Endothelial vasodilator production by ovine uterine and systemic arteries: ovarian steroid and pregnancy control of ERα and ERβ levels

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Pregnancy and the follicular phase are physiological states of elevated oestrogen levels and rises in uterine blood flow (UBF). The dramatic increase in utero-placental blood flow during gestation is required for normal fetal growth and development. Oestrogen exerts its vasodilatory effect by binding to its specific oestrogen receptors (ER) in target cells, resulting in increased expression and activity of endothelial nitric oxide synthase (eNOS) to relax vascular smooth muscle (VSM). However, the regulation of endothelial *versus* VSM ERα and ERβ expression in uterine arteries (UAs) during the ovarian cycle, pregnancy and with exogenous hormone replacement therapy (HRT) are currently unknown. ER mRNA and protein localization was determined by *in situ* hybridization (ISH) using ^{35}S-labelled riboprobes and immunohistochemistry (IHC), respectively. UA endothelial (UAendo), UA VSM, omental artery endothelium (OA endo), and OA VSM proteins were isolated and ERα and ERβ protein expression was determined by Western analysis. We observed by ISH and IHC that ERα and ERβ mRNA and protein were localized in both UAendo and UA VSM. Immunoblot data demonstrated ovarian hormone specific regulation of ERα and ERβ protein in UAendo and UA VSM. Compared to luteal phase sheep, both ERα and ERβ levels in UAendo were elevated in follicular phase sheep. Whereas ERβ was elevated by pregnancy in UAendo and UA VSM, ERα was not appreciably altered. eNOS was increased in UAendo from follicular and pregnant sheep. Ovariectomized ewes (OVEX) had substantially reduced UAendo ERβ, but not UAendo ERα or OAendo ERα and ERβ. In contrast, OVEX increased UA VSM ERα and ERβ and decreased OA VSM ERα and ERβ. Treatment with oestradiol-17β (E2β), but not progesterone or their combination, increased UAendo ERα levels. The reduced ERβ in UAendo from OVEX ewes was reversed by $E_2\beta$ and progesterone treatment. While ERα and eNOS were not elevated in any other reproductive or non-reproductive endothelia tested, ERβ was augmented by pregnancy in uterine, mammary, placenta, and coronary artery endothelia. ERα and ERβ mRNA and protein are expressed in UA endothelium with expression levels depending on the endocrine status of the animal, indicating UA endothelium is a target for oestrogen action *in vivo,* and that the two receptors appear to be differentially regulated in a spatial and temporal fashion with regard to the reproductive status or HRT.

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A pivotal physiological role of oestrogen to up-regulate uterine blood flow (UBF) during specific reproductive states has been demonstrated by studies showing a concomitant increase in circulating endogenous oestrogen levels and UBF in the follicular, but not luteal, phase of the oestrous cycle and during normal pregnancy in many species (Greiss & Anderson, 1969; Ford & Christenson, 1979; Ford *et al.* 1982; Magness *et al.* 1991; Magness, 1998; Gibson *et al.* 2004). In addition, UBF is increased by exogenous oestrogen treatment of ovariectomized and intact non-pregnant and pregnant sheep (Greiss & Anderson, 1970; Rosenfeld *et al.* 1973; Rosenfeld *et al.*

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1974, 1976; Magness & Rosenfeld, 1989*a*,*b*; Magness, 1998; Gibson *et al.* 2004).

During normal pregnancy, a physiological state in which both oestrogen and progesterone are elevated, UBF increases up to 20- to 50-fold in order to provide sufficient oxygen and nutrient supply to meet the developmental needs of the growing fetus (Magness & Zheng, 1996; Magness, 1998). Elevated uterine perfusion is clinically significant as shown in cases where insufficient UBF is associated with intrauterine growth retardation and severe neonatal morbidity due to an inadequate supply of oxygen and nutrients (reviewed in Magness, 1998). Recent evidence also is suggestive that oestrogen regulation of UBF is most likely achieved via direct local actions on the uterine, but not systemic (i.e. omental and renal), artery endothelia via binding to specific oestrogen receptors (ERα and/or ERβ), to increase the expression and/or activity of endothelial nitric oxide synthase (eNOS) for an enhanced production of the potent vasodilator, nitric oxide (NO) (Van Buren *et al.* 1992; Rosenfeld *et al.* 1996; Magness *et al.* 1997, 2005; Vagnoni *et al.* 1998; Vagnoni & Magness, 1998; Salhab *et al.* 2000; Rosenfeld *et al.* 2003). In these studies, vascular smooth muscle (VSM) eNOS levels were quite low and unchanged by oestrogen treatment. Because infusion of oestrogen into ovariectomized (OVEX) ewes begins to increase UBF within 30 min and UBF is elevated more than 10-fold by 2 h (Magness & Rosenfeld, 1989*a*, *b*; Magness *et al.* 1998), these UBF responses to oestrogen may involve both acute non-genomic effects and chronic transcription-based gene expression at the level of the endothelium and VSM (Chen *et al.* 2004; Liao *et al.* 2005). This concept is supported by studies demonstrating that the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), as well as the ER antagonist ICI 182 780 similarly reduced the elevated UBF in oestrogen-treated OVEX non-pregnant (NP) sheep (Van Buren *et al.* 1992; Rosenfeld *et al.* 1996; Magness *et al.* 2005), follicular phase sheep (Gibson *et al.* 2004; Magness *et al.* 2005), as well as late pregnant sheep (Miller *et al.* 1999; Magness *et al.* 2005). Although the effects of oestrogen in these vasodilatory processes are postulated to be mediated by specific ERs, the role of changes in ER levels in uterine arteries is unclear. The presence of ERs in uterine arteries has been documented by several groups (Lantta *et al.* 1983; Giambi *et al.* 1984; Batra & Iosif, 1987; Wu *et al.* 2000; Weihua *et al.* 2000) including our own laboratories (Chen *et al.* 2004; Liao *et al.* 2005).

It has been shown previously that both the originally described ERα (Green *et al.* 1986; Koike *et al.* 1987) and the more recently cloned ERβ (Kuiper *et al.* 1996) are both expressed in whole rat uterine tissue homogenates, with ERα being the predominant isoform (Byers *et al.* 1997). However, it is not known specifically if either or both of these ERs are differentially regulated in uterine or systemic artery endothelium and VSM during physiological states of elevated oestrogen and/or progesterone. Consequently, their potential role in regulating UBF remains unclear.

The hypothesis of the current study is that ERα and ERβ, receptors known to be present in ovine uterine artery endothelium and VSM, are regulated by the reproductive state via alterations in ovarian/placental steroid (oestrogen/progesterone) levels in the animal. The specific objectives are to determine (1) if ERα, ERβ and eNOS levels in uterine *versus* systemic artery endothelium and/or VSM are independently regulated in physiological states of elevated oestrogen (follicular), progesterone (luteal), or both (pregnancy), and (2) if exogenous infusion of ovarian steroids, oestrogen, progesterone or their combination will mimic such ERα and ERβ changes in UAendo *versus* UA VSM.

Methods

Isolation and preparation of arteries from sheep during the ovarian cycle and pregnancy

Mixed Western breed ovary-intact luteal ($n = 12$), follicular ($n = 8$), and pregnant ($n = 12$, gestational age $= 125 \pm 4$) ewes were killed with intravenous pentobarbital sodium ($\sim$50 mg kg^{-1}). Procedures for animal handling and experimental protocols were approved by the University of Wisconsin–Madison Research Animal Care Committees of both the Medical School and the College of Agriculture and Life Sciences, and followed the recommended American Veterinary Medicine Association guidelines for killing of laboratory farm animals. Non-pregnant sheep were synchronized to the luteal (day 10–11) and follicular (day -1 to 0) phase as recently described (Gibson *et al.* 2004).

We used the previously described and validated rapid isolation procedure to obtain endothelial-derived proteins devoid of VSM (Magness *et al.* 1997). Briefly, uterine, mammary, and placental (reproductive), omental, renal, and coronary (non-reproductive) arteries were excised, placed in phosphate-buffered saline (PBS: 8 mM sodium phosphate, 2 mM potassium phosphate, 0.15 M NaCl, pH 7.4; Sigma Chemical Co., St Louis, MO, USA), dissected free of connective tissue and rinsed free of blood. Portions of each artery type were opened longitudinally and the endothelium/tunica intima was gently scraped (3–6 times) from the artery and placed into lysis buffer (50 mM Tris, 0.15 M NaCl and 10 mM EDTA, pH 7.4, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 M phenylmethylsulphonyl floride, 5 μg ml^{-1} leupeptin, and 5 μg ml^{-1} aprotinin; all from Sigma) using a curved-end spatula, as previously described (Magness *et al.* 1997; Vagnoni *et al.* 1998). The remaining 'scraped' vessel was rubbed with a wet cotton swab and any remaining adventitia was completely removed before the denuded artery (VSM) was placed in lysis buffer. The

endothelial-isolated proteins (endo) and denuded arteries (VSM) were snap frozen in liquid nitrogen immediately upon collection and were stored at −20°C. Thus UAendo samples represent an endothelial-enriched preparation that was mechanically scraped from the tunica media; UA VSM samples are denuded vessels that have had their endothelium removed leaving the basal lamina and VSM. Additional intact artery segments were collected for *in situ* hybridization and immunohistochemistry (IHC); they were fixed in 4% formaldehyde in sodium cacodylate buffer (0.1 M, pH = 7.4; EM Science, Fort Washington, PA, USA) for 24 h, and were then stored at 4°C in sodium cacodylate buffer containing 0.01% sodium azide until dehydration and placement into paraffin blocks.

Prolonged oestrogen and progesterone treatment regime

Mixed western breed ewes ($n = 20$) were ovariectomized via a midventral laparotomy as previously described (Magness & Rosenfeld, 1989*a*,*b*; Magness *et al.* 1993; Vagnoni *et al.* 1998), and after at least 8–10 days of recovery, steroid replacement therapy was administered. For the oestrogen-treated ewes, an indwelling 19-gauge polyvinyl catheter was placed into the right ventricle via the jugular vein. Animals then received a 5 μg kg^{-1} bolus of $E_2\beta$ (Sigma) mixed in 3 ml (∼10–11% EtOH) of saline, then flushed with 5 ml of saline, followed immediately by 6 μg kg^{-1} day^{-1} continuous infusion in 9.5% EtOH in isotonic saline (0.0123 ml min^{-1}) for 10 days. $E_2\beta$ was dissolved in 95% ethanol and stored at 4°C at a stock concentration of 1 mg ml^{-1}. The $E_2\beta$ dose for treatment and the time for tissue collection were based on eNOS expression and haemodynamic responses as well as blood levels of $E_2\beta$ as determined in our previous studies (Magness & Rosenfeld, 1989*a*,*b*; Magness *et al.* 1993; Vagnoni *et al.* 1998; Rupnow *et al.* 2001). Progesterone (P4) was simultaneously administered using one controlled internal drug release (CIDR) implant with 0.3 g progesterone (Carter Holt Harvey Ltd, New Zealand) placed into the vagina of OVEX ewes for 10 days. Control ewes received vehicle (ethanol in saline) infusion and/or blank CIDR implants. With this protocol, circulating P4 levels are elevated in P4- and $P_4 + E_2\beta$-treated ewes when compared to vehicle- or E2β-alone-treated ewes and also higher then time 0 prior to beginning the steroid treatments. The levels of P4 achieved approximate luteal phase levels (1–3 ng ml^{-1}). On day 10 of treatment the OVEX ewes were killed and the vessels isolated as described above.

In situ hybridization

Intact artery segments were collected for *in situ* hybridization (Byers *et al.* 1997) by fixation in 4% formaldehyde in sodium cacodylate buffer (0.1 M, pH 7.4; EM Science, Fort Washington, PA, USA) for 24 h and then stored at 4°C in sodium cacodylate buffer containing 0.01% sodium azide until dehydration and placement into paraffin blocks. Then, 5 μm sections were cut and mounted onto slides. Paraffin mounted tissue sections were depariffinized, rehydrated, then permealized by incubation for 30 min in proteinase K (5 μg ml^{-1}). Next, sections were briefly refixed in 4% paraformaldehyde, acetylated in 0.1 M TEA with acetic anhydride (0.25%), and then dehydrated. Antisense and sense [^{35}S]UTP-labelled RNA probes were synthesized using SP6 or T7 RNA polymerase. The ERα probe template was from a partial ovine cDNA sequence and the ERβ template was a partial bovine cDNA sequence (Spencer & Bazer, 1995). The RNA probe (1×10^7 c.p.m. ml^{-1}) in hybridization buffer (ISH buffer; Sigma) was applied to the tissue sections and then overlaid with coverslips. Slides were hybridized in a humidified incubator at 50°C for 16–18 h. After hybridization, the coverslips were removed and sections were treated with RNase A (20 μg ml^{-1}) for 30 min, washed in increasingly stringent solutions of saline–sodium citrate buffer (SSC; down to 0.1 × SSC) at 55°C, and dehydrated through an ethanol series. Slides were exposed to autoradiography film for 2–3 days then processed for liquid emulsion autoradiography using NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA). Slides were developed using Kodak D-19 developer and fixer and stained with haematoxylin. Images are at 10× magnification. Protein localization was confirmed using immunohistochemistry (IHC) using methods we have previously described (Magness *et al.* 1997; Chen *et al.* 2004; Liao *et al.* 2005).

Western blot analysis of ERα, ERβ, and eNOS proteins

Total cell extracts (20 μg protein lane^{-1}) were boiled in SDS sample buffer for 5 min, then electrophoresed on 12.5% SDS-PAGE gels for 100 min at 150 V. Separated proteins were then electrically (100 V, 30 min) transferred to a PVDF membrane (Magness *et al.* 1997). ERα, ERβ, and eNOS protein were detected using specific antibodies of anti-human ERα mAb (1 : 200, Neomarker Ab-7), anti-human ERβ pAb (1 : 3000, Santa Cruz H150) and eNOS mAb (1 : 10 000 Transduction Laboratories). Western blots for the VSM samples were reprobed for smooth muscle myosin (1 : 40 000, NeomarkerSM-M5) as a loading control. Non-specific binding was blocked with 5% fat-free milk in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 2 h at room temperature, after which the membrane was incubated with appropriate amounts of primary antibodies in TBST + 1% BSA at room temperature for 1.5 h. Following five washes (1 × 5, 1 × 15, 3 × 5 min) with TBST, the membrane was incubated with anti-mouse or anti-rabbit peroxidase-conjugated IgG for 1 h, respectively. The

membrane was washed five times with TBST, and bound antibodies were detected with the ECL+ reagents (Amersham) according to the manufacturer's instruction.

Statistical analysis

All applicable data were analysed with 2-tailed Student's *t* tests or ANOVA as appropriate, assuming unequal variance. Data are presented as the means and standard error of the mean (S.E.M).

Results

Localization of ERα and ERβ mRNA and protein

To determine the cellular (endothelium *versus* VSM) localization of ERα and ERβ mRNAs in UAs, *in situ* hybridization was performed ($n = 7$) using ^{35}S-labelled sense (control) or antisense riboprobes synthesized from specific ovine ERα and bovine ERβ cDNAs (Fig. 1*A* and *B*). Hybridization with antisense probes revealed that ERα and ERβ mRNA are both strongly expressed in the ovine UA endothelium seen as concentrated silver grains (darkfield) representing positive mRNA labelling along the lumen of the vessels. Widespread expression of the ERs was also observed throughout the tunica media. Furthermore, the basal lamina, which separates the endothelium and VSM, is shown to be devoid of both ERα and ERβ mRNAs. Tissue sections subjected to hybridization with sense probes showed minimal background levels of ERα and ERβ mRNAs. Because mRNA expression is not necessarily indicative of the mature 'functional' protein, immunohistochemistry (data not shown) was utilized to determine the cellular localization of ERα and ERβ protein. As we have previously reported (Chen *et al.* 2004; Liao *et al.* 2005), ERα and ERβ proteins were clearly seen in both the uterine artery endothelium and the VSM and as was the case in the mRNA localization, the basal lamina is completely devoid of ER protein. ERα protein was expressed uniformly throughout the UA cells (non-nuclear and nuclear staining), whereas ERβ exhibited heavy, preferential nuclear staining, an observation consistent with reports in ovine fetal pulmonary endothelial cells (Chambliss *et al.* 2002) and UAECs (Liao *et al.* 2005).

Effects of reproductive state on the expression of ERα and ERβ in uterine artery endothelium and VSM

We determined if ERα and ERβ protein expression is quantitatively regulated in the UAendo and/or VSM during physiological states of elevated oestrogen (follicular phase and pregnancy) or progesterone (luteal phase and pregnancy) using Western blot analysis. Expression levels of the samples from luteal, follicular and pregnant sheep were expressed as a fold of the average of all luteal phase samples run on the same blots (Fig. 2). UAendo ERα expression was elevated 1.5-fold ($P < 0.05$) in follicular compared to luteal UAendo. This rise in ERα expression was specific to the endothelium since UA VSM levels were unchanged ($P > 0.05$) for follicular phase samples. Pregnancy did not significantly alter ERα expression levels in either the endothelium or VSM, although UAendo ERα levels in pregnancy and follicular phase samples were similar. As seen with ERα, ERβ protein expression (Fig. 2) in the follicular UAendo, but not UA VSM, was significantly elevated. Pregnant UAendo ($P < 0.01$) ERβ expression showed a 1.6-fold rise over luteal controls. ERβ protein levels in VSM were unaltered by the follicular phase, but were significantly increased 3.2-fold of luteal phase UA VSM by pregnancy ($P < 0.01$). The UA VSM immunoblots were reprobed for smooth muscle myosin and showed homogeneity of protein loading. Specifically, smooth muscle myosin for the follicular phase and pregnant UA VSM averaged 1.0- and 1.1-fold of the luteal control, respectively. There was no significant difference between the three groups.

Effects of ovariectomy and hormone replacement therapy (HRT) on the expression of ERα and ERβ protein in uterine artery endothelium and VSM

We then analysed ERα and ERβ expression in UAendo and UA VSM from OVEX animals treated with vehicle or with E2β, P4, and E2β + P4 (Figs 3 and 4). Data are expressed as folds of the average of the luteal samples run on the same blot. Ovariectomy did not alter ERα levels in UAendo; however, E2β treatment resulted in a 2-fold increase ($P < 0.001$) in UAendo ERα protein over luteal control and to levels significantly increased ($P < 0.05$) over the vehicle- and P4-treated sheep (Fig. 3*A*). Combination treatment of P4 and oestrogen partially attenuated the oestrogen-mediated rises in UAendo ERα levels. In contrast to ERα, the OVEX vehicle-treated animals had ERβ expression nearly half that of the luteal control ($P < 0.01$; Fig. 3*B*). ERβ in the UAendo of the E2β, P4, and E2β + P4 animals was significantly higher than that of luteal levels ($P < 0.01$, $P < 0.05$, $P < 0.001$, respectively) as well as the OVEX vehicle-treated sheep ($P < 0.01$, $P < 0.01$, $P < 0.001$, respectively). The E2β + P4-treated animals showed further elevation in UAendo ERβ over both the animals treated with either E2β ($P < 0.01$) or P4 ($P < 0.05$) alone, which were similar ($P > 0.05$).

Figure 4*A* illustrates ERα UA VSM expression in luteal *versus* OVEX sheep treated with vehicle, E2β, P4, or E2β + P4. ERα UA VSM protein expression in vehicle-treated OVEX sheep was nearly 2.5-fold higher than the luteal control ($P < 0.001$). ERα UA VSM was

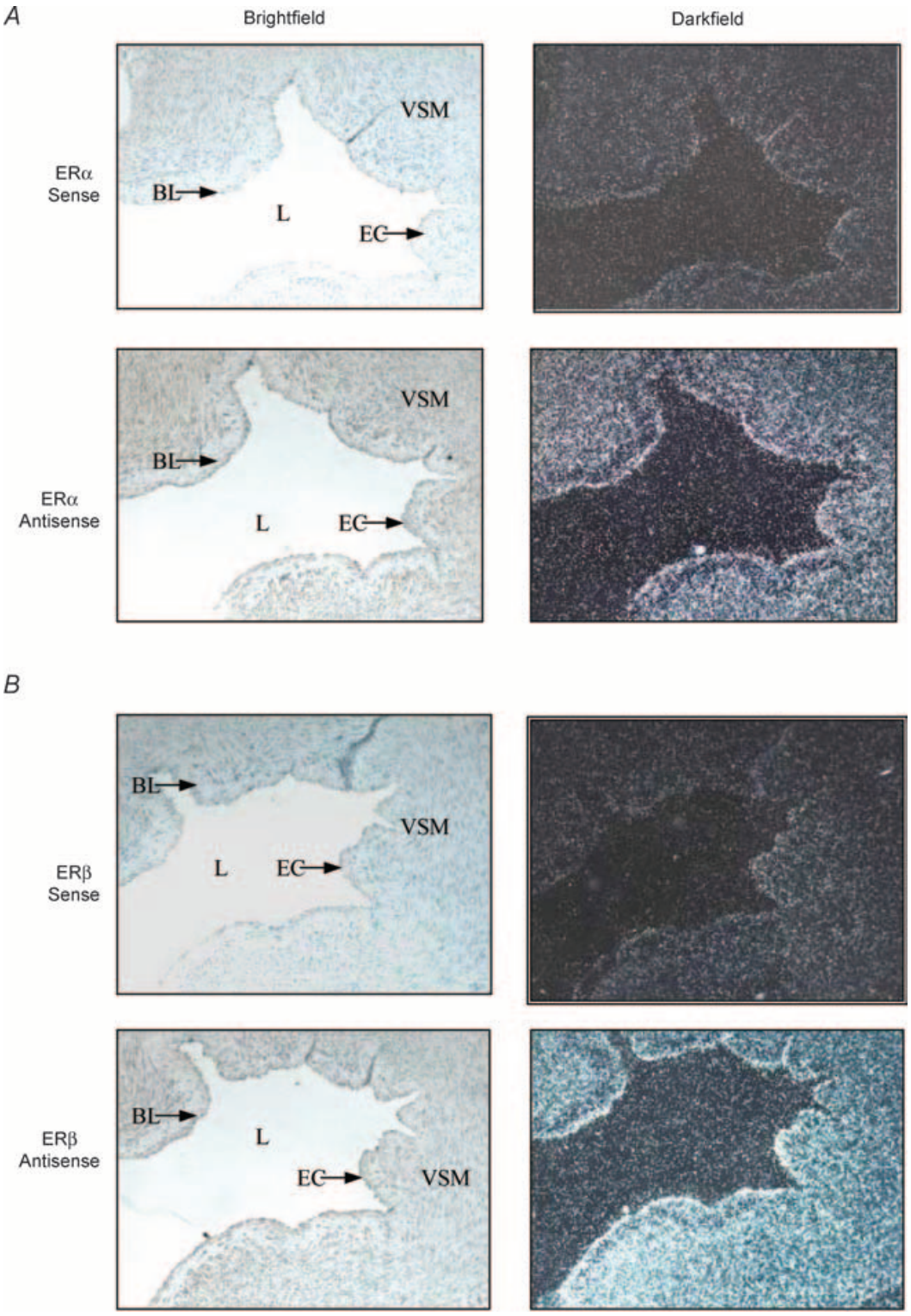

Figure 1. Localization of ERα (*A*) and ERβ (*B*) mRNAs in ovine uterine arteries
Uterine arteries from pregnant sheep were fixed and paraffin-embedded, and whole uterine artery sections (~5 μM) were cut. Localization of ERα and ERβ mRNAs was determined by *in situ* hybridization using ^{35}S-labelled sense (Control) or antisense riboprobes synthesized from a specific ovine ERα cDNA or bovine ERβ cDNA. Silver grains shown in darkfield images represent positive mRNA labelling. Images were taken at 10× magnification. Abbreviations: BL, basal lamina; EC, endothelial cell; L, lumen; VSM, vascular smooth muscle.

unaltered by oestrogen treatment, and thus remained significantly elevated compared to luteal phase UA VSM ($P < 0.05$). By contrast, P4 and P4 + E2β decreased UA VSM ERα ($P < 0.01$) compared to OVEX vehicle and oestrogen treatment reaching luteal phase levels. Figure 4*B* illustrates ERβ VSM expression in luteal *versus* OVEX HRT sheep. As observed with ERα, UA VSM ERβ levels in the OVEX vehicle and E2β were elevated ~2.0-fold over luteal controls ($P < 0.001$). Treatment with P4 significantly decreased UA VSM ERβ compared to both vehicle

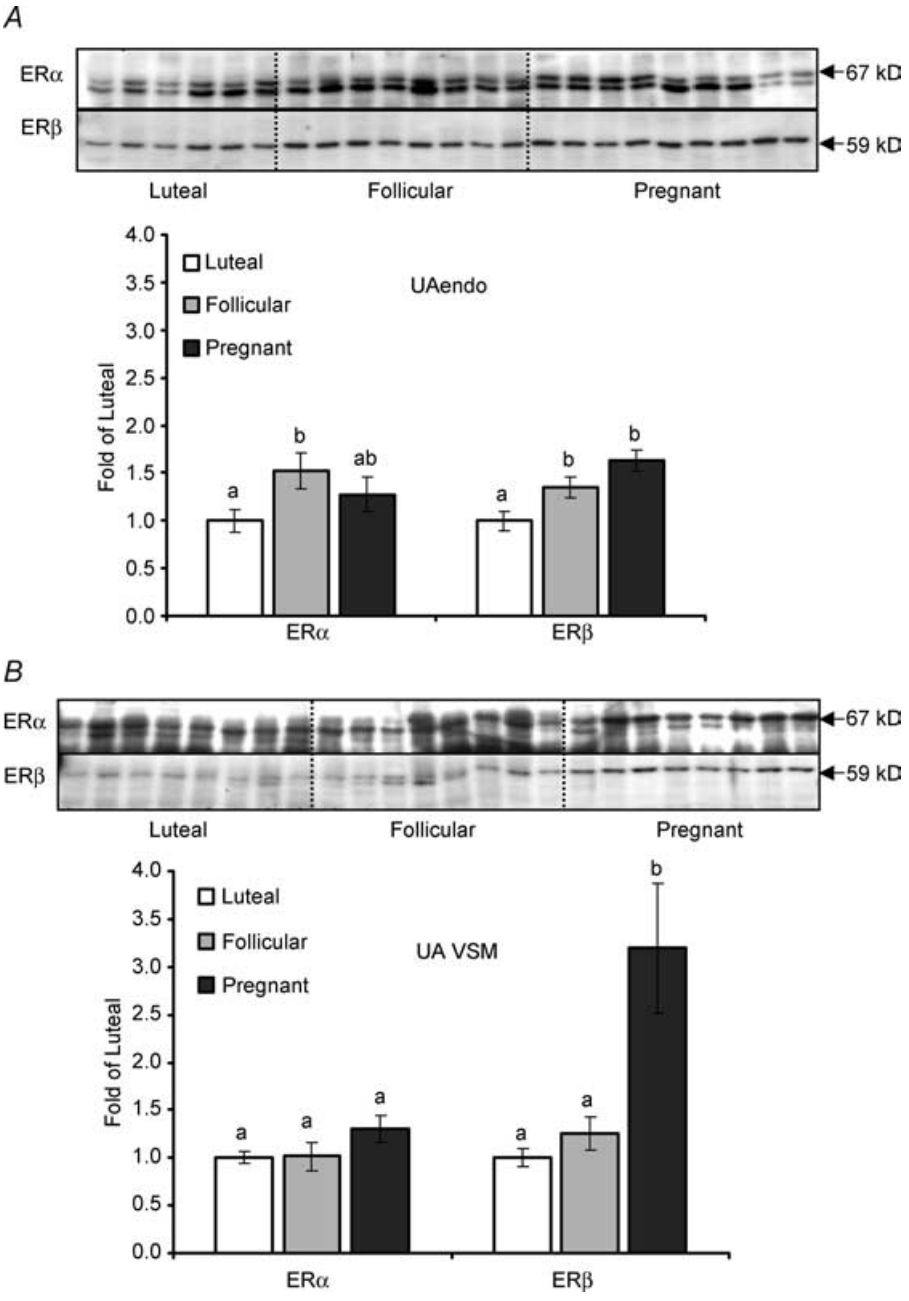

Figure 2. ERα and ERβ protein expression in ovine UAendo (*A*) and UA VSM (*B*) lysates from luteal, follicular and pregnant ewes

Western blot analysis was performed to compare the relative expression levels of ERα and ERβ protein in UAs from luteal ($n = 12$), follicular ($n = 8$), and pregnant ($n = 12$) sheep. *A*, UAendo samples represent endothelium (i.e. mechanically isolated tunica intima). *B*, UA VSM samples are denuded vessels that have had their endothelium removed leaving the basal lamina and VSM. Samples from luteal, follicular and pregnant sheep are expressed as fold of the average of all luteal samples run on the same Western blots. Data are means ± S.E.M. Means with different letter superscripts are statistically different ($P < 0.05$) within a tissue preparation. Representative Western immunoblots are shown above for ERα and ERβ at molecular weights of 67 and 59 kDa, respectively. The UA VSM immunoblots were reprobed for smooth muscle myosin and showed homogeneity of protein loading.

($P < 0.01$) and E2β treatments ($P < 0.05$). UA VSM ERβ remained partially elevated in the E2β + P4-treated animals.

In these OVEX HRT studies we also evaluated OAendo and OA VSM ER levels from the same animals. In contrast to UAendo, OAendo ER levels were unaltered ($P > 0.05$) by OVEX or HRT, ERα and ERβ, respectively, averaging 0.82 ± 0.06 and 1.09 ± 0.04 OVEX as a fold of luteal levels. By contrast to UA VSM, OA VSM ER levels were substantially reduced ($P > 0.01$) in OVEX ewes, ERα and ERβ, respectively, averaging only 0.06 ± 0.01 and 0.28 ± 0.02 OVEX as a fold of luteal levels. HRT did not alter OA VSM ER levels showing that the UA responses were specific to that vascular bed ($P > 0.05$). All of the UA and OA VSM immunoblots were reprobed for smooth muscle myosin and showed homogeneity of protein loading.

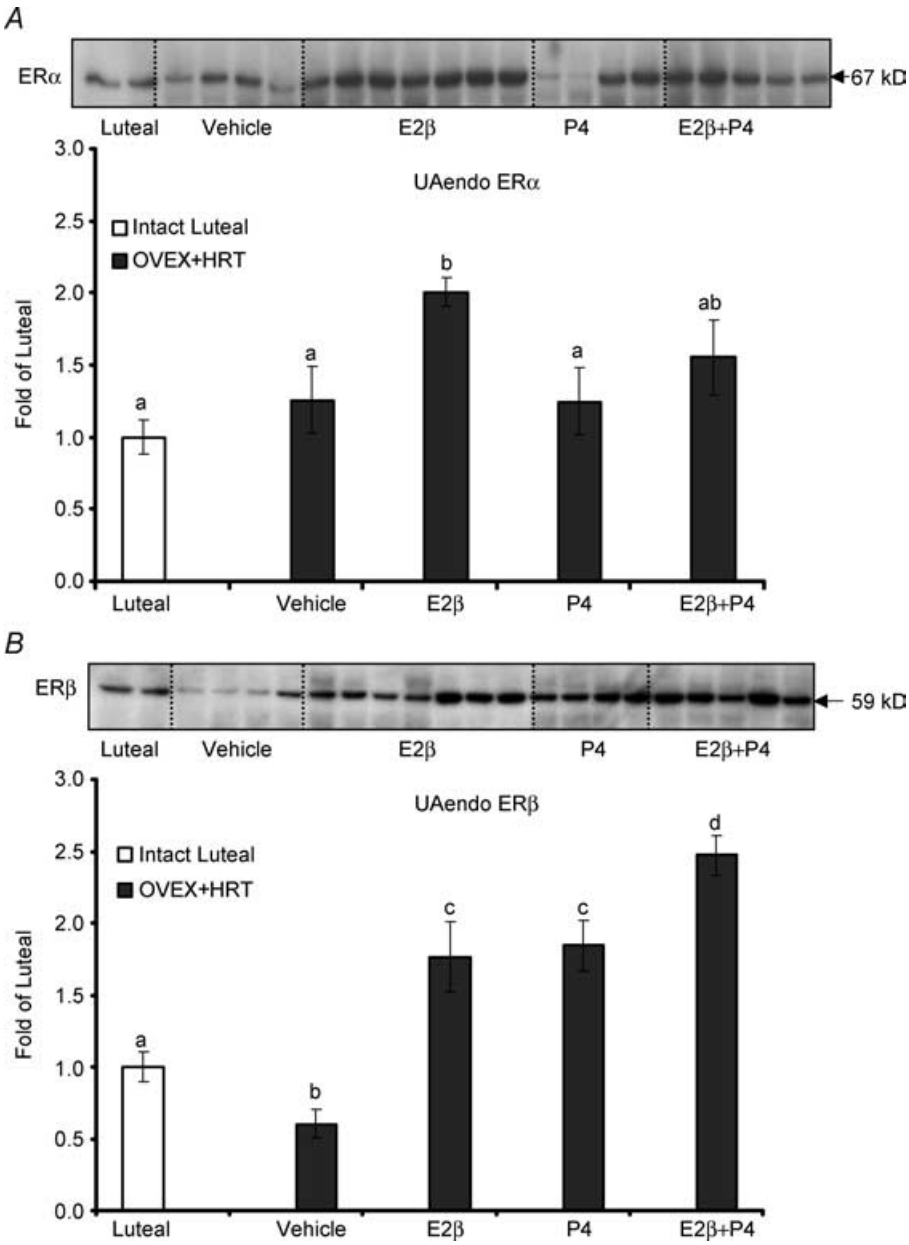

Figure 3. ERα (*A*) and ERβ (*B*) protein expression in ovine uterine artery endothelium of intact luteal phase *versus* OVEX ovarian steroid hormone treated ewes

Western blot analysis was performed to compare the relative levels of ERα and ERβ protein in luteal ($n = 12$) *versus* OVEX sheep treated with vehicle ($n = 4$), E2β ($n = 7$), P4 ($n = 4$) or E2β + P4 ($n = 5$). OVEX samples are expressed as fold of the average of the luteal samples run on the same Western blot. Data are means ± S.E.M. Means with different letters are statistically different ($P < 0.05$). Representative Western immunoblots are shown for ERα and ERβ at molecular weights of 67 and 59 kDa, respectively.

Effects of reproductive state on the expression of ERα, ERβ and eNOS protein in reproductive *versus* non-reproductive endothelium

Changes in UAendo ERα, ERβ, and eNOS protein were also compared to other reproductive endothelia, i.e. mammary (MAendo) and placental (PAendo) as well as various non-reproductive endothelia, omental (OAendo), renal (RAendo), and coronary (CAendo). In contrast to the 1.5-fold higher UAendo ERα in follicular *versus* luteal UAendo there was no significant ERα change in any other endothelial preparation from follicular or pregnant sheep (Fig. 5*A*). Besides UAendo, when compared to luteal endothelium, ERβ was not significantly changed in any of the follicular endothelial preparations ($P > 0.05$) that were

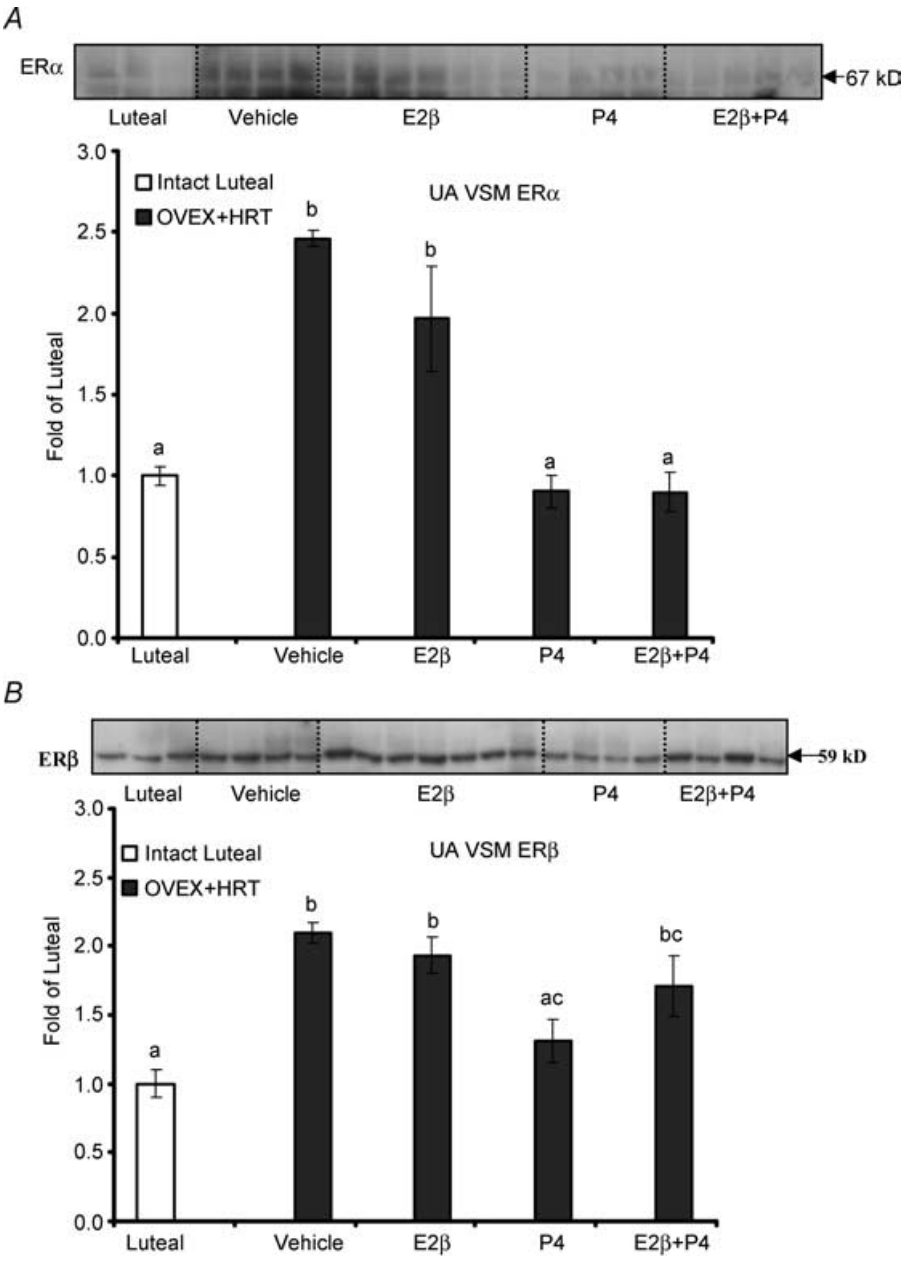

Figure 4. ERα (*A*) and ERβ (*B*) protein expression in ovine uterine artery vascular smooth muscle (VSM) of intact luteal phase *versus* OVEX ovarian steroid hormone treated ewes
Western blot analysis was performed to compare the relative expression of ERα and ERβ protein in luteal ($n = 12$) *versus* OVEX sheep treated with vehicle ($n = 4$), E2β ($n = 7$), P4 ($n = 4$) or E2β + P4 ($n = 5$). OVEX samples are expressed as a fold of the average of all the luteal samples runs on the same Western blot. Data are means ± S.E.M. Means with different letters are statistically different ($P < 0.05$). Representative Western immunoblots are shown for ERα and ERβ at molecular weights of 67 and 59 kDa, respectively. These UA VSM immunoblots were reprobed for smooth muscle myosin (SMM) and show homogeneity of protein loading.

surveyed (Fig. 5*B*). In contrast, during pregnancy, all three reproductive endothelial tissues displayed significantly increased ERβ expression. Specifically, UAendo ERβ from the pregnant group was increased 1.6-fold ($P < 0.01$) and MAendo 1.5-fold ($P < 0.01$) over luteal controls. Placental endothelial ERβ was 2.3-fold higher than luteal UAendo and clearly higher than pregnant UAendo. Since PAendo ERα was lower than luteal UAendo it appears that ERβ may be the primary ER in the placental vasculature. The only non-reproductive tissue in which ERβ changed significantly was CAendo from pregnant sheep, which rose 1.5-fold ($P < 0.05$) over luteal. In MAendo and CAendo follicular phase levels of ERβ were intermediate and thus not significantly different from either luteal or pregnant levels.

With regards to protein levels of eNOS, an endothelial marker that is consistently and substantially higher with physiological states of elevated UBF, in the follicular phase UAendo eNOS was elevated over luteal by 4.2-fold ($P < 0.001$) and during pregnancy by 5.8-fold ($P < 0.001$) (Fig. 6). However, eNOS protein was barely detectable in UA VSM samples, and was not significantly changed with reproductive state (data not shown). No other significant changes in eNOS levels were observed in any of the other reproductive or non-reproductive endothelia. These data are consistent with our previous reports (Magness *et al.* 1997, 2001; Vagnoni *et al.* 1998; Vagnoni & Magness, 1998) in uterne *versus* omental and renal arteries. We did not previously report the regulation of eNOS in PAendo, MAendo, or CAendo in these three physiological states. The fact that eNOS did not change in these vessels speaks of the uniqueness of the uterine vasculature and validates the physiological state of the animals.

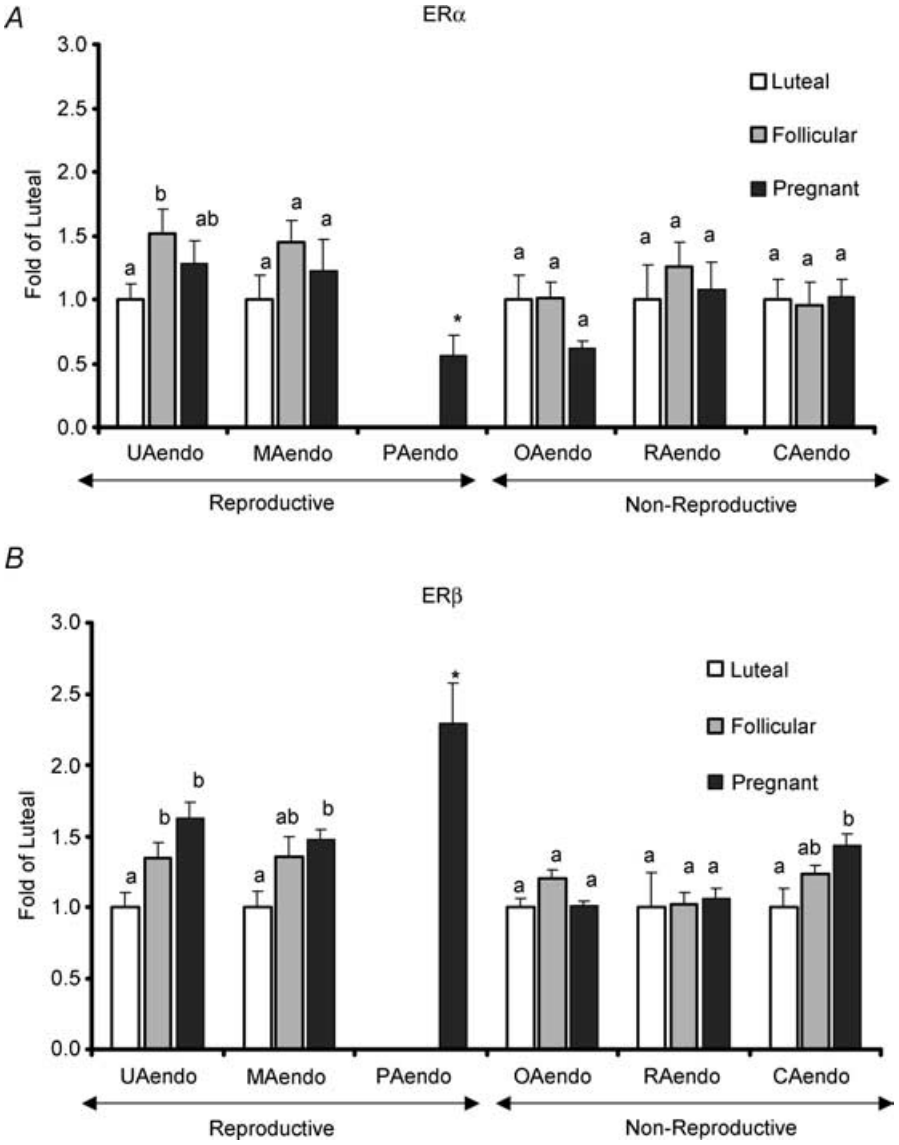

Figure 5. ERα (*A*) and ERβ (*B*) protein expression in reproductive *versus* non-reproductive arterial endothelia
Western blot analysis was performed to evaluate the relative levels of ERα and ERβ protein in luteal, follicular, and pregnant sheep reproductive and non-reproductive endothelia. Samples from luteal, follicular and pregnant sheep are expressed as fold of the average of all luteal samples within an artery type, run on the same Western blots. Expression of PAendo is given as fold of the average luteal UAendo also run on the same blot. Treatment groups: luteal: uterine ($n = 12$), mammary ($n = 5$), omental ($n = 7$), renal ($n = 6$), coronary ($n = 7$). Follicular: uterine ($n = 8$), mammary ($n = 5$), omental ($n = 8$), renal ($n = 6$), coronary ($n = 8$). Pregnant: uterine ($n = 12$), mammary ($n = 6$), placental ($n = 8$) omental ($n = 8$), renal ($n = 8$), coronary ($n = 8$). Data are means ± S.E.M. Means with different letters are statistically different ($P < 0.05$) within a tissue preparation. ERα UAendo: Lut < Fol ($P < 0.05$), For ERβ UAendo: Lut < Fol ($P < 0.05$) and Lut < Preg ($P < 0.001$); MAendo: Lut < Preg ($P < 0.01$); CAendo: Lut < Preg ($P < 0.05$).*For ERα: PAendo < Luteal UAendo but for ERβ PAendo > Luteal UAendo ($P < 0.05$).

Discussion

Rises in UBF with exogenous oestrogen treatment and during the follicular phase and pregnancy appear to be partly mediated by elevations in endogenous oestrogen levels (Anderson *et al.* 1977; Ford *et al.* 1982; Magness & Rosenfeld, 1989*a*; Magness *et al.* 1991; Magness, 1998; Gibson *et al.* 2004). A physiological role for ERs to regulate UBF via an NO-mediated mechanism is deduced from studies demonstrating that the ER receptor antagonist ICI 182 780 and the NOS inhibitor L-NAME both partially, but also similarly, lower the UBF elevated in OVEX sheep with exogenous oestrogen treatment or in follicular and pregnant sheep (Van Buren *et al.* 1992; Rosenfeld *et al.* 1996; Magness *et al.* 1997, 2001; Gibson *et al.* 2004; Magness *et al.* 2005). We have not collected data ourselves about the remaining 30–40% of UBF that cannot be inhibited by L-NAME. However this might be due to calcium-activated potassium channels expressed on uterine VSM myocytes (Rosenfeld *et al.* 2000, 2002). Rosenfeld found that the combination of an inhibitor of BK_{Ca} (calcium-activated potassium channel) tetraethylammonium and L-NAME completely inhibited the $E_2\beta$-induced rise in UBF in OVEX ewes, which suggests the involvement of endothelium-derived hyperpolarizing factor (EDHF).

Treatment of UAECs in culture with E2β increases *de novo* NO production via an ER mediated mechanism, since it is completely blocked by both L-NAME and ICI 182 780 (Chen *et al.* 2004). In the current studies we observed that both ERα and ERβ mRNAs are expressed in UAendo and VSM using *in situ* hybridization (Fig. 1). These data are consistent with ER protein expression observed using IHC in this (data not shown) and our recent reports in ovine UAendo (Chen *et al.* 2004; Liao *et al.* 2005). Thus both the UAendo and UA VSM indeed are target sites for the actions of oestrogen on the uterine vasculature. However because eNOS is localized in the endothelium rather then the VSM (Magness *et al.* 1997; Vagnoni *et al.* 1998), the specific eNOS/NO component of the oestrogen-mediated rises in UBF occurs via an ER-mediated mechanism at the level of the UAendo more so than the VSM. In addition, nuclear and extra-nuclear ER staining *ex vivo* was observed, suggesting both traditional genomic signalling as well as novel, acute extra-nuclear cell

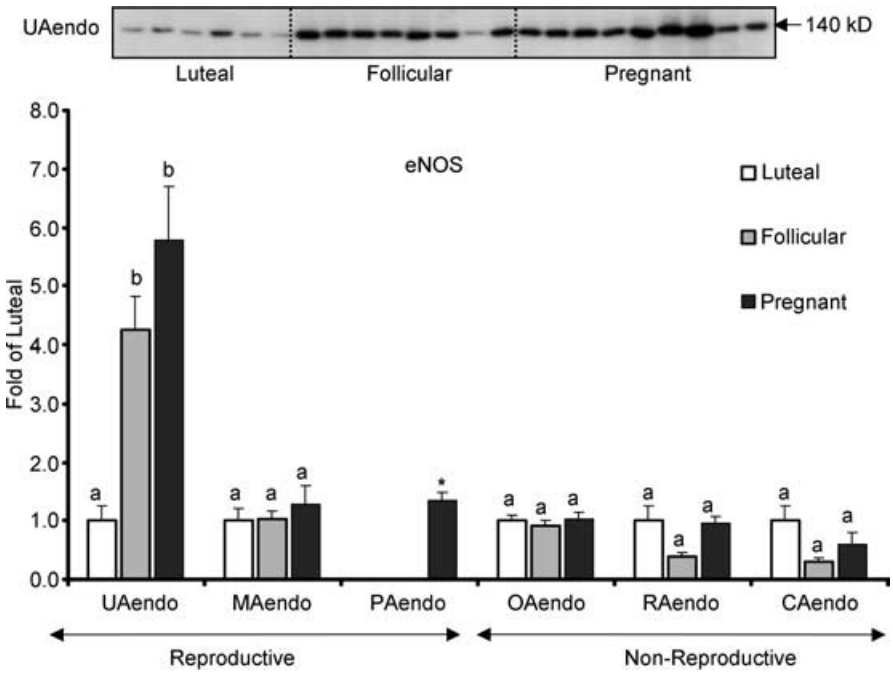

Figure 6. Expression of eNOS in reproductive *versus* non-reproductive arterial endothelia
Western blot analysis was performed to compare the relative expression of eNOS protein in luteal, follicular and pregnant sheep in reproductive and non-reproductive endothelium. Samples from luteal, follicular and pregnant sheep are expressed as fold of the average of all luteal samples within an artery type, run on the same Western blots. Expression of PAendo is given as fold of the average luteal UAendo also run on the same blot. Treatment groups: luteal: uterine ($n = 12$), mammary ($n = 5$), omental ($n = 7$), renal ($n = 6$), coronary ($n = 7$). Follicular: uterine ($n = 8$), mammary ($n = 5$), omental ($n = 8$), renal ($n = 6$), coronary ($n = 8$). Pregnant: uterine ($n = 12$), mammary ($n = 6$), placental ($n = 8$), omental ($n = 8$), renal ($n = 8$), coronary ($n = 8$). Data are means ± S.E.M. Means with different letters are statistically different ($P < 0.05$) within a tissue preparation. UAendo: Lut < Fol ($P < 0.001$), Lut < Preg ($P < 0.001$). A representative Western immunoblot is shown above for eNOS at a molecular weight of 140 kDa.

signalling are present *in vivo* as we have demonstrated in cultured UAEC studies (Chen *et al.* 2004; Liao *et al.* 2005). ERs are after all, transcription factors, and in vascular cells ERs mediate oestrogen regulation of the expression of a number of target genes, which in turn mediate the direct long-term or genomic effect of oestrogen on vascular tissue (Mendelsohn & Karas, 1999). The promoter region of the human (Venema *et al.* 1994) and sheep (Genebank accession no. AY684193) eNOS genes contains multiple half-palindromic oestrogen responsive elements (EREs), suggesting ER-mediated actions on eNOS gene expression in UA endothelial cells. In this regard, we confirmed our previous observations that UAendo eNOS is elevated in the follicular phase (4.2-fold), pregnancy (5.8-fold) (Fig. 6), and with progesterone (∼2.6-fold), oestrogen (∼5.4-fold) and combination treatment (∼7.5-fold) (VEH < P4 ≪ E2B = E2B + P4; not shown) (Magness *et al.* 1997, 2001; Vagnoni *et al.* 1998; Rupnow *et al.* 2001). These data are specifically shown because these well-described changes in eNOS fully validate the UAendo preparations as reflections of the true physiological/HRT status. The data presented herein clearly demonstrate for the first time that the levels of ERs are indeed spatially and differentially regulated in the UAendo and/or UA VSM during physiological states of elevated oestrogen or with HRT.

Western analysis revealed that during the follicular phase ERα and ERβ were elevated in the UAendo, but not UA VSM. These data support the premise that ovarian steroids maintain and regulate UAendo ER expression. We also observed that in UAendo ERβ, but not ERα protein was substantially reduced in OVEX vehicle sheep compared to luteal phase sheep (Fig. 3*B*). Although it is unknown if ERα or ERβ, or both mediate the oestrogen-induced rise in UBF, the fall in UAendo ERβ with OVEX may partly explain the reduced E2β-mediated uterine vasodilatory responses observed in OVEX *versus* ovary intact sheep (Gibson *et al.* 2004). The alteration in UAendo ERs was due to removal of ovarian-derived oestrogen or progesterone since HRT restored and/or elevated the ER levels. Unlike ERα, UAendo ERβ regulation appeared to be responsive to both oestrogen and progesterone and the combination caused an even greater rise in this receptor. Alternatively, Byers *et al.* (1997) reported that the preovulatory gonadotropin surge in cycling rats dramatically down-regulated ERβ mRNA in the ovary. Circulating gonadotropin levels in our OVEX ewes would be elevated, due to a lack of ovarian negative feedback to the hypothalamus (Karsch *et al.* 1973), possibly also down-regulating ERβ levels in UAendo. This is plausible, but untested, given that Toth *et al.* (2001) demonstrated the presence of LH/hCG receptors in UA endothelium.

During pregnancy, a state of elevated oestrogen and progesterone, only minor changes in ERα were noted, whereas ERβ was significantly increased in both UAendo, but even more so in UA VSM. This substantial rise in UA VSM ERβ (in the presence of constitutive levels of ERα) may have functional implications with regard to the fact that the uterine vascular bed has reduced vasoconstrictor responses during pregnancy (Magness, 1998 for review) and also that UA VSM undergoes dramatic concentric local remodelling during gestation via processes of hypertrophy and hyperplasia (Cipolla & Osol, 1994; Hammer & Cipolla, 2005). There are no studies defining the roles of ERα and/or ERβ in these important uterine vascular adaptations noted during pregnancy. The explicit role of ER subtypes in the entire cardiovascular system has been difficult to assess due to the lack of specific ER receptor subtype antagonists. However some information can be gleaned from genetic knock-out (KO) studies. ERβ KO mice develop age-dependent hypertension with males showing greater disease progression, demonstrating a role for ovarian steroids, and in particular oestrogen, to maintain reduced constrictor responses in the vasculature via ERβ (Zhu *et al.* 2002). In other studies, when wild-type mice were subject to carotid vascular injury, the levels of ERβ were elevated in the underlying VSM during the healing/remodelling process (Lindner *et al.* 1998). However when experiments were performed with a truly valid KO model, E2β conferred its vascular protective effect (i.e. reduced medial thickening and VSM proliferation index) via ERα (Pare *et al.* 2002) rather then ERβ (Karas *et al.* 2001).

In contrast to UAendo, UAVSM from OVEX ewes (vehicle and E2β) had elevated ERα and ERβ levels compared to luteal phase sheep, demonstrating spatial regulation different from that of the UAendo. In contrast, OA VSM from OVEX sheep showed substantial reductions in both ERα and ERβ levels which were unaltered by HRT (data not shown). The elevated UA VSM ER levels were reduced by progesterone (Fig. 4). Considering that pregnancy is characterized by high levels of oestrogen and progesterone it was surprising that ERα in the UA VSM was not regulated by pregnancy and ERβ expression was dramatically elevated (Fig. 2). Thus unlike the OVEX HRT model, pregnancy may regulate UA VSM ERs via mechanisms other than placental-derived oestrogen and progesterone. This suggestion needs to also be considered in light of the fact that the OVEX sheep were steroid treated for only 10 days and the UAs from pregnant sheep were from late gestation animals (120–130 days).

There are aspects of the HRT studied that did mimic the endogenous steroid hormone effects. In contrast to the UA VSM, the UAendo showed follicular rises in ERα (Fig. 2) that were similar to the E2β-induced rise in ERα and the latter was partially attenuated by progesterone combination to intermediate levels similar

to luteal-, vehicle- or progesterone-alone-treated OVEX sheep (Fig. 3). Moreover, ERβ is significantly elevated in follicular and pregnant sheep (Fig. 2), and also in OVEX sheep with exposure of oestrogen, progesterone, or its combination for 10 days (Fig. 3). Thus, the ERα and ERβ levels *in vivo* are regulated spatially and differentially in UAendo *versus* UA VSM by ovarian steroids. Our observations are in part consistent with uterine tissue studies by Wu *et al.* (1996), who showed that combination oestrogen and progesterone infusion into OVEX ewes down-regulates ER mRNA in myometrium and endometrium. Similar results were seen in OVEX rats treated with progesterone, i.e. ERα, but not ERβ, mRNA fell in uterine extracts, returning to control levels after removal of the progesterone implant (Rosser *et al.* 1993; Murata *et al.* 2003). Spencer & Bazer (1995) and Zheng *et al.* (1996) also demonstrated ER regulation, finding that in cycling and pregnant ewes, steady state levels of endometrial and myometrial ER mRNA are modulated by the endocrine status of the animal. Collectively, these data suggest that uterine ER gene expression is regulated in a tissue- and even a cell type-specific manner during the oestrous cycle and pregnancy and that local UAendo ER changes may partly regulate responsiveness to the vasoactive effects of exogenous and endogenous oestrogen levels.

While others have shown multiple tissue comparisons (Kuiper *et al.* 1997; Taylor & Al-Azzawi, 2000) or partial surveys of vascular ER expression (Orimo *et al.* 1993; Karas *et al.* 1994; Losordo *et al.* 1994; Venkov *et al.* 1996; Kim-Schulze *et al.* 1996; Caulin-Glaser *et al.* 1996; Hodges *et al.* 2000; Andersson *et al.* 2001), this is the first study in which the endothelial isolated proteins were extensively studied with regard to endocrine status of the animal. Although ERα was readily detectable in the vessels tested, none of the other reproductive or non-reproductive endothelia surveyed except UAendo and PAendo had ERα levels that were different relative to luteal phase expression (Fig. 5). ERβ levels also were not altered by physiological state in RAendo and OAendo. The observation that both ERs are not altered in OAendo by the ovarian cycle, OVEX or HTR may have functional significance in that we have demonstrated that whereas oestrogen profoundly increases UBF 10- to 20-fold in as little as 2 h, omental blood flow was unaltered (Magness & Rosenfeld, 1989*a*; Magness *et al.* 1998, 2005). It is noteworthy that ERβ was indeed elevated by pregnancy in the three reproductive endothelia (UA, MA and PA), but also CAendo and that follicular ERβ levels were similar to pregnancy levels in UA, MA and CA. Thus CA was the only non-reproductive endothelium to show ERβ regulation and suggests a more ubiquitous role for this receptor subtype in the mammalian vasculature especially in lieu of the observation that ERβ KO mice develop age-dependent hypertensive cardiovascular disease (Zhu *et al.* 2002). The finding that ERβ in CAendo is increased in pregnancy is particularly intriguing, given the substantial increases in cardiac output, heart rate and stroke volume and the profound decrease in systemic vascular resistance observed during normal pregnancy (Magness, 1998; Elkayam *et al.* 2001). Furthermore, blood flows to the vascular beds surveyed are differentially affected by exogenous E2β treatment, i.e. elevated in the uterine, mammary, and coronary, but not omental or renal (Magness *et al.* 1998; Rosenfeld *et al.* 1973, 1976). Moreover, oestrogen-induced rises in UBF (Van Buren *et al.* 1992; Rosenfeld *et al.* 1996; Gibson *et al.* 2004), coronary blood flow (Lang *et al.* 2000) and mammary blood flow (Mershon *et al.* 2002) are attenuated by treatment with the NOS inhibitor L-NAME. In this regard, eNOS was only up-regulated in the UAendo during folliculogenesis and pregnancy, though it is present in all the reproductive and non-reproductive endothelia tested (Fig. 6), suggesting elevations in eNOS activation and thus NO production rather then elevations in eNOS capacity may be more important in CA and MA endothelia. These data also reinforce the unique nature of the UAendo *versus* other vascular beds as a tissue highly responsive to oestrogen.

In summary, the current study provides clear evidence of ERα and ERβ expression in various vascular beds implicating direct effects of oestrogen on the vascular wall of reproductive and non-reproductive arteries. We also investigated regulation of ER protein expression in the endothelium *versus* the VSM during physiological states of elevated endogenous oestrogen (i.e. the ovarian cycle and pregnancy) and with exogenous HRT in OVEX sheep. In UAendo, but not UA VSM, ERα and ERβ levels were elevated during the follicular phase. However, pregnancy only elevated ERβ in both UAendo and UA VSM. OVEX substantially reduced UAendo ERβ, but not UAendo ERα or either ER in OAendo. In contrast, ERα and ERβ were increased in UA VSM, but were decreased in OA VSM. In OVEX ewes UAendo ERα levels were increased by E2β treatment whereas UAendo ERβ levels were elevated by $E_2\beta$ and progesterone treatment. During pregnancy, a state of elevated oestrogen and progesterone, ERβ levels were greater in uterine, mammary, placental, and coronary artery endothelia. Thus UA ERα and ERβ expression levels depend on the endocrine status of the animal, demonstrating that UA endothelium is a target for oestrogen action *in vivo,* and that the two receptors appear to be differentially regulated in a spatial and temporal fashion with regard to the reproductive status or HRT. This differential regulation of both ERα and ERβ expression in various reproductive and non-reproductive vascular beds by oestrogen implies an auto-regulation loop with regards to oestrogen actions in the cardiovascular system.

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