Vasorelaxing effects of estetrol in rat arteries

Rob H P Hilgers, Suzanne Oparil, Wout Wouters¹ and Herjan J T Coelingh Bennink¹

Division of Cardiovascular Disease, Department of Medicine, Vascular Biology and Hypertension Program, University of Alabama at Birmingham, 1034 Zeigler Research Building, 703 19th Street South, Birmingham, Alabama 35294-0007, USA

¹Pantarhei Bioscience, Zeist, The Netherlands

(Correspondence should be addressed to S Oparil; Email: soparil@uab.edu)

Abstract

This study compared *ex vivo* relaxing responses to the naturally occurring human hormone estetrol (E₄) vs 17 β -estradiol (E₂) in eight different vascular beds. Arteries were mounted in a myograph, contracted with either phenylephrine or serotonin, and cumulative concentration-response curves (CRCs) to E₄ and E₂ (0.1–100 μ mol/l) were constructed. In all arteries tested, E₄ had lower potency than E₂, although the differential effect was less in larger than smaller arteries. In uterine arteries, the nonselective estrogen receptor (ER) blocker ICI 182 780 (1 μ mol/l) caused a significant rightward shift in the CRC to both E₄ and E₂, indicating that the relaxation responses were ER dependent. Pharmacological blockade of nitric oxide (NO) synthases by N^w-nitro-L-arginine methyl ester (L-NAME) blunted E₂-mediated but not E₄-mediated relaxing responses, while inhibition of prostaglandins and endothelium-dependent

hyperpolarization did not alter relaxation to either E₄ or E₂ in uterine arteries. Combined blockade of NO release and action with L-NAME and the soluble guanylate cyclase (sGC) inhibitor ODQ resulted in greater inhibition of the relaxation response to E₄ compared with E₂ in uterine arteries. Endothelium denudation inhibited responses to both E₄ and E₂, while E₄ and E₂ concentration-dependently blocked smooth muscle cell Ca²⁺ entry in K⁺-depolarized and Ca²⁺-depleted uterine arteries. In conclusion, E₄ relaxes precontracted rat arteries in an artery-specific fashion. In uterine arteries, E₄-induced relaxations are partially mediated via an endothelium-dependent mechanism involving ERs, sGC, and inhibition of smooth muscle cell Ca²⁺ entry, but not NO synthases or endothelium-dependent hyperpolarization.

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Introduction

The hormone estetrol (estra-1,3,5(10)-trien-3,15 α -16 α ,17 β -tetrol or E₄) was discovered in 1965 (Hagen *et al.* 1965). E₄ is produced in nature from 17 β -estradiol (E₂) and estrone (E₃) only by the human fetal liver during pregnancy via 15 α - and 16 α -hydroxylase (Schwers *et al.* 1965, Gursipide *et al.* 1966). The concentration of E₄ increases during pregnancy in both the fetus and the mother, but 24 h after delivery, the level is undetectable.

The chemical structure of E₄ is closely related to that of E₂, but the pharmacokinetic, metabolic, and endocrinological profile of E₄ is substantially different from that of E₂. E₂ has high affinity for the estrogen receptor α (ER α) and ER β (K_i values: 0.2 and 0.05 nmol/l respectively), while E₄ has low-to-moderate affinity for ER α and ER β (K_i values: 4.9 and 19 nmol/l respectively; Coelingh Bennink *et al.* 2008a). E₂ has very low oral availability because over 99% of an oral dose is converted into estrone (E₁) and E₁ sulfate. Most of the circulating E₂ is bound to either albumin or sex hormone binding globulin (SHBG), further decreasing plasma levels of free E₂ by a factor of about 30. By contrast, E₄ does not undergo phase I metabolism by human HepG2 cells and does

not bind to SHBG (Hammond *et al.* 2008). As a result of these favorable metabolic and protein binding properties, E₄ has excellent oral potency despite its low-to-moderate affinity for ERs.

E₄ has been studied in many validated models and has been shown to behave as a full ER agonist, similar to E₂, in most of these (Coelingh Bennink *et al.* 2008b, Heegaard *et al.* 2008, Holinka *et al.* 2008, Visser & Coelingh Bennink 2009). However, important differences between E₄ and E₂ were noted. E₄ did not induce synthesis of SHBG *in vitro* as did E₂ (Hammond *et al.* 2008). Recent studies showed that E₄ behaved as an ER antagonist in *in vitro* and *in vivo* models of estrogen-dependent breast tumors in the presence of E₂. E₂ behaved as full agonist in these models (Coelingh Bennink *et al.* 2008c). E₄ is now undergoing clinical testing as the estrogenic component of a combined oral contraceptive and for its effects on breast tumors.

The physiological function of E₄ is not completely understood, although a role in regulating uterine blood flow has been suggested. Unilateral intrauterine injection of E₄ in nonpregnant oophorectomized ewes has been shown to increase uterine blood flow, although with a 15- to 30-fold lower potency than E₃ (Levine *et al.* 1984). Similarly,

administration of E₂ to ovariectomized ewes results in a rapid uterine vasodilation leading to a rise in uterine blood flow within 30–45 min (Killam *et al.* 1973). This rise in uterine blood flow is partially mediated via the release of nitric oxide (NO) and resultant increases in cGMP secretion, as shown by local infusion of the NO synthase blocker N^ω-nitro-L-arginine methyl ester (L-NAME; Van Buren *et al.* 1992, Rosenfeld *et al.* 1996). The dilating effect of E₂ on ovine uterine arteries is ER dependent, as shown by local infusion of the nonselective ER blocker ICI 182 780 in nonpregnant ewes (Magness *et al.* 2005).

To further profile E₄, this study was designed to study the *in vitro* vasorelaxing effects of E₄ and to compare them with those of E₂. The objectives of this study were 1) to determine the *ex vivo* relaxing effects of E₄ compared to E₂ in rat uterine, thoracic aortic, carotid, mesenteric, pulmonary, renal, middle cerebral, and septal coronary arterial segments; 2) to assess the involvement of ERs in the vasorelaxing response to E₄ in uterine arteries; and 3) to assess the endothelium-dependent and endothelium-independent vasorelaxing effects of E₄ in uterine arteries. The uterine artery was chosen for assessment of the acute vasoactive properties of E₄ and for comparison of these to the effects of E₂ because the uterine arterial circulation is known to be highly sensitive to the vasodilator effects of E₂. The uterine arteries are exposed to high physiological levels of E₂ during the follicular and luteal phases of the menstrual cycle and during pregnancy, resulting in major increases in uterine blood flow (Magness 1998).

Materials and Methods

Animals

A total of 40 female nulliparous Sprague Dawley rats (age 12 weeks) were obtained from Charles River Breeding Laboratories, maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (0600–1800 h), and fed a standard rat pellet diet (2016 Teklad Global 16% Protein Rodent Diet (Harlan Laboratories, Teklad Diets, Madison, WI, USA) *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Vessel preparation

Rats were killed by CO₂ inhalation and the uterus, thoracic aorta, left common carotid artery, mesentery, lungs, left kidney, brains, and heart were removed and placed in cold Krebs–Ringer buffer (KRB) with the following composition (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 5.5 glucose (all purchased from Sigma–Aldrich). Tissue samples were pinned down onto a 90 mm glass petri dish coated with black Sylgard and soaked in cold KRB. Main uterine arterial segments running along

both uterine horns were cleared of adipose and connective tissues. The thoracic aorta and left common carotid artery were cleaned of connective tissue and cut into 2.5–3 mm segments. A fourth-order branch segment of the superior mesenteric artery was dissected from the mesentery; a small (200–300 µm in diameter) pulmonary arterial segment was dissected from the lung; a segment of the left main renal artery was dissected, and a segment of the middle cerebral artery was dissected from the brain. The atria and the left ventricle were removed to expose the interventricular septum of the heart, and a segment of the septal coronary artery was dissected. Aortic and carotid artery segments were mounted between two stainless steel pins, whereas the other segments (all 2 mm long) were mounted between two stainless steel jaws connected with two wires (40 µm in diameter) through the lumen of the segment in a myograph chamber (Danish Myo Technology, Inc., Aarhus, Denmark) filled with 5 ml KRB solution, maintained at 37 °C, and continuously aerated with 95% O₂ and 5% CO₂. Four arterial segments were analyzed in parallel. The remaining segments were temporarily stored at 4 °C.

Determination of optimal diameters

Thoracic aortae and carotid and pulmonary arteries were passively stretched according to a procedure first described by Mulvany & Halpern (1977). Briefly, the segments were distended stepwise in 100 µm increments measured with a built-in micrometer and the wall tension (N/m) was recorded using data acquisition (Powerlab 8/35, ADInstruments, Colorado Springs, CO, USA) and recording Software (ChartLab7, Colorado Springs, CO, USA). Thoracic aortic segments and carotid arteries were stretched at wall tension corresponding to a pressure of 90 mmHg, whereas pulmonary arteries were stretched at a wall tension corresponding to 40 mmHg. At this passive wall tension, segments were contracted with high K⁺ KRB (60 mmol/l KCl in KRB solution; replacing equimolar NaCl with KCl), thus generating active wall tension, which was set to a 100% contraction level.

All other arterial segments were progressively and actively stretched to the internal diameter at which the largest contractile response to 10 µmol/l norepinephrine (NE) or 60 mmol/l K⁺ KRB (middle cerebral arteries) was obtained. This internal diameter was referred to as the optimal diameter and the corresponding active wall tension was set to a 100% contraction level.

Arterial integrity was assessed by contracting arterial segments to either 1 µmol/l phenylephrine (PHE; for uterine, thoracic aorta, carotid, mesenteric, pulmonary, and renal arteries) or 0.1–1 µmol/l serotonin (5-HT; for middle cerebral and septal coronary arteries), followed by endothelium-dependent relaxation with 1 µmol/l acetylcholine (ACh). Arteries that relaxed immediately (> 50% of relaxation) were considered to have a functional endothelium.

Experimental protocols

Cumulative concentration-response curves (CRCs) were constructed with PHE (0.16–20 $\mu\text{mol/l}$) or 5-HT (0.001–10 $\mu\text{mol/l}$). Arterial segments were then washed with KRB and after 10 min were contracted with a single concentration of the appropriate contractile agent, resulting in a near maximal contraction (80–100% of active wall tension obtained with 10 $\mu\text{mol/l}$ NE or 60 mmol/l K^+ depolarization). During a stable contraction, a CRC to E_4 (0.1–100 $\mu\text{mol/l}$) was performed. After a 30-min washout period, segments were again contracted with the appropriate contractile agent and the CRC was repeated with E_2 (0.1–100 $\mu\text{mol/l}$). The order of application of the estrogenic compounds was altered on every experimental day. In a subset of uterine arteries, CRCs to E_4 and E_2 were run in parallel. Relaxing responses to E_2 and E_4 were unaltered when run in series compared with parallel application.

We constructed CRCs to the selective $\text{ER}\alpha$ agonist propyl-[1H]-pyrazole-1,3,5-triyl-triphenol (PPT; 0.1–30 $\mu\text{mol/l}$; [Stauffer et al. 2000](#)) and the selective $\text{ER}\beta$ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; 0.1–30 $\mu\text{mol/l}$; [Meyers et al. 2001](#)) and compared their uterine arterial relaxing responses to E_4 and E_2 . To study the involvement of ERs ($\text{ER}\alpha$ and $\text{ER}\beta$) in the uterine arterial relaxing responses to E_4 in comparison to E_2 , arteries were incubated for 30 min with the nonselective ER antagonist ICI 182 780 (7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; 1 $\mu\text{mol/l}$; [Wakeling et al. 1991](#)), after which the arterial segments were contracted with a single concentration of PHE (1–10 $\mu\text{mol/l}$) followed by a CRC to either E_4 or E_2 .

To assess whether E_4 triggered the release of vasodilator and/or contractile prostaglandins in uterine arteries, the cyclooxygenase inhibitor indomethacin (INDO; 10 $\mu\text{mol/l}$) was used. The involvement of endothelium-derived NO in uterine arterial relaxing responses to E_4 was assessed using the nonselective NO synthase blocker L-NAME (100 $\mu\text{mol/l}$). The combined application of L-NAME and INDO was used to assess the role of endothelium-derived hyperpolarizing factor (EDHF) in E_4 -mediated relaxing responses in uterine arteries. The involvement of cGMP in E_4 -mediated relaxation was assessed by incubating uterine arteries with L-NAME, INDO, and the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 $\mu\text{mol/l}$).

The hyperpolarization response, involving both release of EDHF and spread of a hyperpolarizing current, is initiated in the endothelium via activation of small and intermediate calcium-activated K^+ channels (SK_{Ca} and IK_{Ca} respectively; [Burnham et al. 2002](#)). Blockade of both K_{Ca} channels results in complete blockade of the EDHF response in rat mesenteric arteries ([Crane et al. 2003](#)). However, in some arteries, such as skeletal arterioles and coronary arteries, the large-conductance K_{Ca} (BK_{Ca}) is involved in EDHF-mediated responses ([Feher et al. 2010](#)). We did not attempt to study the role of the BK_{Ca} channel blocker iberiotoxin in E_4 - and E_2 -mediated

relaxing responses in uterine arteries. 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-trietheno-1,1,7-metheno-7H-dibenzo [b,n] [1,5,12,16]tetraazacyclotricosine-5,13-diiium dibromide (UCL 1684; 1 $\mu\text{mol/l}$; [Campos Rosa et al. 2000](#)) was used to block SK_{Ca} channels and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; 1 $\mu\text{mol/l}$; [Wulff et al. 2000](#)) was used to block IK_{Ca} channels. All inhibitors were applied 30 min before the addition of PHE. After washing with KRB, arterial segments were again incubated with the appropriate pharmacological blocker(s) and subsequently (after 10 min) contracted with PHE (1–10 $\mu\text{mol/l}$) followed by a CRC to either E_4 or E_2 .

The role of endothelium in E_4 -mediated relaxing responses was assessed using endothelium-denuded uterine arteries. The endothelium was mechanically removed by gently rubbing the lumen with a human hair ([Osol et al. 1989](#)). Successful denudation was achieved when relaxation to 1 $\mu\text{mol/l}$ ACh was absent. Endothelium-independent relaxing responses to the NO donor sodium nitroprusside (SNP; 0.1–10 000 nmol/l) were performed in PHE (10 $\mu\text{mol/l}$)-contracted endothelium-intact arteries.

The potential inhibitory effect of E_4 on voltage-operated smooth muscle cell Ca^{2+} entry was assessed by incubating endothelium-intact uterine arteries with three different concentrations (10, 30, and 100 $\mu\text{mol/l}$) of E_4 or vehicle (ethanol) for 10 min in Ca^{2+} -free and high K^+ KRB solution, followed by a cumulative addition of CaCl_2 (0.01–2.5 mmol/l). To rule out modulating effects of other endothelium-derived relaxing factors, arteries were incubated with L-NAME (100 $\mu\text{mol/l}$), INDO (10 $\mu\text{mol/l}$), and ODQ (10 $\mu\text{mol/l}$). Results were compared with three different concentrations (3, 10, and 30 $\mu\text{mol/l}$) of E_2 .

Drugs

E_4 was provided by Pantarhei Bioscience B.V. (Zeist, The Netherlands) and dissolved in ethanol (stock solution of 10 mmol/l). ACh, endothelin-1, L-NAME, NE, PHE, serotonin, and SNP were purchased from Sigma-Aldrich and dissolved in distilled H_2O . INDO and E_2 (Sigma-Aldrich) were dissolved in ethanol (stock solutions of 10 mmol/l). TRAM-34 (Sigma), ODQ (EMD Chemicals, Gibbstown, NJ, USA), PPT, DPN, and UCL 1684 (all from Tocris Bioscience, Ellisville, MO, USA) were dissolved in DMSO.

Data and statistical analysis

Contractile responses were expressed as a percentage of the maximal contractile response to 10 $\mu\text{mol/l}$ NE or 60 mmol/l K^+ before the administration of any pharmacological inhibitor. Relaxing responses were expressed as a percentage of the maximal contractile response to PHE or 5-HT. Individual CRCs were fitted to a sigmoid regression curve (GraphPad Prism 5.0). As the sigmoidal curve of E_4 could not be fully defined, a constant plateau value (set to 100%) was defined in order for GraphPad Prism to calculate

Table 1 Optimal diameters and active wall tensions to NE (10 μmol/l) or depolarizing potassium (K⁺) solution (60 mmol/l KCl in Krebs–Ringer solution) for rat uterine, aortae, carotid, mesenteric, pulmonary, renal, middle cerebral, and septal coronary arteries. Uterine, mesenteric, pulmonary, and renal arteries were contracted with NE. Aortae, carotid, middle cerebral, and septal coronary arteries were contracted with depolarizing K⁺ solution. When more arteries of the same type were isolated from one rat, the values were averaged per rat. Values are shown as mean ± s.e.m.

Artery type	Uterine	Aorta	Carotid	Mesenteric	Pulmonary	Renal	Middle cerebral	Septal coronary
Optimal diameter (μm)	317 ± 3	1467 ± 17	587 ± 7	228 ± 5	217 ± 9	478 ± 19	232 ± 4	247 ± 7
Active wall tension (N/m)	4.81 ± 0.10	5.24 ± 16	2.07 ± 0.11	2.41 ± 0.09	1.51 ± 0.16	4.92 ± 0.29	1.41 ± 0.10	0.88 ± 0.07
<i>n</i>	40	6	27	27	11	24	24	19

NE, norepinephrine. *n* denotes the total number of rats used.

a LOGEC₅₀ (pEC₅₀ value indicating sensitivity). Maximal relaxation to the highest concentration of E₄ or E₂ (E_{max}) and pEC₅₀ values are shown as mean ± s.e.m. Statistical significance of effects and differences were analyzed using either one-way ANOVA (comparison of pEC₅₀ and E_{max}) or two-way ANOVA (comparison of CRCs). A Bonferroni *post hoc* test was used to compare multiple groups. A *P* value < 0.05 was considered statistically significant.

Results

Arterial integrity

Optimal diameters of uterine arteries measured in the wire myograph averaged 317 ± 3 μm and arteries developed an average active wall tension of 4.81 ± 0.10 N/m in response to 10 μmol/l NE. Optimal diameters and active wall tensions for all artery types are summarized in Table 1. Sensitivity (pEC₅₀) and maximal contraction to PHE (uterine, carotid, mesenteric, pulmonary, and renal artery) or 5-HT (middle cerebral and septal coronary artery) and sensitivity and maximal

relaxations to 1 μmol/l ACh and the NO donor SNP for all arteries are shown in Table 2.

Vasorelaxing responses to E₄

In uterine arteries, pEC₅₀ for the control estrogenic compound E₂ averaged 5.44 ± 0.05 and reached a near maximal relaxation (E_{max}: 93 ± 1%) in response to the highest concentration tested (100 μmol/l; Tables 3 and 4 and Fig. 1A). pEC₅₀ and E_{max} for E₄ were significantly lower (4.36 ± 0.07 and 74 ± 4% respectively; Tables 3 and 4 and Fig. 1A). Hence, in uterine arteries, E₄ was a 14-fold less potent vasodilator compared with E₂. Vasorelaxing properties of E₄ compared with E₂ for the other seven arterial types are shown in Fig. 1B through H and are summarized in Tables 3 and 4. E₄ resulted in much lower variability than E₂ in pEC₅₀ and E_{max} values for all artery types tested (Tables 3 and 4). Larger arteries (aorta, carotid, and renal) had lower pEC₅₀ and E_{max} than smaller arteries (mesenteric, pulmonary, middle cerebral, septal coronary, and uterine).

Table 2 Contractile characteristics to phenylephrine or serotonin, responses to a single concentration of ACh (1 μmol/l), and cumulative concentrations of SNP in contracted rat uterine, carotid, mesenteric, middle cerebral, pulmonary, renal, and septal coronary arteries. Sensitivity (pEC₅₀) to contractile agents and to the NO donor SNP is calculated by GraphPad Prism Software as described in the Materials and Methods section and represents the negative logarithmic concentration of the contractile agent that induces a 50% tension level compared to E_{max}. Maximal contraction to contractile agent (E_{max}) is calculated at the percentage tension compared to the AWT corresponding to the artery's optimal diameter. The percentage contraction before ACh application is calculated as the percentage tension compared to the AWT corresponding to the artery's optimal diameter. Values are shown as mean ± s.e.m.

Artery type	Uterine	Carotid	Mesenteric	Pulmonary	Renal	Middle cerebral	Septal coronary
Contractile agent	Phenylephrine	Phenylephrine	Phenylephrine	Phenylephrine	Phenylephrine	Serotonin	Serotonin
<i>n</i>	10	9	10	6	10	9	7
pEC ₅₀	5.78 ± 0.03	6.57 ± 0.52	6.07 ± 0.04	5.98 ± 0.36	6.11 ± 0.02	7.14 ± 0.13	6.56 ± 0.10
E _{max} (% of AWT)	100 ± 2	99 ± 12	105 ± 2	56 ± 13	109 ± 2	81 ± 5	121 ± 16
<i>n</i>	21	19	17	6	18	18	15
Percentage of contraction before ACh	85 ± 3	69 ± 4	92 ± 3	65 ± 8	80 ± 5	66 ± 4	95 ± 8
Percentage of relaxation to ACh	47 ± 4	57 ± 6	83 ± 4	80 ± 8	59 ± 5	22 ± 3	63 ± 7
<i>n</i>	10	8	10	6	10	9	6
SNP (pEC ₅₀)	7.30 ± 0.04	8.13 ± 0.03*	6.92 ± 0.07	7.17 ± 0.08	6.91 ± 0.08*	6.50 ± 0.10*	7.62 ± 0.08*
E _{max} (%)	79 ± 4	99 ± 1*	83 ± 4	83 ± 11	65 ± 4	77 ± 5	99 ± 1*

ACh, acetylcholine; SNP, sodium nitroprusside; NO, nitric oxide; AWT, active wall tension; *n*, number of experiments. **P* < 0.05 vs uterine.

Table 3 Vasorelaxing properties (sensitivity and maximal relaxing responses) of E₂ compared with E₄ in isolated and endothelium-intact rat uterine, aorta, carotid, and mesenteric (fourth-order) arteries. Arteries were contracted with the appropriate contractile agent before application of the estrogenic compound. The contraction before application of E₄ or E₂ is expressed as percentage of the contraction in response to 10 µmol/l NE. The sensitivity (pEC₅₀) of E₄ or E₂ is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E₄ or E₂ that induces 50% relaxation compared with the maximal relaxation (E_{max}). Potency of E₂ over E₄ is calculated as 10(pEC₅₀(E₂) – pEC₅₀(E₄)) from paired arteries only. Values are shown as mean ± S.E.M.

Artery type	Uterine		Aorta		Carotid		Mesenteric	
	E ₂	E ₄	E ₂	E ₄	E ₂	E ₄	E ₂	E ₄
Control (%)	100 ± 4	95 ± 3	95 ± 11	96 ± 10	101 ± 7	97 ± 7	89 ± 3	92 ± 2
pEC ₅₀	5.44 ± 0.05	4.36 ± 0.07*	4.61 ± 0.08	4.30 ± 0.15	4.91 ± 0.05	4.39 ± 0.15*	5.78 ± 0.13	4.62 ± 0.08*
E _{max} (%)	93 ± 1	74 ± 4*	59 ± 7	68 ± 8	62 ± 4	65 ± 6	99 ± 1	91 ± 3
Potency (fold)	14 ± 2		2 ± 1		5 ± 2		18 ± 4	
n	16	16	6	6	10	10	8	8

E₂, 17β-estradiol; E₄, estetrol; NE, norepinephrine; n, number of experiments. *P < 0.05 vs E₂.

Role of ERs in uterine and carotid arterial relaxing responses to E₄

To study the contributions of ER subtypes α and β in relaxing responses in uterine arteries, selective agonists for ERα and ERβ were first tested. pEC₅₀ values for the ERα agonist PPT were significantly greater than those for the ERβ agonist DPN (5.43 ± 0.10 vs 4.77 ± 0.25 respectively; Fig. 2A).

The nonselective ER blocker ICI 182 780 (1 µmol/l) resulted in significant rightward shifts in the CRCs to E₄ and E₂ in uterine (Fig. 2B) arteries. For E₄, pEC₅₀ averaged 4.54 ± 0.08 in vehicle and 4.05 ± 0.11 in ICI 182 780-treated uterine arteries (Fig. 2B). For E₂, pEC₅₀ averaged 5.56 ± 0.07 in vehicle and 5.12 ± 0.05 in ICI 182 780-treated uterine arteries (Fig. 2B).

Role of endothelium-derived relaxing factors in uterine arterial relaxing responses to E₄

The cyclooxygenase inhibitor INDO (10 µmol/l) did not significantly alter sensitivity to E₄ or E₂ (Fig. 3A). The NO synthase blocker L-NAME (100 µmol/l) caused a significant rightward shift in the CRC to E₂, but not to E₄ (Fig. 3B).

The same trend as with L-NAME alone was observed when uterine arteries were incubated with both L-NAME and INDO (Fig. 3C). We next tested the role of cGMP in mediating the response to E₄ and E₂ by preventing NO release and action by blocking the sGC with ODQ (10 µmol/l) in combination with L-NAME and INDO. Interestingly, blockade of cGMP release blunted the response to E₄ but not to E₂ (Fig. 3D). The role of EDHF in mediating relaxing responses was pharmacologically tested by the addition of TRAM-34 (1 µmol/l) and UCL 1684 (1 µmol/l), inhibitors of SK_{Ca} and IK_{Ca} respectively. Inhibition of SK_{Ca} and IK_{Ca} channels in the combined presence of L-NAME and INDO did not alter the response to E₄ or E₂ (Fig. 3E).

The contribution of the endothelium to relaxing responses to E₄ was assessed by mechanical removal of the endothelium. Successful denudation was confirmed by the absence of relaxation to 1 µM ACh in PHE-contracted arteries (0 ± 1%). A significant rightward shift in the CRCs to E₄ and E₂ was observed in endothelium-denuded compared with endothelium-intact uterine arteries (Fig. 3F). Table 5 summarizes the effects of pharmacological inhibitors and endothelial denudation on the vasodilator properties of E₄ compared with E₂ in rat uterine arteries.

Table 4 Vasorelaxing properties (sensitivity and maximal relaxing responses) of E₂ compared to E₄ in isolated and endothelium-intact rat pulmonary, renal, middle cerebral, and septal coronary arteries. Arteries were contracted with the appropriate contractile agent before application of the estrogenic compound. The contraction before application of E₄ or E₂ is expressed as percentage of the contraction in response to 10 µmol/l NE. The sensitivity (pEC₅₀) of E₄ or E₂ is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E₄ or E₂ that induces 50% relaxation compared to the maximal relaxation (E_{max}). Potency of E₂ over E₄ is calculated as 10(pEC₅₀(E₂) – pEC₅₀(E₄)) from paired arteries only. Values are shown as mean ± S.E.M.

Artery type	Pulmonary		Renal		Middle cerebral		Septal coronary	
	E ₂	E ₄	E ₂	E ₄	E ₂	E ₄	E ₂	E ₄
Control (%)	93 ± 14	97 ± 6	81 ± 2	84 ± 3	77 ± 6	79 ± 4	94 ± 5	99 ± 12
pEC ₅₀	5.60 ± 0.09	4.35 ± 0.06*	5.25 ± 0.11	4.30 ± 0.05*	5.49 ± 0.07	4.22 ± 0.07*	5.68 ± 0.10	4.74 ± 0.04*
E _{max} (%)	99 ± 1	80 ± 8	81 ± 2	66 ± 4*	97 ± 1	71 ± 4*	100 ± 0	98 ± 2
Potency (fold)	20 ± 6		11 ± 3		22 ± 5		10 ± 2	
n	4	4	8	8	8	8	4	4

E₂, 17β-estradiol; E₄, estetrol; NE, norepinephrine; n, number of experiments. *P < 0.05 vs E₂.

Figure 1 Relaxing responses to 17 β -estradiol (E_2 ; 0.1–100 μ mol/l; closed circles) and estrol (E_4 ; 0.1–100 μ mol/l; open circles) in contracted uterine (A), aorta (B), carotid (C), fourth-order mesenteric (D), pulmonary (E), renal (F), middle cerebral (G), and septal coronary (H) arteries. Values are expressed as mean $\pm$ S.E.M. * P < 0.05 vs E_2 .

Inhibition of calcium entry by E_4 in K^+ -depolarized uterine arteries

From Fig. 3F, it is clear that there is a significant endothelium-independent relaxation response to both E_4 and E_2 . We therefore analyzed whether E_4 could inhibit the entry of Ca^{2+} via voltage-operated Ca^{2+} channels on smooth muscle

cells. Uterine arteries were first depleted of intracellular Ca^{2+} by washing them three times with 60 mmol/l Ca^{2+} -free K^+ KRB, followed by the addition of either vehicle (ethanol), or three different concentrations of E_4 or E_2 . Cumulative addition of $CaCl_2$ resulted in a contraction that was not significantly blocked by the lowest concentrations tested, namely 10 μ mol/l E_4 (Fig. 4A) and 3 μ mol/l E_2 (Fig. 4B). Next, we compared the inhibitory effects of higher concentrations of E_4 (30 and 100 μ mol/l) and E_2 (10 and 30 μ mol/l). A concentration-dependent inhibitory effect of both E_4 and E_2 was observed. Overall, the results in Fig. 4 show that E_4 has tenfold lower potency than E_2 in inhibiting Ca^{2+} entry in depolarized smooth muscle cells.

Discussion

This study assessed the *ex vivo* relaxing potency of the steroid hormone E_4 in comparison to E_2 in eight arterial beds, i.e. uterine artery, thoracic aorta, the left common carotid artery, the fourth-order branch of the superior mesenteric artery, pulmonary artery, left main renal artery, middle cerebral artery, and septal coronary artery of the rat. In all arteries tested, E_4 had a weaker relaxing potency than E_2 . Pharmacological blockade experiments revealed that E_4 caused relaxation of precontracted rat uterine arteries via both an endothelium-dependent (involving ER) and an ODQ-sensitive mechanism. Furthermore, E_4 inhibited smooth muscle cell Ca^{2+} entry and contraction, albeit with tenfold lower potency than E_2 .

Estrogens exert their biological effects by binding to specific ERs, primarily ER α and ER β . Previous *in vitro* binding studies showed that E_4 has moderate affinity for human ERs, with four to five times higher affinity for the ER α compared with the ER β (Visser *et al.* 2008). The same study reported that E_4 has tenfold lower affinity for the ER α .

Figure 2 Relaxing responses to the ER α agonist PPT (0.1–30 μ mol/l; circles) and the ER β agonist DPN (0.1–30 μ mol/l; squares) in PHE-contracted uterine arteries (A). Relaxing responses to 17 β -estradiol (E_2 ; 0.1–100 μ mol/l; closed symbols) and estrol (E_4 ; 0.1–100 μ mol/l; open symbols) in PHE-contracted uterine arteries in the presence of the nonselective estrogen receptor blocker ICI 162 780 (1 μ mol/l) or the absence of ICI 162 780 (VEH; 10 μ l DMSO; B). Values are expressed as mean $\pm$ S.E.M. * P < 0.05 vs PPT (A). * P < 0.05 vs E_4 VEH; ** P < 0.05 vs E_2 VEH (B).

Figure 3 Relaxing responses to 17 β -estradiol (E_2 ; 0.1–100 μ M/l; closed symbols) and estetrol (E_4 ; 0.1–100 μ M/l; open symbols) in PHE-contracted uterine arteries in the absence of any pharmacological inhibitor (VEH), in the presence of the cyclooxygenase inhibitor IND (10 μ M/l; A); the NO synthase blocker L-NAME (100 μ M/l; B); the combined presence of L-NAME and IND (L-N+I; C); the combined presence of L-NAME, IND, and the selective sGC blocker ODQ (10 μ M/l; L-N+I+O; D); the combined presence of L-NAME, IND, and the selective IK_{Ca} channel blocker TRAM-34 (1 μ M/l) and the selective SK_{Ca} channel blocker UCL 1684 (1 μ M/l; E); and in the presence (+ENDO) and absence (–ENDO) of endothelium (F). Values are expressed as mean $\pm$ S.E.M. * P < 0.05 vs E_4 VEH; ** P < 0.05 vs E_2 VEH.

compared with the reference compound diethylstilbestrol (DES) and 100-fold lower affinity for the $ER\beta$ compared with DES (Visser *et al.* 2008). Kitazawa *et al.* (1997) showed that DES has threefold higher potency than E_2 in relaxing rat femoral arteries. Based on these observations, it was postulated that E_4 would have weaker vasorelaxant potency than E_2 . The current *ex vivo* arterial reactivity study clearly showed that this is indeed the case for a broad spectrum of rat artery types. E_4 was a much weaker ($\sim$ 14-fold) vasorelaxant than E_2 in

PHE-contracted endothelium-intact uterine arteries in the absence of any pharmacological inhibitor. Thus, substitution of two hydrogen atoms for two hydroxyl groups on the carbon 15 and 16 positions of the E_2 molecule appears to result in lower receptor affinity and a reduced functional response.

In smaller resistance arteries, e.g. the middle cerebral artery and fourth-order mesenteric artery, E_4 was an even weaker vasorelaxant compared with E_2 . Surprisingly, in larger elastic arteries, such as the thoracic aorta and common carotid artery, the difference in potency between E_4 and E_2 was smaller, mainly because of a reduction in sensitivity to E_2 compared with its sensitivity in smaller arteries. This reduced sensitivity to E_2 in larger arteries is in agreement with findings of others (Lindsey *et al.* 2011). Furthermore, the differential sensitivities to E_2 of the mesenteric, renal, and uterine arteries observed in this study are similar to values reported by others (Naderali *et al.* 1999, Leung *et al.* 2005, Scott *et al.* 2007). Scott *et al.* observed a pEC_{50} value of 5.47 ± 0.05 in endothelium-denuded uterine arteries, while Naderali *et al.* observed a pEC_{50} value of 6.04 ± 0.06 in the superior mesenteric artery, and Leung *et al.* (2005) observed a pEC_{50} value of 5.54 ± 0.22 in the renal artery. Hence, great inter-arterial variability exists in vasomotor responses to estrogenic hormones.

Previous studies have shown that *in vivo* administration of the nonselective ER antagonist ICI 182 780 into one uterine artery of ovariectomized and E_2 -treated nonpregnant sheep blunted the E_2 effect on uterine blood flow, confirming its ER dependence (Magness *et al.* 2005). Here, we show that ICI 182 780 resulted in a significant rightward shift in the CRCs to E_4 and E_2 , suggesting the contribution of ER. However, E_2 -mediated relaxing responses were not inhibited by ICI 182 780 in endothelium-denuded rat uterine arteries (Scott *et al.* 2007), most likely because the endothelial layer, which expresses ERs, had been mechanically removed. *In vitro* studies showed that $ER\alpha$ are localized in caveolae in isolated endothelial cells (Chambliss *et al.* 2000), supporting the conclusion that the lack of inhibitory effect of ICI 182 780 in denuded uterine arteries was due to the mechanical removal of the endothelial layer.

We compared relaxing responses to E_4 and E_2 with responses to selective agonists for $ER\alpha$ and $ER\beta$ in uterine arteries. We showed that the $ER\alpha$ agonist PPT has a more potent relaxing effect on uterine arteries compared with the $ER\beta$ agonist DPN. This finding is consistent with the study by Montgomery *et al.* (2003) in which the relative potency of PPT was greater than DPN in relaxing rat mesenteric arteries *ex vivo*. The order of potency of the estrogenic compounds and ER agonists in relaxing the rat uterine artery is $E_2 = PPT > DPN > E_4$.

The current study demonstrated that the relaxing action of E_4 in uterine arteries was L-NAME insensitive, suggesting that endothelium-derived NO plays a marginal role in E_4 -mediated relaxing responses in the rat uterine vasculature. By contrast, relaxing responses to E_2 were partially L-NAME sensitive in rat uterine arteries, similar to earlier observations

Table 5 Influence of pharmacological inhibitors and endothelial denudation on the vasodilator properties of E₂ compared with E₄ in rat uterine arteries. The sensitivity (pEC₅₀) of E₄ or E₂ is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E₄ or E₂ that induces 50% relaxation compared to the maximal relaxation (E_{max}). See text for inhibitor concentrations used. When more arteries of the same type were isolated from one rat, the values were averaged and regarded as n=1 per rat. Values are shown as mean±S.E.M.

Treatment	E ₂			E ₄		
	pEC ₅₀	E _{max} (%)	n	pEC ₅₀	E _{max} (%)	n
Control (none)	5.69±0.10	94±1	9	4.66±0.15 [†]	75±3 [†]	9
ICI 182 780	5.12±0.05*	83±2*	5	4.05±0.11 ^{*,†}	49±4 ^{*,†}	5
Indomethacin (INDO)	5.75±0.07	96±1	7	4.40±0.06 [†]	79±3 [†]	7
N ^ω -nitro-L-arginine methyl ester (L-NAME)	5.32±0.08*	93±1	8	4.39±0.06 [†]	76±4 [†]	8
L-NAME+INDO	5.26±0.09*	95±2	7	4.43±0.10 [†]	78±5	7
L-NAME+INDO+ODQ	5.34±0.05*	94±1	9	3.68±0.08 ^{*,†}	35±4 ^{*,†}	8
L-NAME+INDO+TRAM-34+UCL 1684	5.53±0.11	88±4	6	4.33±0.08 [†]	70±2 [†]	6
Endothelial denudation	5.07±0.06*	89±1	7	3.96±0.05 ^{*,†}	52±4 ^{*,†}	7

E₂, 17β-estradiol; E₄, estetrol; n, number of experiments. *P<0.05 vs control, [†]P<0.05 vs E₂.

in the *in vivo* uterine circulation (Van Buren *et al.* 1992, Rosenfeld *et al.* 1996). Our data do not provide a clear mechanistic explanation for the L-NAME insensitivity of the uterine arterial relaxing responses to E₄. Neither prostacyclin (PGI₂) nor EDHF contributed to the relaxing response to either estrogenic hormone. Surprisingly, pharmacological inhibition of sGC resulted in marked blunting of E₄-mediated relaxation in uterine arteries, an effort that was not observed in E₂-treated vessels.

Both NO synthase and large-conductance Ca²⁺-activated K⁺ channels (BK_{Ca}) are involved in acute E₂-induced vasodilation in oophorectomized nonpregnant ewes (Khan *et al.* 2000, Rosenfeld *et al.* 2000). NO activates cGMP-dependent protein kinase G, which subsequently opens BK_{Ca} on smooth muscle cells, thus decreasing Ca²⁺ influx via voltage-gated Ca²⁺ channels, leading to hyperpolarization and relaxation. A more detailed mechanistic study is needed to determine directly whether E₄ can induce the release of cGMP from smooth muscle cells or whether L-NAME-insensitive intracellular NO stores play a role. Endothelial denudation blunted relaxations to E₄ and E₂ in the uterine artery, probably by disrupting the ER in caveolae on the plasma membrane of endothelial cells.

Both E₄ and E₂ elicited large endothelium-independent relaxation responses, suggesting that estrogenic compounds may interfere with excitation–contraction coupling, thus inhibiting contraction. Excitatory agonists, such as adrenergic agonists and contractile peptides like angiotensin II, can stimulate smooth muscle contraction through three distinct signaling pathways: Ca²⁺ influx through membrane Ca²⁺ channels, IP₃-induced Ca²⁺ release from the sarcoplasmic reticulum, and Ca²⁺ sensitizing mechanisms of the contractile machinery (Somlyo & Somlyo 1994, Hilgers & Webb 2005). The contraction-modulating effects of estrogens have been attributed to their Ca²⁺ antagonistic properties. E₂ can inhibit voltage-dependent calcium inward currents (L-type voltage-operated Ca²⁺ channels or L-VOCC) located on smooth muscle cells, but not on endothelial cells (Shan *et al.*

1994, Zhang *et al.* 1994, Nakajima *et al.* 1995). This leads to reduced intracellular Ca²⁺ concentration and subsequent lower Ca²⁺-calmodulin-dependent myosin light-chain phosphorylation and contraction. By contrast, E₂ effects have not been ascribed to modulating Ca²⁺ release from intracellular stores or by IP₃ (Kitazawa *et al.* 1997). High K⁺ concentrations (>25 mmol/l) can depolarize the smooth muscle cell membrane and stimulate L-VOCC and Ca²⁺ influx independent of the endothelium. In Ca²⁺-free conditions, no contraction can occur during high K⁺ (60 mmol/l) depolarization. Titration of CaCl₂ into the organ bath results in a Ca²⁺-dependent contraction. Using this approach in endothelium-intact uterine arteries, in conditions where NO release and action were blocked, we tested the Ca²⁺ antagonistic effects of E₄ and found that E₄ and E₂ concentration-dependently blocked L-VOCC-dependent contractions in uterine arteries. E₄ was roughly

Figure 4 Contractile responses to cumulative addition of calcium chloride (CaCl₂; 0.01–2.5 mmol/l) during 60 mmol/l K⁺ depolarization in uterine arteries that were incubated with L-NAME (100 μmol/l) and INDO (10 μmol/l). (A) Effects of 10 μmol/l estetrol (E₄; open circles), 30 μmol/l E₄ (open squares), and 100 μmol/l E₄ (open diamonds) compared to vehicle (VEH; closed circles). (B) Effects of 3 μmol/l 17β-estradiol (E₂; closed circles), 10 μmol/l E₂ (closed squares), and 30 μmol/l E₂ (closed diamonds) compared to vehicle (VEH; open circles). E₄ or E₂ was applied 10 min before the cumulative application of CaCl₂. Values are expressed as mean±S.E.M. *P<0.05 vs VEH; **P<0.05.

tenfold less potent than E_2 in inhibiting these contractions. Interestingly, 3 $\mu\text{mol/l}$ E_2 did not block L-VOCC-dependent contractions (Fig. 4A), but resulted in $\sim 50\%$ relaxation of PHE-contracted uterine arteries (Fig. 1A). The fact that these Ca^{2+} antagonistic effects of E_4 and E_2 are seen at higher concentrations might suggest that these Ca^{2+} antagonistic effects are unrelated to their endothelium and ER-dependent estrogenic effects.

In conclusion, we have demonstrated that E_4 is capable of inducing a relaxing response in arteries from a variety of vascular beds. E_4 was several fold less potent than E_2 in relaxing these arteries, but the differential relaxing potency of E_4 vs E_2 decreased in larger arteries, mainly because of reduced potency of E_2 . E_4 caused relaxation of contracted uterine arteries via an endothelium-dependent mechanism involving ERs, as well as smooth muscle-dependent inhibition of Ca^{2+} entry. The mechanism of the discrepancy between the micromolar range estrogenic effects on *ex vivo* vasorelaxation and the nanomolar range of circulating estrogen levels *in vivo* is unclear, but the observation does suggest that the effects of estrogens on *ex vivo* vasorelaxation are pharmacological in nature. The relatively low vasorelaxing potency of E_4 might be beneficial in clinical use in that E_4 will provide beneficial effects associated with estrogens without altering hemodynamics. Furthermore, previous studies have shown that E_4 (compared with E_2) has a low first-pass liver metabolism, no SHBG binding, ER antagonistic effects on breast cancer models, and anti-thrombotic properties, suggesting that E_4 has promise for clinical use as a component hormone in a combined oral contraceptive and as therapy for breast tumors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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