Vasorelaxing effects of estetrol in rat arteries

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Abstract

This study compared *ex vivo* relaxing responses to the naturally occurring human hormone estetrol (E_4) vs 17β-estradiol (E_2) in eight different vascular beds. Arteries were mounted in a myograph, contracted with either phenylephrine or serotonin, and cumulative concentration-response curves (CRCs) to E_4 and E_2 (0·1–100 μmol/l) were constructed. In all arteries tested, E_4 had lower potency than E_2, although the differential effect was less in larger than smaller arteries. In uterine arteries, the nonselective estrogen receptor (ER) blocker ICI 182 780 (1 μmol/l) caused a significant rightward shift in the CRC to both E_4 and E_2, indicating that the relaxation responses were ER dependent. Pharmacological blockade of nitric oxide (NO) synthases by N^{ω}-nitro-L-arginine methyl ester (L-NAME) blunted E_2-mediated but not E_4-mediated relaxing responses, while inhibition of prostaglandins and endothelium-dependent hyperpolarization did not alter relaxation to either E_4 or E_2 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 E_4 compared with E_2 in uterine arteries. Endothelium denudation inhibited responses to both E_4 and E_2, while E_4 and E_2 concentration-dependently blocked smooth muscle cell Ca^{2+} entry in K^+-depolarized and Ca^{2+}-depleted uterine arteries. In conclusion, E_4 relaxes precontracted rat arteries in an artery-specific fashion. In uterine arteries, E_4-induced relaxations are partially mediated via an endothelium-dependent mechanism involving ERs, sGC, and inhibition of smooth muscle cell Ca^{2+} entry, but not NO synthases or endothelium-dependent hyperpolarization.

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Introduction

The hormone estetrol (estra-1,3,5(10)-trien-3,15α-16α,17β-tetrol or E_4) was discovered in 1965 (Hagen *et al.* 1965). E_4 is produced in nature from 17β-estradiol (E_2) and estrol (E_3) only by the human fetal liver during pregnancy via 15α- and 16α-hydroxylase (Schwers *et al.* 1965, Gurpide *et al.* 1966). The concentration of E_4 increases during pregnancy in both the fetus and the mother, but 24 h after delivery, the level is undetectable.

The chemical structure of E_4 is closely related to that of E_2, but the pharmacokinetic, metabolic, and endocrinological profile of E_4 is substantially different from that of E_2. E_2 has high affinity for the estrogen receptor α (ERα) and ERβ (K_i values: 0·2 and 0·05 nmol/l respectively), while E_4 has low-to-moderate affinity for ERα and ERβ (K_i values: 4·9 and 19 nmol/l respectively; Coelingh Bennink *et al.* 2008*a*). E_2 has very low oral availability because over 99% of an oral dose is converted into estrone (E_1) and E_1 sulfate. Most of the circulating E_2 is bound to either albumin or sex hormone binding globulin (SHBG), further decreasing plasma levels of free E_2 by a factor of about 30. By contrast, E_4 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, E_4 has excellent oral potency despite its low-to-moderate affinity for ERs.

E_4 has been studied in many validated models and has been shown to behave as a full ER agonist, similar to E_2, in most of these (Coelingh Bennink *et al.* 2008*b*, Heegaard *et al.* 2008, Holinka *et al.* 2008, Visser & Coelingh Bennink 2009). However, important differences between E_4 and E_2 were noted. E_4 did not induce synthesis of SHBG *in vitro* as did E_2 (Hammond *et al.* 2008). Recent studies showed that E_4 behaved as an ER antagonist in *in vitro* and *in vivo* models of estrogen-dependent breast tumors in the presence of E_2. E_2 behaved as full agonist in these models (Coelingh Bennink *et al.* 2008*c*). E_4 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 E_4 is not completely understood, although a role in regulating uterine blood flow has been suggested. Unilateral intrauterine injection of E_4 in nonpregnant oophorectomized ewes has been shown to increase uterine blood flow, although with a 15- to 30-fold lower potency than E_3 (Levine *et al.* 1984). Similarly,

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administration of E_2 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 E_2 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 E_4, this study was designed to study the *in vitro* vasorelaxing effects of E_4 and to compare them with those of E_2. The objectives of this study were 1) to determine the *ex vivo* relaxing effects of E_4 compared to E_2 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 E_4 in uterine arteries; and 3) to assess the endothelium-dependent and endothelium-independent vasorelaxing effects of E_4 in uterine arteries. The uterine artery was chosen for assessment of the acute vasoactive properties of E_4 and for comparison of these to the effects of E_2 because the uterine arterial circulation is known to be highly sensitive to the vasodilator effects of E_2. The uterine arteries are exposed to high physiological levels of E_2 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 ($60 \pm 5\%$), 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 CO_2 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 $CaCl_2$, 1·2 $MgSO_4$, 1·2 KH_2PO_4, 25·0 $NaHCO_3$, 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% O_2 and 5% CO_2. 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 μmol/l) or 5-HT (0·001–10 μmol/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 μmol/l NE or 60 mmol/l K^+ depolarization). During a stable contraction, a CRC to E_4 (0·1–100 μmol/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 μmol/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 ERα agonist propyl-[1H]-pyrazole-1,3,5-triy-triphenol (PPT; 0·1–30 μmol/l; Stauffer *et al.* 2000) and the selective ERβ agonist 2,3-*bis*(4-hydroxyphenol)-propionitrile (DPN; 0·1–30 μmol/l; Meyers *et al.* 2001) and compared their uterine arterial relaxing responses to E_4 and E_2. To study the involvement of ERs (ERα and ERβ) 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 μmol/l; Wakeling *et al.* 1991), after which the arterial segments were contracted with a single concentration of PHE (1–10 μmol/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 μmol/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 μmol/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 μmol/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-diium dibromide (UCL 1684; 1 μmol/l; Campos Rosa *et al.* 2000) was used to block SK_{Ca} channels and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; 1 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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 μmol/l), INDO (10 μmol/l), and ODQ (10 μmol/l). Results were compared with three different concentrations (3, 10, and 30 μmol/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 μmol/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
n	40	6	27	27	11	24	24	19

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

a $LOGEC_{50}$ (pEC_{50} value indicating sensitivity). Maximal relaxation to the highest concentration of E_4 or E_2 (E_{max}) and pEC_{50} values are shown as mean±S.E.M. Statistical significance of effects and differences were analyzed using either one-way ANOVA (comparison of pEC_{50} 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 (pEC_{50}) 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 E_4

In uterine arteries, pEC_{50} for the control estrogenic compound E_2 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). pEC_{50} and E_{max} for E_4 were significantly lower (4·36±0·07 and 74±4% respectively; Tables 3 and 4 and Fig. 1A). Hence, in uterine arteries, E_4 was a 14-fold less potent vasodilator compared with E_2. Vasorelaxing properties of E_4 compared with E_2 for the other seven arterial types are shown in Fig. 1B through H and are summarized in Tables 3 and 4. E_4 resulted in much lower variability than E_2 in pEC_{50} and E_{max} values for all artery types tested (Tables 3 and 4). Larger arteries (aorta, carotid, and renal) had lower pEC_{50} 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 (pEC_{50}) 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
n	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
n	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
n	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 E_2 compared with E_4 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 E_4 or E_2 is expressed as percentage of the contraction in response to 10 μmol/l NE. The sensitivity (pEC_{50}) of E_4 or E_2 is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E_4 or E_2 that induces 50% relaxation compared with the maximal relaxation (E_{max}). Potency of E_2 over E_4 is calculated as 10(pEC50(E2) − pEC50(E4)) from paired arteries only. Values are shown as mean ± S.E.M.

	Uterine		Aorta		Carotid		Mesenteric	
Artery type	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

E_2, 17β-estradiol; E_4, estetrol; NE, norepinephrine; *n*, number of experiments. *$P<0·05$ vs E_2.

Role of ERs in uterine and carotid arterial relaxing responses to E_4

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. pEC_{50} 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 E_4 and E_2 in uterine (Fig. 2B) arteries. For E_4, pEC_{50} averaged 4·54±0·08 in vehicle and 4·05±0·11 in ICI 182 780-treated uterine arteries (Fig. 2B). For E_2, pEC_{50} 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 E_4

The cyclooxygenase inhibitor INDO (10 μmol/l) did not significantly alter sensitivity to E_4 or E_2 (Fig. 3A). The NO synthase blocker L-NAME (100 μmol/l) caused a significant rightward shift in the CRC to E_2, but not to E_4 (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 E_4 and E_2 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 E_4 but not to E_2 (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 E_4 or E_2 (Fig. 3E).

The contribution of the endothelium to relaxing responses to E_4 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 E_4 and E_2 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 E_4 compared with E_2 in rat uterine arteries.

Table 4 Vasorelaxing properties (sensitivity and maximal relaxing responses) of E_2 compared to E_4 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 E_4 or E_2 is expressed as percentage of the contraction in response to 10 μmol/l NE. The sensitivity (pEC_{50}) of E_4 or E_2 is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E_4 or E_2 that induces 50% relaxation compared to the maximal relaxation (E_{max}). Potency of E_2 over E_4 is calculated as 10(pEC50(E2) − pEC50(E4)) from paired arteries only. Values are shown as mean ± S.E.M.

	Pulmonary		Renal		Middle cerebral		Septal coronary	
Artery type	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

E_2, 17β-estradiol; E_4, estetrol; NE, norepinephrine; *n*, number of experiments. *$P<0·05$ vs E_2.

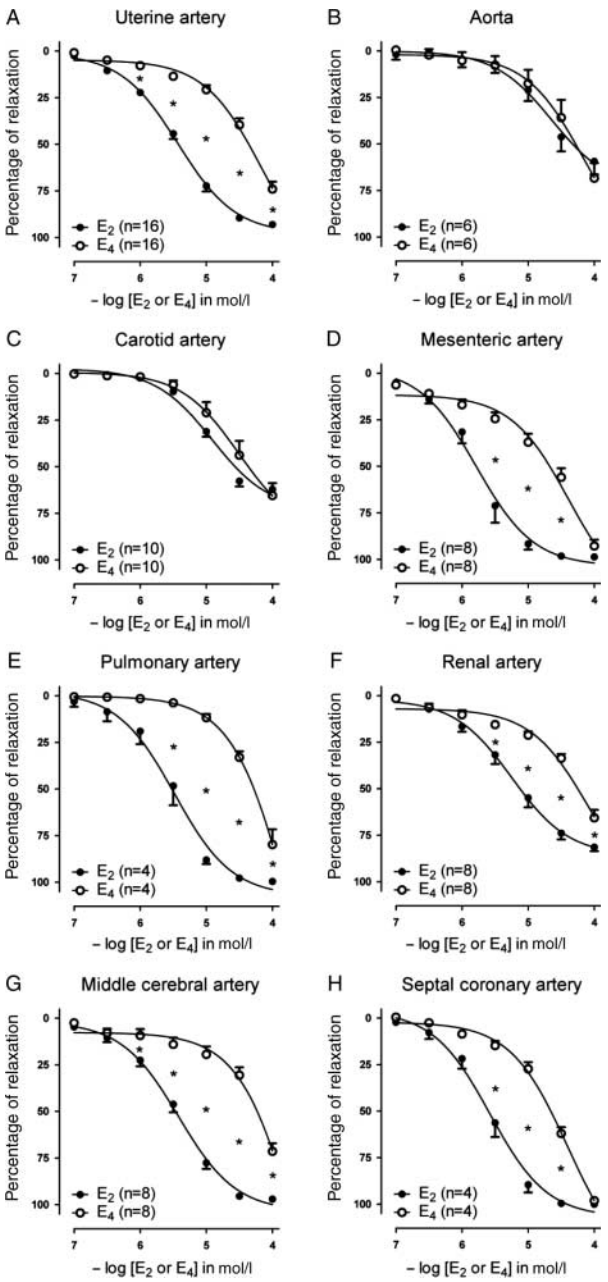

Figure 1 Relaxing responses to 17β-estradiol (E_2; 0·1–100 μmol/l; closed circles) and estetrol (E_4; 0·1–100 μmol/l; open circles) in contracted uterine (A), aorta (B), carotid (C), fourth-order mesenteric (D), pulmonary (E), renal (F), middle cerebral (G), and septal coronary (H) arteries. Values are expressed as mean ± 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 μmol/l E_4 (Fig. 4A) and 3 μmol/l E_2 (Fig. 4B). Next, we compared the inhibitory effects of higher concentrations of E_4 (30 and 100 μmol/l) and E_2 (10 and 30 μmol/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 ERα and ERβ. Previous *in vitro* binding studies showed that E_4 has moderate affinity for human ERs, with four to five times higher affinity for the ERα compared with the ERβ (Visser *et al.* 2008). The same study reported that E_4 has tenfold lower affinity for the ERα

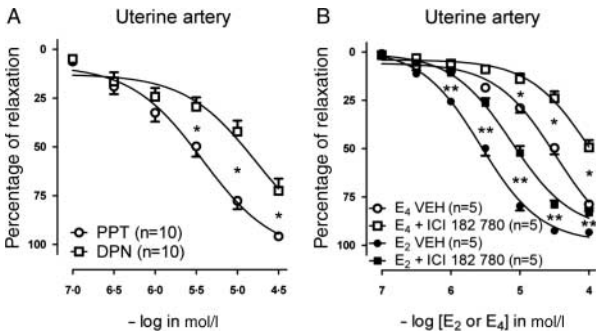

Figure 2 Relaxing responses to the ERα agonist PPT (0·1–30 μmol/l; circles) and the ERβ agonist DPN (0·1–30 μmol/l; squares) in PHE-contracted uterine arteries (A). Relaxing responses to 17β-estradiol (E_2; 0·1–100 μmol/l; closed symbols) and estetrol (E_4; 0·1–100 μmol/l; open symbols) in PHE-contracted uterine arteries in the presence of the nonselective estrogen receptor blocker ICI 182 780 (1 μmol/l) or the absence of ICI 182 780 (VEH; 10 μl DMSO; B). Values are expressed as mean ± S.E.M. *$P<0·05$ vs PPT (A). *$P<0·05$ vs E_4 VEH; **$P<0·05$ vs E_2 VEH (B).

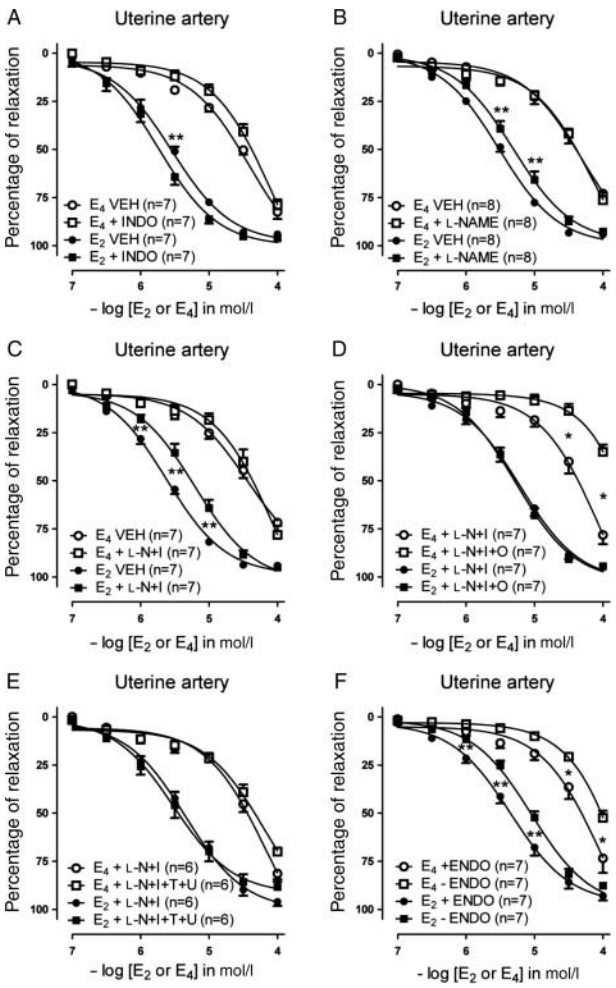

Figure 3 Relaxing responses to 17β-estradiol (E_2; 0·1–100 μmol/l; closed symbols) and estetrol (E_4; 0·1–100 μmol/l; open symbols) in PHE-contracted uterine arteries in the absence of any pharmacological inhibitor (VEH), in the presence of the cyclooxygenase inhibitor INDO (10 μmol/l; A); the NO synthase blocker L-NAME (100 μmol/l; B); the combined presence of L-NAME and INDO (L-N+I; C), the combined presence of L-NAME, INDO, and the selective sGC blocker ODQ (10 μmol/l; L-N+I+O; D); the combined presence of L-NAME, INDO, and the selective IK_{Ca} channel blocker TRAM-34 (1 μmol/l) and the selective SK_{Ca} channel blocker UCL 1684 (1 μmol/l; E); and in the presence (+ENDO) and absence (−ENDO) of endothelium (F). Values are expressed as mean±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 ERβ 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 (~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 ERα 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 ERα and ERβ in uterine arteries. We showed that the ERα agonist PPT has a more potent relaxing effect on uterine arteries compared with the ERβ agonist DPN. This finding is consistent with the study by Montgomory *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 E_2 compared with E_4 in rat uterine arteries. The sensitivity (pEC_{50}) of E_4 or E_2 is calculated with GraphPad Prism Software as described in the Materials and Methods section and denotes the negative logarithmic concentration of E_4 or E_2 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.

	E_2			E_4		
Treatment	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

E_2, 17β-estradiol; E_4, estetrol; *n*, number of experiments. *$P<0·05$ vs control, †$P<0·05$ vs E_2.

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 E_4. Neither prostacyclin (PGI_2) nor EDHF contributed to the relaxing response to either estrogenic hormone. Surprisingly, pharmacological inhibition of sGC resulted in marked blunting of E_4-mediated relaxation in uterine arteries, an effort that was not observed in E_2-treated vessels.

Both NO synthase and large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are involved in acute E_2-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 Ca^{2+} influx via voltage-gated Ca^{2+} channels, leading to hyperpolarization and relaxation. A more detailed mechanistic study is needed to determine directly whether E_4 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 E_4 and E_2 in the uterine artery, probably by disrupting the ER in caveolae on the plasma membrane of endothelial cells.

Both E_4 and E_2 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: Ca^{2+} influx through membrane Ca^{2+} channels, IP_3-induced Ca^{2+} release from the sarcoplasmatic reticulum, and Ca^{2+} sensitizing mechanisms of the contractile machinery (Somlyo & Somlyo 1994, Hilgers & Webb 2005). The contraction-modulating effects of estrogens have been attributed to their Ca^{2+} antagonistic properties. E_2 can inhibit voltage-dependent calcium inward currents (L-type voltage-operated Ca^{2+} 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 Ca^{2+} concentration and subsequent lower Ca^{2+}-calmodulin-dependent myosin light-chain phosphorylation and contraction. By contrast, E_2 effects have not been ascribed to modulating Ca^{2+} release from intracellular stores or by IP_3 (Kitazawa *et al.* 1997). High K^+ concentrations (>25 mmol/l) can depolarize the smooth muscle cell membrane and stimulate L-VOCC and Ca^{2+} influx independent of the endothelium. In Ca^{2+}-free conditions, no contraction can occur during high K^+ (60 mmol/l) depolarization. Titration of $CaCl_2$ into the organ bath results in a Ca^{2+}-dependent contraction. Using this approach in endothelium-intact uterine arteries, in conditions where NO release and action were blocked, we tested the Ca^{2+} antagonistic effects of E_4 and found that E_4 and E_2 concentration-dependently blocked L-VOCC-dependent contractions in uterine arteries. E_4 was roughly

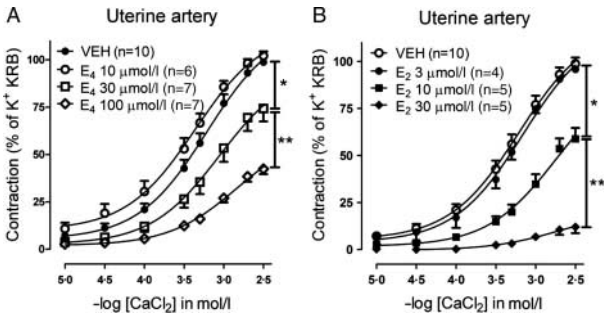

Figure 4 Contractile responses to cumulative addition of calcium chloride ($CaCl_2$; 0·01–2·5 mmol/l) during 60 mmol/l K^+ depolarization in uterine arteries that were incubated with L-NAME (100 µmol/l) and INDO (10 µmol/l). (A) Effects of 10 µmol/l estetrol (E_4; open circles), 30 µmol/l E_4 (open squares), and 100 µmol/l E_4 (open diamonds) compared to vehicle (VEH; closed circles). (B) Effects of 3 µmol/l 17β-estradiol (E_2; closed circles), 10 µmol/l E_2 (closed squares), and 30 µmol/l E_2 (closed diamonds) compared to vehicle (VEH; open circles). E_4 or E_2 was applied 10 min before the cumulative application of $CaCl_2$. 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 μmol/l E_2 did not block L-VOCC-dependent contractions (Fig. 4A), but resulted in ∼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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