ONLINE SUPPLEMENT

Estradiol-17β, and its CYP450- and COMT-Derived Metabolites Stimulate Proliferation in Uterine Artery ECs: Role of ER-α vs. ER-β

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Expanded Materials and Methods:

Materials:

$E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 were purchased from Steraloids Inc., Newport, RI. BrdU Cell Proliferation Assays and rabbit anti-CYP3A4 were obtained from EMD Chemicals Inc., Gibbstown, NJ. ICI 182,780 was purchased from Sigma-Aldrich, St. Louis, MO. 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyleth oxy)phenol]-1H-pyrazole dihydrochloride (MPP) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrim idin-3-yl]phenol (PHTPP) were purchased from Tocris Bioscience, Ellisville, MO. Mouse anti-CYP1A1 and rabbit anti-CYP1A2 were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Rabbit anti-CYP1B1 was purchased from Abcam Inc., Cambridge, MA, rabbit anti-COMT was purchased from Chemicon International Inc., Temecula, CA and rabbit anti-ER-β antibody was purchased from Novus Biologicals, Littleton, CO.

Cell Preparation and Culture:

All procedures and protocols for experimental procedures were approved by the University of Wisconsin-Madison School of Medicine and Public Health Research Animal Care and Use Committee. UAECs were isolated from late gestation ewes (P); (120-130 days; term= 147 days; n=6) and nonpregnant (NP; luteal n= 5 and follicular n=2) ewes by collagenase digestion, cultured in growth media (DVal MEM with 20% FCS, 100 mg/ml penicillin, and 100 mg/ml streptomycin) as previously described.[1,2] Validations were conducted on each cell preparation for functional endothelial cell markers, PECAM-1, eNOS, LDL-uptake and smooth muscle myosin (negative control) as previously described.[1,2] UAECs (passages 3-4) were plated in T75 flasks containing phenol free Endothelial Basal Medium (EBM) serum free without growth factors (Lonza, Walkersville, MD), 20% FBS and 1% penicillin-streptomycin. Cells were grown to ~ 70% confluence and were at passage 5 when transferred to chambered coverglass slides, or 96 well plates, or lysed for protein extraction as needed for the respective experiments described below.

Protein Extraction and Western Immunoblotting:

Protein extraction was performed on UAECs by lysing them in 400 µl of lysis buffer (0.5 M Tris + 0.1 M EDTA + 0.15 M NaCl + 0.1% Tween-20 + 5 mg/ml aprotinin + 5 mg/ml leupeptin + 0.001 M PMSF). Total protein content was determined using BCA Protein Assay (Thermo Scientific, Rockford, IL). For Western blotting, 20 µg protein/lane were boiled in SDS sample buffer for 5 min and electrophoresed on 4-20% gradient SDS-PAGE gels (Bio-Rad, Hercules, CA) for 100 min at 150 V. Separated proteins were then electrically (100 V, 30 min) transferred to a PVDF membrane. 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 120 min and incubated with primary antibodies (1 µg/ml; 1:500) in TBST + 1% BSA for 120 min. CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT protein were detected using mouse anti-CYP1A1, rabbit anti-CYP1A2, rabbit anti-CYP1B1, rabbit anti-CYP3A4 or rabbit anti-COMT. GAPDH was utilized as a loading control. Additionally, ER-β expression in NP-UAECs, P-UAECs and P-UAECs treated with 0.1 nmol/L of estrogen or its metabolites was detected using 1 µg/ml (1:500) rabbit anti- ER-β antibody (Figure S1). After washing, the membrane was incubated with the

corresponding peroxidase-conjugated IgG for 60 min and detected with the Pierce ECL detection kit (Thermo Scientific, Waltham, MA).

Immunofluorescence Confocal Microscopy:

UAECs were plated at ~ 40% confluence on Lab-Tek chambered coverglass slides (Thermo Scientific, Waltham, MA) in EBM + 20% FBS and 1% penicillin-streptomycin. Cells were briefly washed twice with ice cold PBS and fixed for 15 min with 3% paraformaldehyde. Fixed cells were rinsed with 50 mM glycine solution, permeabilized with 0.1% Tritox-X for 3 min and blocked for 30 min with goat serum. Cells were incubated for 30 mins in goat serum containing primary antibodies of 1 µg/ml (1:500) of mouse anti-CYP1A1, rabbit anti-CYP1A2, rabbit anti-CYP1B1, rabbit anti-CYP3A4, or rabbit anti-COMT. Subsequently, cells were incubated with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG of 1 µg/ml or Alexa Fluor 488 goat anti-rabbit IgG of 1 µg/ml (Invitrogen, CA). Wells were set up with corresponding concentrations of secondary antibodies to serve as non-specific binding controls. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, CA). Imaging was performed using Bio-Rad Radiance 2100 MP Rainbow confocal/multiphoton microscope (Bio-Rad Laboratories, Hercules, CA).

Cell Proliferation Assays:

BrdU label was added for 16 hrs during the 24 hrs of steroid treatment and this *in vitro* index of proliferation was evaluated. Plates were read using Synergy HT Multi-Mode Microplate Reader. Results were expressed as fold increases over untreated control after subtracting the value of the blank (wells incubated without BrdU loading).

Validation of cell number increase and cytoxicity after treatment with estrogen and its metabolites was performed using ViaLight Plus High Sensitivity Cell Proliferation and Cytotoxicity Kit (Lonza Inc., Rockland, ME) according to manufacturer's instructions. After 24 hour starvation and subsequent treatment with estrogen and its metabolites in white opaque 96-well plates (24-hours), cells were lysed with Lysis Reagent (10 mins) to extract ATP from cells. Then the appropriate amount of ATP Monitoring Reagent Plus was added (2 mins) in each well to generate luminescent signal. Plates were read using Synergy HT Multi-Mode Microplate Reader to determine luminescence and results expressed in Relative Light Units as fold increases over untreated control after subtracting the value of the blank against an ATP standard curve.

References:

1. Liao WX, Magness RR, Chen DB. Expression of estrogen receptors-alpha and -beta in the pregnant ovine uterine artery endothelial cells in vivo and in vitro. *Biol Reprod.* 2005; 72:530-537.
2. Bird IM, Sullivan JA, Di T, Cale JM, Zhang L, Zheng J, Magness RR. Pregnancy-dependent changes in cell signaling underlie changes in differential control of vasodilator production in uterine artery endothelial cells. *Endocrinology.* 2000; 141:1107-1117.

Results:

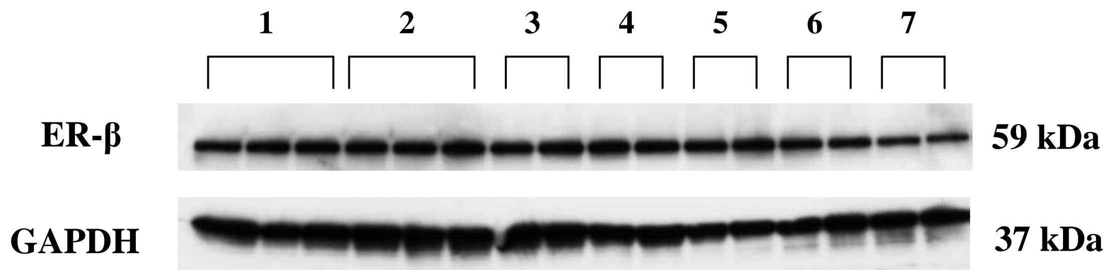

Figure S1: (A) Western immunoblot showing the expression of ER-β and GAPDH (loading control) in: (1) NP-UAECs, (2) P-UAECs and in P-UAECs treated with 0.1 nmol/L of (3) $E_2\beta$, (4) 2-OHE_2, (5) 4-OHE_2, (6) 2-ME_2 and (7) 4-ME_2. Densitometric quantification (data not shown) demonstrated that the levels of ER-β expression were not different between treatment groups (P=0.943). The blots are representative of two independent experiments and each well/blot within a bracket represents a different cell line.