

# Human uterine and placental arteries exhibit tissue-specific acute responses to 17 $\beta$ -estradiol and estrogen-receptor-specific agonists

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**ABSTRACT:** The discrete regulation of vascular tone in the human uterine and placental circulations is a key determinant of appropriate uteroplacental blood perfusion and pregnancy success. Humoral factors such as estrogen, which increases in the placenta and maternal circulation throughout human pregnancy, may regulate these vascular beds as studies of animal arteries have shown that 17 $\beta$ -estradiol, or agonists of estrogen receptors (ER), can exert acute vasodilatory actions. The aim of this study was to compare how acute exposure to ER-specific agonists, and 17 $\beta$ -estradiol, altered human placental and uterine arterial tone *in vitro*. Uterine and placental arteries were isolated from biopsies obtained from women with uncomplicated pregnancy delivering a singleton infant at term. Vessels were mounted on a wire myograph, exposed to the thromboxane receptor agonist U46619 (10<sup>-6</sup> M), and then incubated with incremental doses (5 min, 0.03–30  $\mu$ M) of either 17 $\beta$ -estradiol or agonists specific for the ERs ER $\alpha$  (PPT), ER $\beta$  (DPN) or the G-protein-coupled estrogen receptor GPER-1 (G1). ER $\alpha$  and ER $\beta$  mRNA expression was assessed. 17 $\beta$ -estradiol, PPT and DPN each relaxed myometrial arteries ( $P < 0.05$ ) in a manner that was partly endothelium-dependent. In contrast, 17 $\beta$ -estradiol or DPN relaxed placental arteries (maximum relaxation to 42  $\pm$  1.1 or 47.6  $\pm$  6.53% of precontraction, respectively) to a lesser extent than myometrial arteries (to 0.03  $\pm$  0.03 or 8.0  $\pm$  1.0%) and in an endothelial-independent manner whereas PPT was without effect. G1 exposure did not inhibit the constriction of myometrial nor placenta arteries. mRNA expression of ER $\alpha$  and ER $\beta$  was greater in myometrial arteries than placental arteries. ER-specific agonists, and 17 $\beta$ -estradiol, differentially modulate the tone of uterine versus placental arteries highlighting that estrogen may regulate human uteroplacental blood flow in a tissue-specific manner.

**Key words:** estrogen / ER agonists / human uterine arteries / human placental arteries / vascular tone

## Introduction

A key determinant of appropriate fetal growth during human pregnancy is the transfer across the placental epithelium of oxygen and nutrients between the uterine and fetal circulations. As the two circulations are separate physical entities—blood from the maternal uterine circulation that traverses through the lumen of terminal open-ended spiral arterioles is directed towards the syncytial surface of the placenta but does not come in to direct contact with the enclosed fetoplacental circulatory flow—then materno-fetal nutrient exchange will be influenced by changes in either the uterine or placental vasculatures. This makes it important to understand how the blood vessels from each circulation

respond to physiological vasoactive factors. The placenta lacks autonomic innervations (Myatt, 1992), which suggests that factors within the local environment may be crucial for this purpose.

Estrogen may be one such modulator of uteroplacental vascular function. Estrogen's effect on vascular tone has been documented in many experimental and clinical contexts but not in relation to human pregnancy. Estrogen action via two receptors, ER $\alpha$  (also known as ESR1) and ER $\beta$  (also known as ESR2), classically initiates transcriptional changes (Miller and Duckles, 2008). However, acute vasodilatory actions of estrogen have pointed to non-genomic actions of ER $\alpha$  and ER $\beta$ . These may be mediated via endothelial-dependent and/or -independent mechanisms (Shaw *et al.*, 2000; Hisamoto and Bender,

2005) with the former involving regulation of nitric oxide (NO) bioavailability. Interestingly, a transmembrane G-protein-coupled receptor, GPER-1 (also known as GPR30), has also been identified as an ER capable of mediating rapid non-genomic signalling (Revankar and Prossnitz, 2005; Feldman and Gros, 2011) and it is of interest to unravel whether estrogenic vasoactive actions may be mediated, in part or wholly, via activation of this receptor.

In sheep *in vivo*, the uterine circulation exhibits a rapid vasodilatory response to 17 $\beta$ -estradiol administration (Pastore et al., 2012). Such actions may in part reflect activation of endothelial NO synthase (NOS; Chen et al., 2004) and in rat uterine arteries isolated from non-pregnant rats, but not those from late pregnant rats, 17 $\beta$ -estradiol-mediated relaxations were partly blunted by NOS inhibition (Scott et al., 2007). The involvement of ERs in these actions is a matter of considerable debate. Certainly, reports of variations in the relative abundance of mRNA encoding ER $\alpha$  or ER $\beta$  in uterine endothelial cells (Critchley et al., 2001; Liao et al., 2005; Pastore et al., 2012) points to the possibility of receptor-specific estrogenic responses in the uterine circulation. In comparison to these data, however, it is striking that there is little information about the estrogenic effects on placental blood vessels. The placenta is an important source of estrogen, evinced by the substantial increase in maternal plasma level (You et al., 2006) and the fetal adrenal gland contributes to estrogen within the placental circulation (Smith et al., 2009). Thus, locally derived and/or locally acting estrogen may modulate the vasoreactivity of the human uterine and placental circulations. Of note, it has been suggested that the endothelium of human fetoplacental blood vessels express ER $\beta$  protein but that ER $\alpha$  is absent or expressed at much less levels (Su et al., 2007, 2009).

The availability of agonists specific for ER $\alpha$  (PPT: (4,4',4''-(4-propyl-[<sup>1</sup>H]-pyrazole-1,3,5-triyl) tris-phenol)), ER $\beta$  (DPN: (2,3-bis(4-hydroxyphenyl)-propionitrile)), or GPER-1 (G1) has enabled mapping of the non-genomic vasodilatory actions resulting from stimulation of the different ERs across many vascular beds (Montgomery et al., 2003; Hass et al., 2009; Feldman and Gros, 2011). However, a direct comparison of the acute estrogenic influences on arteries isolated from two human vasculatures, and from patients with no medical complications, has not been examined.

Therefore, we have investigated the effect of 17 $\beta$ -estradiol and ER-specific agonists on the tone of human placental and uterine (myometrial) arteries isolated from biopsies of normal pregnant women with no underlying pathophysiology. Our data point to a new understanding of human uteroplacental vascular regulation by reporting that estrogen regulates the tone of arteries isolated from these circulations in ER- and tissue-specific manners. This also alerts us to the possibility that variations in estrogenic responsiveness may exist across other human arterial tissues.

## Materials and Methods

### Chemicals and solutions

ER agonists were purchased from R&D systems (UK); other chemicals, unless stated, were from Sigma (UK) or VWR (UK).

### Sample collection and tissue microdissection

The study was approved by Local Research Ethics Committees and tissue collected following written, informed consent from women with

uncomplicated, singleton term pregnancies (38–42 weeks gestation). Arteries were dissected as described previously (Corcoran et al., 2012). Chorionic plate arteries (diameter  $284 \pm 18 \mu\text{m}$ ) were dissected from placental biopsies obtained within 30 min of vaginal or elective Caesarean (not in labour) delivery and placed directly into ice-cold tissue collection buffer ((TCB); modified Krebs solution – 154 mM NaCl/5.4 mM KCl/1.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O/10 mM MOPS/5.5 mM glucose/1.6 mM CaCl<sub>2</sub>·2H<sub>2</sub>O; pH 7.4). Myometrial arteries (diameter  $209 \pm 23 \mu\text{m}$ ) were obtained from biopsies ( $\sim 1 \text{ cm}^3$ ) taken at the time of Caesarean section from the upper segment of the uterine incision and placed into ice-cold TCB.

### Myography experiments and analysis

Four segments from each artery were mounted onto a wire myograph (610M; Danish Myotechnologies, Denmark). After a 20 min equilibration period, the arteries were stretched by 100  $\mu\text{m}$  and allowed to equilibrate for 2 min and tension was recorded using a Myodaq software package (Danish Myotechnologies, Denmark). The vessels were stretched a further 100  $\mu\text{m}$  and the procedure was repeated in at least three steps until the passive tension exceeded 5.1 kPa for human placental arteries [vessel tension expressed as active wall tension ( $\Delta T$  in mN/mm) can be transformed to active effective pressure ( $\Delta T/(\text{diameter}/2000)$ ) denoted by kPa] or 13.3 kPa for human myometrial arteries. Arteries were then normalized to a resting diameter equivalent to 0.9 of L<sub>5.1kPa</sub> (placental) or L<sub>13.3kPa</sub> (myometrial) as described previously (Corcoran et al., 2012), and equilibrated in physiological salt solution (PSS: 127 mM NaCl, 4.7 mM KCl, 2.4 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 mM NaHCO<sub>3</sub>, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 0.07 mM EDTA, 1.6 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 6.05 mM glucose at pH 7.4) at 37°C. Myometrial arteries were bubbled with a 95%air–5%CO<sub>2</sub> mixture and placental arteries with 5% O<sub>2</sub>–5% CO<sub>2</sub>–90% N<sub>2</sub> (Corcoran et al., 2012).

Arteries were precontracted with 1  $\mu\text{M}$  of the thromboxane receptor agonist U46619 (9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F<sub>2a</sub>, Merck Millipore, UK) and vasoactive actions of estrogenic compounds examined by adding incremental doses (5 min duration each, of 0.05, 0.15, 0.3, 0.6, 1, 2, 5, 10, 30  $\mu\text{M}$ ) of either 17 $\beta$ -estradiol, PPT, DPN, G1 or vehicle (ethanol). The range of doses was chosen to be akin to that reported in studies on other vessel types (Shaw et al., 2000; Montgomery et al., 2003; Hisamoto and Bender, 2005; Scott et al., 2007). In some experiments, the role of the endothelium was investigated by abrading the arterial lumen to render the endothelium dysfunctional or preincubating with the NOS inhibitor L-NNA (10  $\mu\text{M}$ ). Arteries were subsequently constricted with 1  $\mu\text{M}$  U46619 and cumulative dose–response curves to estrogenic compounds assessed. Endothelial functional integrity, determined by the relaxant actions of 1  $\mu\text{M}$  bradykinin on U46619 precontractions in myometrial arteries, was  $81 \pm 10\%$  before and  $19 \pm 12\%$  after abrasion. In another set of experiments, placental arteries were preincubated with the L-type Ca<sup>2+</sup> channel agonist Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester), further constricted with U46619 and the effects of 17 $\beta$ -estradiol or DPN examined.

In a subset of experiments, arteries were treated with  $\alpha$ -toxin so as to render the plasmalemma permeable to small solutes and ions and enable direct investigation of the Ca<sup>2+</sup>-sensitivity of contraction by directly controlling the level of activating Ca<sup>2+</sup> surrounding the myofilaments. The procedure followed was as reported previously (Wareing et al., 2005; Dordea et al., 2013). In brief, intact PSS-equilibrated vessels were exposed to a mock intracellular solution (G1: sodium creatine phosphate 10 mM; Na<sub>2</sub>ATP 5.2 mM; magnesium methanesulphonate 7.3 mM; potassium methane sulphonate, 74 mM; K<sub>2</sub>EGTA 1 mM buffered to pH 7.1 with 30 mM of the chemical buffer PIPES and addition of KOH) for 15 min and then treated with a 25  $\mu\text{l}$  droplet of permeabilizing cocktail [pCa 6.7 solution (see below) supplemented with 500 U/ml  $\alpha$ -toxin plus 10  $\mu\text{mol/l}$  A23187] for 30 min. The cocktail was removed by washing the vessels with G1 solution followed

by equilibration of the permeabilized arteries in pCa9 (relaxing) solution ( $[Ca^{2+}]_i = 0.001 \mu\text{M}$ ; sodium creatine phosphate, 10 mM;  $Na_2ATP$ , 5.2 mM; magnesium methane sulfonate, 7.3 mM, potassium methane sulfonate, 74 mM;  $K_2EGTA$  1 mM; PIPES, 30 mM; pH 7.1 with KOH). Permeabilization was assessed by exposing vessels to a high calcium activating solution (pCa4.5 ( $[Ca^{2+}]_i = 31.5 \mu\text{M}$ )) followed by relaxation with pCa9 solution. Vessels were exposed to a sub-maximal calcium activating solution (pCa6.7 ( $[Ca^{2+}]_i = 0.2 \mu\text{M}$ )) and thence U46619 (1  $\mu\text{M}$ ) in the continued presence of pCa6.7 solution. Any additional constriction was thus due to sensitization of the contractile filaments to the sub-maximal activating  $Ca^{2+}$  by the presence of U46619. The experiment was completed by exposure to a maximal dose (30  $\mu\text{M}$ ) of either 17- $\beta$  estradiol or DPN to examine any effect of these drugs on agonist-dependent  $Ca^{2+}$ -sensitization of contraction.

Data recorded (Myodaq; Danish Myotechnologies, Denmark) as active wall tension ( $\Delta T$  in mN/mm) was transformed to active effective pressure ( $\Delta T/(\text{diameter}/2000)$ ) denoted by kPa. Data were Arcsine transformed to enable statistical analysis of dose-response curves by two-way repeated-measures analysis of variance with Bonferroni *post hoc* testing (significance taken as  $P < 0.05$ ) and presented graphically as mean  $\pm$  SEM.  $E_{\text{max}}$  (maximal effect) and pIC50 ( $-\text{Log}(IC_{50})$ ) of drugs were calculated and effects of endothelial abrasion and NOS inhibition analysed using *t*-test.

### Quantitative polymerase chain reaction

Vessels were homogenized in lysis buffer and total RNA extracted as described (Dordea *et al.*, 2013). 100 ng RNA from each sample was reverse transcribed using AffinityScript cDNA synthesis kit (Stratagene, USA) including a DNase treatment step to eliminate genomic DNA contamination. ER $\alpha$  and ER $\beta$  were amplified using specific primers (ER $\alpha$  nm\_000125: forward—gct gct ggc tac atc; reverse—agg act cgg tgg ata tgg; ER $\beta$  nm\_001040275: forward—ata ctt tcc tcc tat gta gac; reverse—tgt gat aac tgg cga tgg) and quantified using a Stratagene MX3000P machine and Stratagene Brilliant SYBR Green I QPCR mastermix. cDNA from human myometrial artery and placental artery samples were assessed on the same 96-well plate and for each gene of interest data from each sample were expressed relative to the internal reference cDNA sample [reverse transcribed from human reference total RNA (Stratagene Human Reference Total RNA, La Jolla, USA)] also assessed on every 96-well plate and analysed using an independent *t*-test;  $P < 0.05$  was considered significant.

### Western blotting

Arteries were homogenized in (1 mg per 5  $\mu\text{l}$ ) 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EGTA (pH7.4) with 2% (vol/vol) protease inhibitor and 0.5% (vol/vol) phosphatase inhibitor (Sigma) and prepared for western blotting as described by Dordea *et al.* (2013). Twenty to 30  $\mu\text{g}$  protein from each sample was resolved by SDS-PAGE and transferred to PVDF membranes for western blotting with anti-serum against ER $\beta$  (Abcam #ab288 mouse monoclonal) or ER $\alpha$  (Santa Cruz Biotechnology #H-184 rabbit polyclonal 1:1000; Santa Cruz Biotechnology #MC-20 rabbit polyclonal 1:1000; Vector Labs #VP613 mouse monoclonal, 1:1000) and membranes visualized chemiluminescently following probing with a HRP-goat anti-mouse-IgG (1:5000; DakoCytomation, #PO447) or HRP-goat anti-rabbit-IgG (1:5000; DakoCytomation, #PO448) antibody. The optical densities from the western blot scans were determined using Intelligent Quantifier Software (BioImage Systems Inc.). Protein expression was quantified by comparing the optical density of the arterial band of interest to the optical density of the positive control following the subtraction of background signal. PVDF membranes were stained with naphthol blue black in order to visualize actin expression and assess for equal protein loading between lanes.

## Results

### 17 $\beta$ -estradiol and ER agonists have differing relaxant actions on human myometrial and placental arteries

17 $\beta$ -estradiol induced significant vasodilation of precontracted human myometrial arteries from 0.3 to 30  $\mu\text{M}$  (relaxations to  $59 \pm 5.1\%$ – $0.03 \pm 0.03\%$  of precontraction, Fig. 1A and D). DPN also induced acute vasodilation from 1 to 30  $\mu\text{M}$  (relaxations to  $87 \pm 3.1\%$ – $8.0 \pm 1.0\%$  of precontraction, Fig. 1B and D) and PPT from 2 to 30  $\mu\text{M}$  (relaxations to  $77 \pm 3.3\%$ – $37 \pm 3.9\%$  of precontraction, Fig. 1C and D). In contrast, human placental arteries (Fig. 1E–H) exhibited less vasodilation to 17 $\beta$ -estradiol (from 1 to 30  $\mu\text{M}$ :  $76 \pm 7.1\%$ – $42 \pm 1.1\%$  of precontraction;  $P < 0.001$ ) or DPN (from 2 to 30  $\mu\text{M}$ :  $72 \pm 7.05\%$ – $47.6 \pm 6.53\%$  precontraction;  $P < 0.006$ ) than myometrial arteries and showed no relaxation to PPT (Fig. 1G and H). G1 had no relaxant actions in myometrial or placental arteries (Fig. 1D and H).

### Acute relaxatory effects are partly endothelial- and NO-mediated in human myometrial arteries but are endothelial-independent in human placental arteries

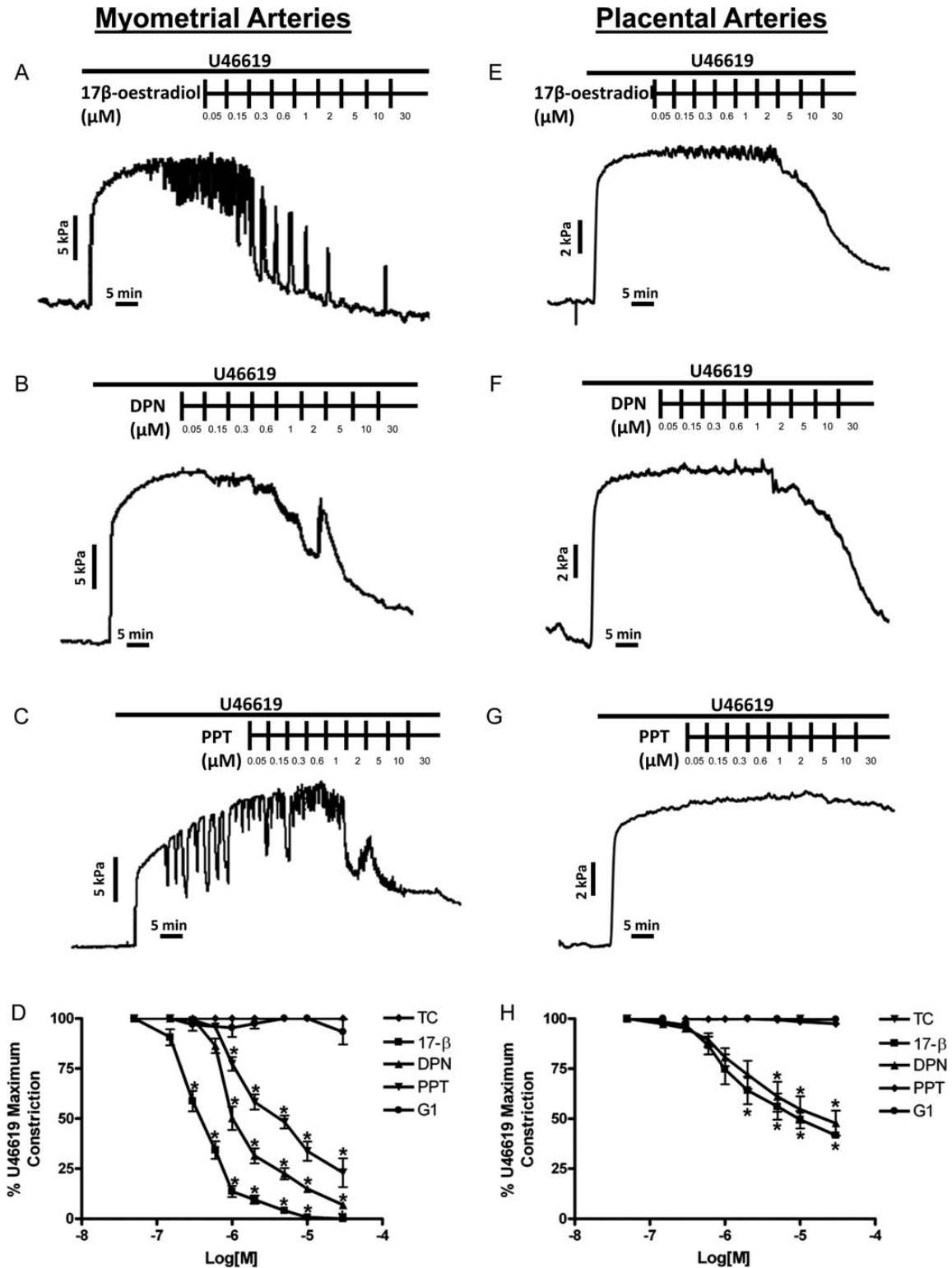
The vasodilatory effects of 17 $\beta$ -estradiol, DPN or PPT on human myometrial arteries were each blunted following endothelial abrasion (Fig. 2A–C). In endothelial abraded vessels, the relaxation to 17 $\beta$ -oestradiol was  $71 \pm 4.8\%$ – $26 \pm 1.8\%$  (0.3–30  $\mu\text{M}$ ), to DPN was  $49 \pm 10\%$ – $17 \pm 2.3\%$  (2–30  $\mu\text{M}$ ) and to PPT was  $83 \pm 4.5\%$ – $57 \pm 4.9\%$  (5–30  $\mu\text{M}$ ) of precontraction. Similar results were obtained in endothelial-intact arteries exposed to L-NNA (Fig. 2A–C).

The data in Table I shows that either treatment—endothelial abrasion or preincubation with L-NNA—in myometrial arteries significantly reduced the maximal relaxation ( $E_{\text{max}}$ ) induced by each agonist and mostly resulted in a reduction in sensitivity (pIC50) to each agent.

In contrast to the effects in intact human myometrial arteries, the vasodilatory actions of 17 $\beta$ -estradiol or DPN on human placental arteries were unaffected by endothelial abrasion (Fig. 3A) or by exposure of endothelial-intact arteries to L-NNA (Fig. 3B).

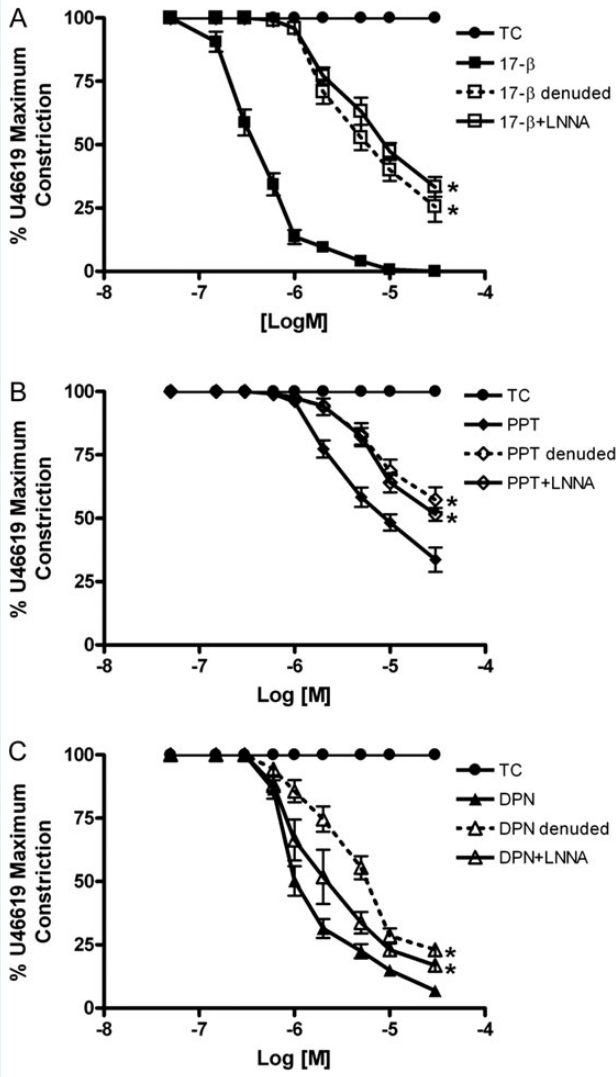
### Differential expression of ER $\alpha$ and ER $\beta$ between human myometrial and placental arteries

Analysis of ER mRNA demonstrated that both types of artery express ER $\alpha$  (Fig. 4A) and ER $\beta$  (Fig. 4B). In myometrial arteries the level of mRNA encoding ER $\alpha$  ( $P < 0.01$ ) and ER $\beta$  ( $P < 0.04$ ) was greater than placental arteries. The Ct values of ER $\alpha$  in the myometrial and placental arteries were, respectively,  $24.35 \pm 0.11$  ( $n = 6$ ) and  $25.43 \pm 0.21$  ( $n = 6$ ) (corresponding calibrator RNA  $25.43 \pm 0.11$ ). The Ct values of ER $\beta$  in the myometrial and placental arteries were, respectively,  $25.54 \pm 0.34$  and  $25.32 \pm 0.02$  (corresponding calibrator RNA  $28.67 \pm 0.15$ ). Western blotting also showed that ER $\beta$  protein was greater in myometrial than placental arteries (Fig. 4C and D). However, in spite of using three separate anti-ER $\alpha$  antibodies (detailed in the Materials and Methods) we were unable to consistently visualize



**Figure 1** Differential effects of 17β-estradiol and ER agonists on human myometrial and placental arteries. Myometrial (A–D) or placental (E–H) arteries were precontracted with U46619 and exposed to incremental doses of estrogenic compounds. Representative raw tracings are displayed in A–C, E–G, summary data (mean ± SEM,  $n = 6$ ) in D and H. 17β-estradiol or ERβ agonist, DPN relaxed myometrial arteries to a greater extent than placental arteries whereas ERα agonist, PPT relaxed only myometrial arteries. GPER-I agonist (G1) was without effect in either artery type. \*Significant differences from time control (TC).





**Figure 2** Relaxatory actions of estrogenic compounds are partly endothelial- and NO-dependent in human myometrial arteries. The relaxation of precontracted myometrial arteries by 17β-estradiol (A), PPT (B) or DPN (C) was blunted following endothelial abrasion (open symbols, dashed lines) or by pretreatment with nitric oxide synthase inhibitor (L-NNA) (open symbols, solid lines). \*Significant differences from intact artery responses. Data are presented as mean ± SEM (n = 6).

staining that could be specifically attributable to ERα in myometrial or placental arteries for the purposes of quantitative comparisons.

### Vasodilatory effects of estrogen do not involve altered Ca<sup>2+</sup>-sensitization of contraction but are enhanced in the presence of an L-type Ca<sup>2+</sup> channel agonist

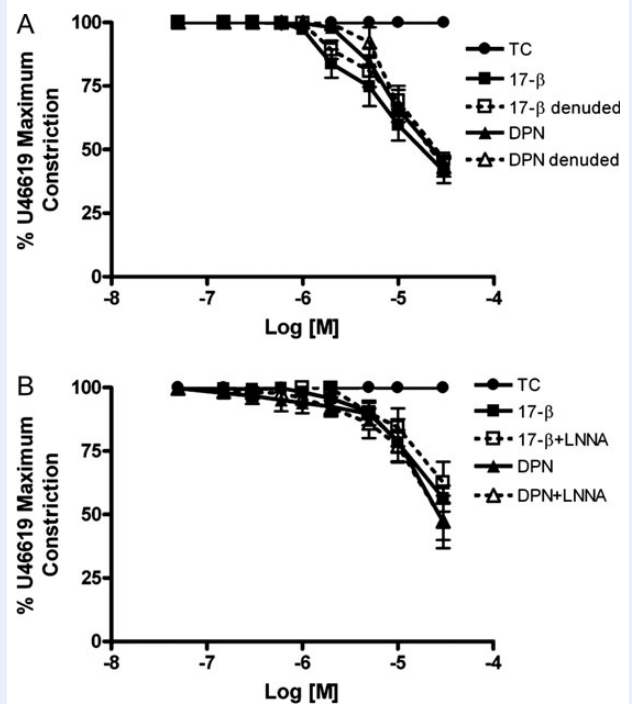
Figure 5A indicates a representative raw tracing of an α-toxin permeabilized placental artery exhibiting U46619-induced Ca<sup>2+</sup>-sensitization of contraction at sub-maximal activating Ca<sup>2+</sup> (pCa6.7). This was of similar magnitude to the preceding contraction induced by maximally

**Table I** Effects of endothelial abrasion or NOS inhibition on the estrogenic relaxatory responses of human myometrial arteries.

|                         | 17β-estradiol            | PPT                      | DPN                      |
|-------------------------|--------------------------|--------------------------|--------------------------|
| <i>E</i> <sub>max</sub> |                          |                          |                          |
| Intact                  | 0.03 ± 0.03              | 33.7 ± 4.8               | 6.9 ± 1.7                |
| Denuded                 | 25.5 ± 5.9 <sup>a</sup>  | 57.3 ± 4.9 <sup>a</sup>  | 23.0 ± 2.4 <sup>a</sup>  |
| +LNNa                   | 33.3 ± 3.9 <sup>a</sup>  | 51.5 ± 2.6 <sup>a</sup>  | 16.8 ± 2.3 <sup>a</sup>  |
| pIC50                   |                          |                          |                          |
| Intact                  | 6.43 ± 0.04              | 5.42 ± 0.06              | 5.99 ± 0.04              |
| Denuded                 | 5.47 ± 0.10 <sup>a</sup> | 5.17 ± 0.07 <sup>a</sup> | 5.48 ± 0.10 <sup>a</sup> |
| +LNNa                   | 5.34 ± 0.13 <sup>a</sup> | 5.14 ± 0.07 <sup>a</sup> | 5.84 ± 0.11              |

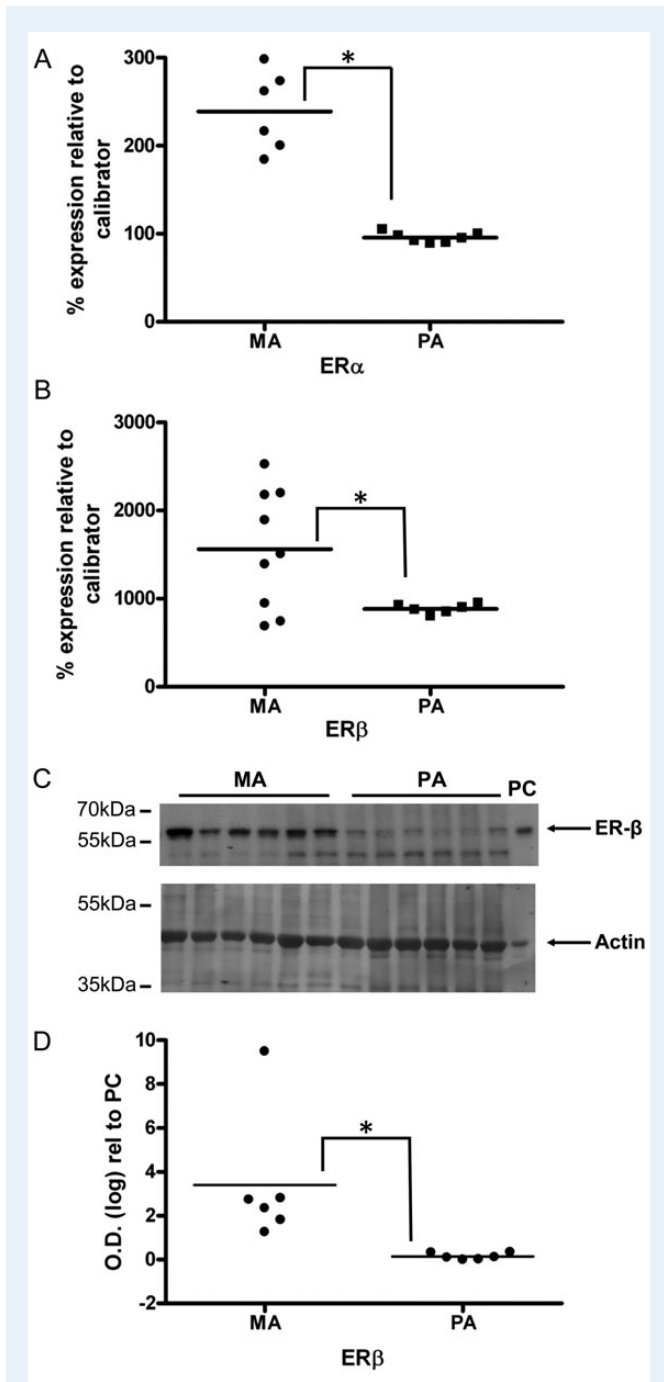
The maximum (*E*<sub>max</sub>) and half-maximal (pIC50) relaxation values of precontracted myometrial arteries by 17β-estradiol, DPN or PPT.

<sup>a</sup>Significant difference from paired intact artery (t-test). Data are presented as mean ± SEM (n = 6).



**Figure 3** Vasodilatory actions of estrogenic compounds are endothelial-independent in human placental arteries. The relaxation of precontracted placental arteries by 17β-estradiol or DPN were unaltered by endothelial abrasion (A) or by pretreatment with L-NNA (B). Data are presented as mean ± SEM (n = 6).

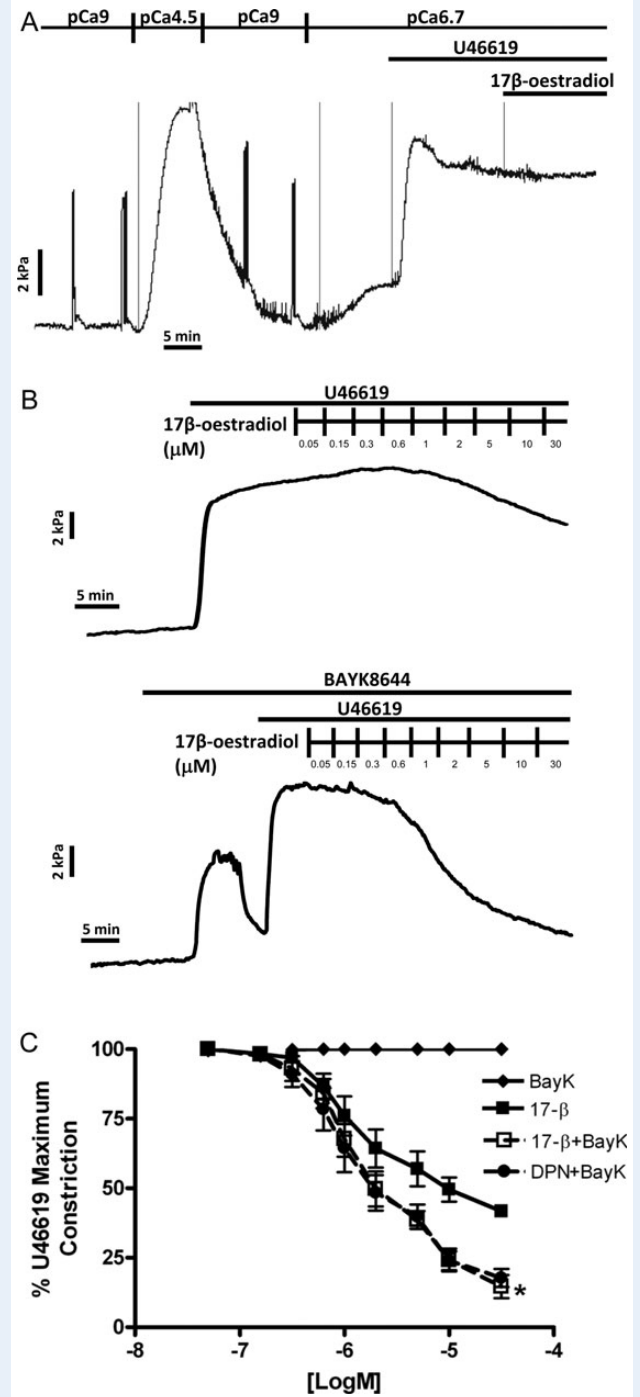
activating pCa4.5 solution. Addition of 30 μM 17β-estradiol was without effect on this agonist-mediated Ca<sup>2+</sup>-sensitization (n = 4). In intact precontracted placental arteries, the relaxation by 17β-estradiol (B) was enhanced by preincubation with the L-type Ca<sup>2+</sup> channel agonist Bay K8644 (C and D). Similar effects were observed on DPN-mediated relaxation (D).



**Figure 4** ER expression in human arteries. Expression of mRNA encoding for ER $\alpha$  (A) or ER $\beta$  (B). ER $\alpha$  mRNA expression was greater in myometrial arteries (MA) than placental arteries (PA). Western blotting indicated that ER expression was greater in myometrial than placental arteries (C and D). PC, refers to the positive control of human myometrium. \*Significant differences between MA and PA.

## Discussion

Efficient uteroplacental blood perfusion is required for a successful pregnancy outcome yet the factors and mechanisms involved in controlling vascular tone within the human uterus and placenta are



**Figure 5** Vasodilatory effects of estrogenic compounds are unrelated to changes in Ca $^{2+}$ -sensitization of contraction but are enhanced in the presence of an L-type Ca $^{2+}$  channel agonist. Permeabilized placental arteries exhibited U46619-induced Ca $^{2+}$ -sensitization of contraction at sub-maximal activating Ca $^{2+}$  (pCa6.7) that was similar in magnitude to contraction in pCa4.5 solution alone (representative raw data tracing of A). 17 $\beta$ -estradiol (30  $\mu$ M) did not affect this agonist-mediated Ca $^{2+}$ -sensitization. In intact preconstricted placental arteries, 17 $\beta$ -estradiol-mediated relaxation (B) was enhanced by preincubation with the L-type Ca $^{2+}$  channel agonist Bay K8644 (C and D). Similar effects were observed on DPN-mediated relaxation (D). \*Significant differences between 17 $\beta$ -estradiol alone and in the presence of Bay K8644.

poorly understood. This study is the first to investigate the potential role of estrogen and estrogen receptors in acutely regulating the vaso-reactivity of arteries isolated from these circulations at the end of pregnancy. Our results demonstrate that the relaxatory influences of 17 $\beta$ -estradiol or ER-specific agonists differ between myometrial and placental arteries. This suggests that estrogen regulation of human uteroplacental vascular tone occurs in a tissue-specific manner and opens the possibility that similar variations may occur between other human vascular beds.

17 $\beta$ -estradiol acutely relaxed precontracted human myometrial arteries. The ER $\beta$ -specific agonist DPN and ER $\alpha$ -specific agonist PPT also exerted vasodilatory effects. The GPER-1 agonist G1 was without effect. These data point to estrogenic vasodilation of human myometrial arteries via ER $\beta$  and ER $\alpha$ . In our study, endothelial abrasion, or NOS inhibition, resulted in a blunting of relaxatory responses to all three agents thus indicating that estrogen relaxation of myometrial arteries is mediated via endothelial-dependent and -independent mechanisms. In sheep, uterine artery endothelial cells express ER $\beta$  and ER $\alpha$  and several estrogenic influences occur in an ER-specific manner (Byers *et al.*, 2005; Pastore *et al.*, 2012). ER $\beta$  and ER $\alpha$  have previously been suggested to mediate non-genomic stimulation of NO production (Wu *et al.*, 2011) and this may underlie the endothelial-dependent relaxant action of estrogen.

Human placental arteries relaxed to a lesser extent to 17 $\beta$ -estradiol or DPN than myometrial arteries. However, there was no relaxation to PPT or G1. Furthermore, the influence of 17 $\beta$ -estradiol or DPN in placental arteries was unaffected by endothelial abrasion or NOS inhibition. This implicates a prominent role for ER $\beta$ , but not ER $\alpha$  or GPER-1, in acute endothelial-independent estrogenic relaxation of human placental arteries and illustrates profound differences to human myometrial arteries. The lack of endothelial-dependence in estrogen action in placental arteries may be related to a relatively weak stimulation of NO-mediated relaxation by endothelial-dependent agonists in this vessel type (Sweeney *et al.*, 2008) but is unlikely to be related to impaired sensitivity of placental artery smooth muscle cells to NO production (Hayward *et al.*, 2013). In addition, in sheep uterine arterial endothelial cells the relative expression of endothelial NOS is greater than that of placental arteries (Byers *et al.*, 2005). The endothelial-independent mechanisms of actions of estrogen in other vessels have been suggested to arise via inhibition of voltage-gated Ca<sup>2+</sup> entry to smooth muscle cells (Shaw *et al.*, 2000; Montgomery *et al.*, 2003). Although in human myometrial and placental arteries this requires further elucidation we have identified two important facets of the possible smooth muscle-related mechanisms of estrogenic action in placental arteries. First, the relaxations are unlikely to involve the modulation of intracellular pathways affecting myofilament Ca<sup>2+</sup>-sensitization. Secondly, that voltage-gated Ca<sup>2+</sup> entry is likely to be a target of acute estrogenic actions because, in the presence of an L-type Ca<sup>2+</sup> channel activator, which one may anticipate to have facilitated plasmalemmal Ca<sup>2+</sup> entry because contractility is increased, the vasodilatory estrogenic influences are actually enhanced. Further such work, in alliance with approaches such as siRNA-mediated receptor knockdown, or the application of ER-specific antagonists, is needed to establish whether the tissue-specific responses we observe are due entirely to ER expression patterns (see below) or the tissue-specific activation of other post-receptor intracellular signalling modalities. This has recently been suggested, for example, to be important in

these human tissues' distinct responses to IGF-I or protein kinase G stimulation (Corcoran *et al.*, 2012; Dordea *et al.*, 2013).

The lack of vasodilatory influence of the GPER-1 agonist G1 on either artery type was surprising given reports indicating a relaxant action on human internal mammary artery tone (Hass *et al.*, 2009). This may reflect tissue-specific actions of G1; however, we investigated the acute effects (within 5 min of each dose) of G1 whereas the vasodilatory influence on mammary arteries was observed over a longer time-course (Hass *et al.*, 2009).

Maternal estradiol levels rise continually throughout gestation with peak values reported to be ~60 nM (Norman *et al.*, 1989; O'Leary *et al.*, 1991; Smith *et al.*, 2009). Assessments of estradiol in the uterine or placental circulations have suggested greater values than in the systemic maternal circulation with peaks of ~350–500 nM (Smith *et al.*, 1979). Notwithstanding the possibility that the concentration of estradiol at the cellular site of action may be greater than that measured in the circulation(s), these *in vivo* circulating estradiol levels are within the lower-to-mid-range of our dose–response curves designed to investigate the acute influences on precontracted tone. A caveat to our study, of course, is that *in vivo* the human uterine or placental arteries will be chronically, rather than acutely, exposed to estrogens.

The differential ER-mediated responses in human myometrial and placental arteries supports a number of animal studies suggesting ER- and tissue-specific estrogenic vasodilatory actions (Montgomery *et al.*, 2003; Al Zubair *et al.*, 2005; Bolego *et al.*, 2005; Ma *et al.*, 2010; Patker *et al.*, 2011). Herein, we found that mRNA encoding ER $\alpha$  and ER $\beta$  are present in both artery types. The expression of ER $\beta$  mRNA or protein was significantly greater in human myometrial than placental arteries. In addition, ER $\alpha$  mRNA was greater in myometrial than placental arteries. Of note, the vasodilatory influence of DPN was less in placental arteries than myometrial vessels and PPT was an ineffective relaxant in placental arteries. ER $\beta$  has been suggested to be the prominent ER expressed in endothelial cells of stem villous placental arteries and endometrial uterine vessels (Critchley *et al.*, 2001; Su *et al.*, 2007). These arteries are both downstream of the resistance-like arteries used in the present study and points, therefore, to a need to consider if there are functionally relevant ER expression differences across the human uterine or placental vascular trees. The unreliability (in our hands) of a number of anti-ER $\alpha$  antibodies to give definitive identification by western blotting, coupled to the small amount of vascular material available for extensive optimization procedures, has hampered our ability to fully examine the ER tissue- and cell-specific protein expression patterns. Nonetheless, the lack of endothelial-dependence of DPN- or 17 $\beta$ -estradiol-mediated relaxation of placental arteries suggests a predominating influence of ER $\beta$ -related events at the level of placental smooth muscle cells where, indeed, ER $\beta$  appears to be expressed (Su *et al.*, 2011). In uterine arteries, on the other hand, the vasodilations to 17 $\beta$ -estradiol, DPN or PPT involved activation of signalling pathways in both endothelial and smooth muscle cells.

The present study is important for identifying (i) that estrogen differentially regulates human uterine and placental vascular tone; (ii) the potential of ER-specific agonists to act as tissue-specific modulators of human uteroplacental vascular tone; (iii) adding to an evolving narrative in the literature whereby circulatory, or locally produced, factors may differentially alter the tone of human myometrial or placental arteries (Hemmings *et al.*, 2006; Hudson *et al.*, 2007; Corcoran *et al.*, 2012). It is of interest that subcutaneous arteries isolated from post-menopausal

healthy women relaxed more to PPT than 17 $\beta$ -estradiol and the effects of either agent were unaltered by NOS inhibition (Cruz et al., 2008)—results that were different to that which we observed in maternal myometrial arteries. These data, therefore, also alerts us to the possibility that, more generally, estrogenic modulation of human arterial tone may occur in a tissue-specific manner and thereby contribute to distributing blood flow in an organ-specific fashion.

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## Authors' roles

J.J.C., C.N., M.S. and J.C.C. performed the experiments and analysed the data. S.C.R. facilitated human tissue provision. M.W. and M.J.T. conceived and designed the study and prepared the manuscript. All authors approved the final version.

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## Conflict of interest

None declared.

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