonists was similar (−log $EC_{50}$ = 5.62 ± 0.13 for $17\alpha$-E2 and 5.47 ± 0.05 for $17\beta$-E2; not significant).

Preincubation of arterial segments with 20 μmol/l ICI-182780, an ESR antagonist, did not affect the vasorelaxant responses to $17\beta$-E2 and $17\alpha$-E2 (Fig. 2). Similar results were obtained with tamoxifen, an ESR antagonist (data not shown). Moreover, $17\beta$-E2:BSA conjugates, in concentrations similar to those of $17\beta$-E2, did not produce any relaxation in endothelium-denuded uterine arteries (data not shown), but $17\beta$-E2 then applied confirmed the relaxation capacity of the arterial segments.

We measured NOS expression by Western blot analysis in whole uterine and mesenteric vascular beds, the latter being representative of the systemic circulation in NP and P rats. Figure 3, A and B, show that, in both vasculatures, NOS3 expression was increased at the end of pregnancy, this difference seeming to be relatively more significant in the uterine compared with the mesenteric circulation (−4.5X vs. −1.5X). NOS2 expression in both vasculatures was undetectable (Fig. 3C; mesenteric not shown). In contrast, we observed that NOS1 was significantly decreased (Fig. 3E) during pregnancy in the uterine but not in the mesenteric vasculature (Fig. 3D).

**DISCUSSION**

The purpose of this investigation was to characterize the vasorelaxant effects of estrogens on the VSM of uterine arteries and their difference during pregnancy. We wanted to evaluate the involvement of the different NOS and of ESR in relation to the acute relaxation induced by estrogens.

First, we observed that uterine artery diameter doubled before parturition compared with NP rats, indicating that this vessel undergoes considerable remodeling during pregnancy, as occurs in all arteries of this circulatory bed (5, 39). Second, we demonstrated that $17\beta$-E2 was able to induce concentration-dependent acute relaxation in endothelium-denuded main uterine arteries. Maximum vasodilatation was greater for NP than P rats (Fig. 1). Furthermore, this larger relative relaxation in arteries of NP rats was reduced after preincubation with L-NAME to the level found in arterial segments from P rats (Fig. 1). These observations demonstrate that E2 induces direct acute VSM relaxation of the uterine artery in rats, and they suggest some involvement of nonendothelial NO production under $17\beta$-E2 application, since the arteries were denuded of their endothelium. Moreover, this NO production in uterine artery VSM is obliterated during gestation.

We have also disclosed that $17\alpha$-E2 induced smaller maximal relaxations than $17\beta$-E2 in uterine arteries. The effect of $17\alpha$-E2 relative to $17\beta$-E2 is a controversial issue in the...
literature. Indeed, some laboratories have reported that 17β-E₂ relaxed pig coronary arteries without achieving complete dilation when contracted with Ca²⁺ (34), while others noted that 17β-E₂ generated relaxation similar to that of 17α-E₂ in the mesenteric arteries of rats (26). On the other hand, 17α-E₂ displayed only minor effects on mesenteric artery diameter over a concentration range similar to the effective vasodilatory concentration of 17β-E₂ in the same strain of rats (37). Our work reveals that the maximal relaxation induced by 17β-E₂ is around two-thirds of that generated by 17α-E₂. Comparison remains difficult, since our experiments were carried out in different arteries (uterine) from animals of a different sex (female). Various vasculatures may present distinct contractile properties. For example, reactivity to Phe in mesenteric arteries is decreased during gestation (24, 38), whereas it is increased in uterine arcuate arteries (39). Various vasculatures may present distinct contractile properties. For example, reactivity to Phe in mesenteric arteries is decreased during gestation (24, 38), whereas it is increased in uterine arcuate arteries (39). However, our comparative data on the effects of 17α-E₂ and 17β-E₂ indicate that the former acts as a partial agonist compared with the latter, because they have different efficacies with similar potencies. In the present study, maximal vascular effects of estrogens require supraphysiological concentrations of free hormone. We must take into consideration that in order to measure vasorelaxant effects of estrogen the tone of the isolated blood vessels should be increased to supraphysiological levels. High concentrations of vasorelaxant are then needed to induce measurable relaxation. It is now generally accepted that concentrations of estrogen higher than those found in plasma are needed (43).

We also wanted to investigate whether the acute vasodilatory action of 17β-E₂ was mediated via intracellular or membrane-localized interactions. An earlier study reported that E₂:BSA was able to relax the pressurized mesenteric arteries of rats with intact endothelium (37). The authors suggested that estrogens may have nongenomic effects that appear to be mediated via stereospecific interactions at the plasma membrane level. In our experiments, two different membrane-impermeant, BSA-conjugated 17β-estradiols (BSA on C17 or C6) were used, and both failed to produce vasorelaxation of endothelium-denuded uterine arteries (data not shown), indicating that estrogen must at least penetrate the plasma membrane of smooth muscle cells to mediate its effects when endothelial cells are removed. To further evaluate ESR involvement in the vasodilatation observed in endothelium-denuded arteries, we performed the same protocol in arterial segments pretreated with ICI-182780 (Fig. 2) or tamoxifen (data not shown) before measuring the concentration-relaxation curves to 17β-E₂ and 17α-E₂. These ESR antagonists did not affect relaxation generated by either 17α-E₂ or 17β-E₂. This finding suggests that E₂-induced relaxation of uterine artery VSM uses a pathway different from ESR. It has been reported that ICI-182780 was also unable to block estrogen-mediated relaxation in other vasculatures, such as the mesenteric arteries of rats (37) and coronary arteries of dogs (40), while the vasorelaxant effects of 17β-E₂ were partially inhibited by ICI-182780 in rat aortas (6). This indicates that the
mechanism by which estrogen exerts its relaxing action could be different in distinct vascular beds and different when the endothelium is present or absent. To our knowledge, our observation is original for the uterine artery. These considerations lead us to postulate that estrogens induce vasodilatation of endothelium-denuded uterine arteries via a mechanism that does not require ESR. However, it shows stereospecificity because 17β-E2 also relaxed the uterine arteries with potency similar to that of 17β-H9252-E2, but with about two-thirds of the latter’s efficacy.

As mentioned above, GPR30 is a candidate effector for the nongenomic actions of E2. It has been determined that GPR30 is located in the cytoplasm and that it initiates cellular signaling from inside the cell (30). Does the absence of relaxation to E2:BSA and the incapacity of ICI-182780 to block E2 in our study mean that these responses might be mediated by GPR30? First, we should demonstrate the presence of these molecules in uterine artery VSMC. With growing interest in effectors similar to GPR30, new tools (agonists) (3) should be developed, and it will then be imperative to see whether this receptor is involved in the vasorelaxant effects of E2 observed in uterine arteries.

We showed by Western blotting that NOS3 expression was increased in mesenteric and uterine vessels during gestation. These data are in agreement with several other studies in animals (23, 28) and humans (27). Moreover, the relative increase of NOS3 in uterine vessels was higher than in mesenteric vessels, as also reported in ewes (23). Despite these observations, augmented NOS3 expression cannot be evoked to explain the higher relaxation generated by 17β-E2 in NP compared with P rats, since our myotropic experiments were carried out in uterine arteries denuded of their endothelium, and NOS3 was found exclusively in the endothelium of uterine arteries of sheep and ewes (8, 35). In addition, we were unable to detect NOS2 in uterine and mesenteric vessels. These results concur with several studies in which NOS2 was not seen in the uterine or resistance vessels of animals (35, 42) and humans (27). Finally, we showed that NOS1 expression did not vary in mesenteric vessels, while it was markedly decreased in uterine vessels at the end of gestation. Another investigation reported
that NOS1 expression tended to be reduced during pregnancy in human uterine arteries but did not reach significance (27). In contrast, Rosenfeld et al. (31) noted increased NOS1 expression in the uterine arteries of ewes after daily 17β-E2 administration. Our data indicate that NOS1 is responsible for NO production in the VSMC of uterine arteries in NP rats. This NO production allows the larger vasorelaxation induced by 17β-E2 and is lost during pregnancy when NOS1 expression is decreased.

In conclusion, in the present study, we demonstrate that 17β-E2 and 17α-E2 induced relaxation of the uterine arteries by an endothelium-independent mechanism. Also, 17β-E2 produced larger relative relaxation in endothelium-denuded uterine arteries of NP compared with P rats because of higher NO production, believed to derive from NOS1. Because E2:BSA conjugates did not relax the uterine arteries, we think that estrogen can relax them by some intracellular interactions in smooth muscle cells, this response being ESR independent, since ESR antagonists were unable to block 17β-E2, and 17α-E2-induced relaxation. However, this acute effect of estrogens manifested some stereosepecificity, indicating that a putative, receptive, specific structure is responsible for the effect. A better understanding of the mechanisms by which estrogens influence uterine vascular function and remodeling during pregnancy may help us to know what causes reduced perfusion of the utero-placental unit in clinical conditions such as intrauterine growth restriction.

GRANTS
This work was supported by Canadian Institutes of Health Research (CIHR) Grant MOP 62790 to J. St. Louis, and P.-A. Scott was the recipient of a studentship from the CIHR.

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