ONLINE SUPPLEMENT

A NOVEL ROLE FOR AN ENDOTHELIAL ADRENERGIC RECEPTOR SYSTEM IN MEDIATING CATECHOLESTRADIOL-INDUCED PROLIFERATION OF UTERINE ARTERY ENDOTHELIAL CELLS

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Expanded Materials and Methods:

Materials:

$E_2\beta$, 2-OHE_2 and 4-OHE_2 were purchased from Steraloids Inc., Newport, RI. BrdU Cell Proliferation Assays was obtained from EMD Chemicals Inc., Gibbstown, NJ. Propranolol, Yohimbine, ICI 118,551, SR 59230A, Formoterol and BRL 37344 were purchased from Tocris Bioscience, Ellisville, MO. Norepinephrine, epinephrine and phentolamine was purchased from Sigma-Aldrich, St. Louis, MO. Mouse anti-α_1-AR, rabbit anti-α_2-AR, rabbit anti-β_1-AR, rabbit anti-β_2-AR and rabbit anti-β_3-AR were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Cell Preparation and Culture:

All procedures and protocols for animal use 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= 3 and follicular n=3) 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) 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 4 when lysed for protein extraction/Western blotting or transferred to 96 well plates as needed for the respective experiments described below.

Protein Extraction and Western Immunoblotting:

Protein extraction was performed on NP-UAECs or P-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. α_1-AR, α_2-AR, β_1-AR, β_2-AR and β_3-AR proteins were detected using mouse anti-α_1-AR, rabbit anti-α_2-AR, rabbit anti-β_1-AR, rabbit anti-β_2-AR and rabbit anti-β_3-AR. GAPDH and/or β-actin were utilized as a loading control. 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).

Cell Proliferation Assays:

5-Bromodeoxyuridine was added after 4 hours (i.e. 20 hours BrdU incubation) during the 24 hours of treatment and this *in vitro* index of proliferation was evaluated. Plates were read using Synergy HT Multi-Mode Microplate Reader. Results are expressed as the fold increases over untreated control after subtracting the "blank"(wells incubated without 5-bromodeoxyuridine). Validation of cell number increase and cytoxicity after treatment was performed using ViaLight Plus High Sensitivity Cell Proliferation and Cytotoxicity Kit (Lonza Inc., Rockland, ME) according to manufacturer's instructions.[2] After 24 hour starvation and subsequent treatment 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.

Experimental Treatments: Blockade and Activation of α-ARs and/or β-ARs:

P-UAEC proliferation experiments were performed in quadruplicates and replicated in ≥ four different P-UAEC preparations. For concentration-response studies, P-UAECs in 96-well plates were serum starved (24 hours) in EBM, washed with serum free EBM and medium was replaced with EBM or EBM containing 0.1, 1, 10 or 100 nmol/L of norepinephrine or epinephrine (24 hours) or 0.1 nmol/L of 2-OHE_2 or 4-OHE_2. We specifically chose to study the concentration of 0.1 nmol/L for the catecholestradiols based on the dose-response curves from our previous study.[2] For specificities of all antagonists and agonist used in this study, please see table S1. The α-ARs and/or β-ARs were blocked nonselectively by pretreating P-UAECs (10 μmol/L; 1 hour) with either the α-AR blocker phentolamine or the β-AR blocker propranolol followed by treatments with 0.1 nmol/L of norepinephrine and epinephrine or 2-OHE_2 or 4-OHE_2 (24 hours). Additional concentration studies to investigate AR activation with catecholamines alone or their interactive effects with catecholestradiols were evaluated by combining treatments of 0.1 nmol/L 2-OHE_2 or 4-OHE_2 with 0.1 nmol/L norepinephrine or epinephrine. Based on Western analyses expression of specific AR subtypes in P-UAECs, we conducted α-ARs or β-ARs subtype specific blockade by selectively blocking (10 μmol/L; 1 hr) with the α_2-AR inhibitor yohimbine, β_2-AR antagonist ICI 118,551, and β_3-AR inhibitor SR 59230A followed by catecholestradiol treatments (0.1 nmol/L for 24 hours). We then performed validation studies of AR-subtype specific inhibition by evaluating P-UAEC mitogenic concentration responses using 0, 0.1, 1, 10, 100 nmol/L of the specific β_2-AR agonist Formoterol as well as the β_3-AR agonist BRL 37344. We also studied the effects of 1 μmol/L of ICI 118,551 or SR 59230A on 100 nmol/L of Formoterol and BRL 37344 in order to further evaluate the mitogenic effects of receptor activation and specificity of β_2 and β_3-AR selective agonists in P-UAECs.

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Table S1:

Ligand Compound	**α-AR/β-AR**	**Agonist/Antagonist**	**Binding Selectivity Relative to other ARs**	**Reference**
Phentolamine	α-ARs	Antagonist	N/A	Meier et al, 1949.[3]
Propranolol	β-ARs	Antagonist	N/A	Stoschitzky et al, 1995.[4]
Yohimbine	α_2-ARs	Antagonist	N/A	Doxey et al, 1984.[5]
ICI 118,551	β_2-ARs	Antagonist	≥ 100-fold	Bilski et al, 1983.[6]
SR59230A	β_3-ARs	Antagonist	≥ 10-fold	Manara et al, 1996.[7]
Formoterol	β_2-ARs	Agonist	≥ 330-fold	Decker et al, 1982.[8]
BRL37344	β_3-ARs	Agonist	≥ 4-fold	Oriowo et al, 1996.[9]

N/A means negligible binding selectivity and/or complete binding affinity to AR subtype