A Novel Role for an Endothelial Adrenergic Receptor System in Mediating Catecholestradiol-Induced Proliferation of Uterine Artery Endothelial Cells

Sheikh O. Jobe, Sean N. Fling, Jayanth Ramadoss, Ronald R. Magness

Abstract—Sequential conversion of estradiol-17β to its biologically active catecholestradiol, 2-hydroxyestradiol (OHE2) and 4-OHE2, contributes importantly to its angiogenic effects on uterine artery endothelial cells (UAECs) derived from pregnant, but not nonpregnant ewes via an estrogen receptor-independent mechanism. Because catecholestradiols and catecholamines exhibit structural similarities and have high affinity for α- and β-adrenergic receptors (ARs), we investigated whether the endothelial α- or β-ARs mediate catecholestradiol-induced proliferation of P-UAECs and whether catecholamines alter these responses. Western analyses revealed expression of specific AR subtypes in nonpregnant UAECs and P-UAECs, including α2-, β2-, and β3-ARs but not α1- and β1-ARs. Levels of β2-ARs and β3-ARs were unaltered by pregnancy, whereas α2-ARs were decreased. Norepinephrine and epinephrine increased P-UAEC, but not nonpregnant UAEC proliferation, and these effects were suppressed by propranolol (β-AR blocker) but not phentolamine (α-AR blocker). Catecholamines combinations with 2-OHE2 or 4-OHE2 enhanced P-UAEC mitogenesis. Catecholestradiol-induced P-UAEC proliferation was also inhibited by propranolol but not phentolamine. β2-AR and β3-AR antagonists (ICI 118 551 and SR 59230A, respectively) abrogated the mitogenic effects of both 2-OHE2 and 4-OHE2. Stimulation of β2-ARs and β3-ARs using formoterol and BRL 37344 dose-dependently stimulated P-UAEC proliferation, which was abrogated by ICI 118 551 and SR 59230A, respectively. Proliferation effects of both catecholamines and catecholestradiols were only observed in P-UAECs (not nonpregnant UAECs) and were mediated via β2-ARs and β3-ARs. We demonstrate for the first time convergence of the endothelial AR and estrogenic systems in regulating endothelial proliferation, thus providing a distinct evolutionary advantage for modulating uterine perfusion during stressful pregnancies. (Hypertension. 2011;58:874-881.) • Online Data Supplement

Key Words: catecholamines ■ catecholestradiols ■ adrenergic receptors ■ endothelial proliferation

Estradiol-17β (E2β) has physiological/pathophysiologic effects on the cardiovascular system via diverse mechanisms, including local conversion to catecholestradiols 2-hydroxyestradiol (OHE2) and 4-OHE2 by cytochrome P450s.1,2 Catecholestradiols improve endothelial function and alleviate hypertension in ZSF1 rats, an animal model for hypertension and metabolic syndrome.2,3 We reported that catecholestradiols stimulate P-UAEC proliferation,1,2 but not phentolamine (β-AR blocker). Catecholestradiols compete with high affinity for binding to neuroendoctrine enzymes, α-adrenergic receptors (ARs) and β-ARs in the hypothalamus, anterior pituitary, corpus striatum, and liver.5-10 3D structural and functional analyses demonstrate that the catecholestradiol catechol moiety is functionally important in AR activation.11,12 Thus, it is conceivable that the estrogen receptor–independent mitogenic effects of 2-OHE2 and 4-OHE2 on the uterine endothelium may be mediated by α-ARs or β-ARs, and they may directly interact with the catecholamines that endogenously activate ARs. However, there are no reports on the potential role of an endothelial AR system in catecholestradiol-induced proliferation.

We hypothesized that catecholestradiols stimulate P-UAEC proliferation via α-ARs and/or β-ARs and that catecholamines that directly activate these ARs will alter these responses. We investigated the following: (1) NP-
UAEc versus P-UAEc subtype-specific expression of α₁-, α₂-, β₁-, β₂-, and β₃-ARs; (2) whether norepinephrine and epinephrine stimulate NP-UAEc versus P-UAEc proliferation and the interactive effects of catecholamines and catecholestradiol in mitogenesis; (3) whether catecholamines and catecholestradiols stimulate P-UAEc proliferation via α-ARs or β-ARs; (4) whether ARs exhibit subtype specificity in catecholestradiol-induced P-UAEc proliferation; and (5) subtype-specific pharmacological activation of ARs on P-UAEc proliferation. We report for the first time specific AR subtype-specific inhibition for P-UAEc mitogenic responses.

Figure 1. Adding 1 hydroxy (OH) group modifies the estrogenic phenolic A ring forming “catechol” moieties, potentially making it an adrenergic receptor (AR)-mimetic agent for inducing estrogen receptor (ER)-independent angiogenesis. The boxes outline the shared phenolic A ring catechol moiety among 2-hydroxyestradiol, 4-hydroxyestradiol, norepinephrine, and epinephrine. ?? indicates hypotheses tested.

**Methods**

For complete details, please see the online Data Supplement at http://hyper.ahajournals.org.

**Cell Preparation and Culture**

Protocols were approved by the University of Wisconsin-Madison Animal Care Committee.⁴ NP-UAEcs and P-UAEcs were isolated and cultured from nonpregnant (n = 6) and late-gestation (n = 6) ewes.⁴ At passage 4 (~70%) confluence, UAEcs were lysed for Western analyses or transferred to 96-well plates for experimental treatments.

**Western Analyses**

Western analyses were performed⁴ using rabbit anti–α₁-AR, anti–α₂-AR, anti–β₁-AR, anti–β₂-AR, or anti–β₃-AR antibodies (1:500) and secondary antibodies (1:2000). β-Actin and GAPDH were used as loading controls.

**5-Bromodeoxyuridine Cell Proliferation Assays**

5-Bromodeoxyuridine assay was performed and validated as described previously.⁴

**Experimental Treatments**

Proliferation experiments were performed in quadruplicates and replicated in ≥4 different preparations. NP-UAEcs and P-UAEcs in 96-well plates were serum starved (24 hours) and washed in endothelial basal media, and medium was replaced with endothelial basal media containing 0.0, 0.1, 1.0, 10.0, or 100.0 nmol/L of norepinephrine or epinephrine (24 hours) Additional P-UAEc studies investigating interactions were performed by combination treatments (0.1 nmol/L) of 2-OHE₂ or 4-OHE₂ with norepinephrine or epinephrine. α-ARs and/or β-ARs were blocked nonselectively by pretreating P-UAEcs (10 µmol/L; 1 hour) with phentolamine (α-AR blocker) or propranolol (β-AR blocker) followed by norepinephrine, epinephrine, 2-OHE₂, or 4-OHE₂ (0.1 nmol/L; 24 hours). Based on Western analyses of specific AR subtypes, we conducted AR subtype-specific inhibition for P-UAEc mitogenic responses (0.0, 0.1, 1.0, 10.0, and 100.0 nmol/L) using specific β₂-AR agonist formoterol and β₂-AR agonist BRL 37344. ICI 118 551 and SR 59230A (β₃-AR) followed by catecholestradiol treatments (0.1 nmol/L; 24 hours). We validated AR subtype-specific inhibition for P-UAEc mitogenic responses (0.0, 0.1, 1.0, 10.0, and 100.0 nmol/L) using specific β₂-AR agonist formoterol and β₂-AR agonist BRL 37344. ICI 118 551 and SR 59230A (10 µmol/L) effects, respectively on, formoterol and BRL 37344 (100 nmol/L) were utilized for specificity validation of β₂-AR and β₂-AR agonists in P-UAEcs. For antagonists/agonist specificities, please see the online Data Supplement.

**Statistical Analysis**

Data means±SEM are presented as a fold of untreated control. Overall group differences were determined by 1-way or 2-way
Results

P-UAECs Express Distinct AR Subtypes
Western immunoblotting showed α2-AR, β2-AR, and β3-AR but not α1-AR or β1-AR subtypes in P-UAECs (Figure 2A). Positive controls show protein expressions of α1-ARs, α2-ARs, β1-ARs, β2-ARs, and β3-ARs in vascular smooth muscle cells, left ventricular cardiomyocytes, and adipose tissue, thus validating the absence of α1-ARs or β1-ARs. Western analyses and densitometric analyses (data not shown) showed the equal expression of β2-ARs and β3-ARs between NP-UAECs and P-UAECs; however, α2-ARs were higher in NP-UAECs versus P-UAECs.

Catecholamines Increase P-UAEC but Not NP-UAEC Proliferation and Augment Catecholestradiol-Induced P-UAEC Proliferation
Neither norepinephrine nor epinephrine stimulated NP-UAEC proliferation (Figure 3A). Concentration-dependent norepinephrine responses were observed in P-UAEC (Figure 3B) with maximum proliferation (2.08 ± 0.03-fold) observed at 100 nmol/L. At a low physiological concentration (0.1 nmol/L), norepinephrine significantly elevated proliferation (1.78 ± 0.028-fold). The highest P-UAEC proliferative responses to epinephrine were 1.89 ± 0.035-fold observed at 1 nmol/L; however, at 10 and 100 nmol/L, there was no further increase in proliferation to epinephrine, and this was slightly less than that observed with norepinephrine.

We then determined their interactive effects using combination treatments (0.1 nmol/L) of norepinephrine or epinephrine with 2-OHE2 or 4-OHE2 (Figure 3C). At this dose, the magnitude of P-UAEC proliferative responses to catecholamines was similar to catecholestriols (1.86 ± 0.02-fold versus 1.81 ± 0.02-fold, respectively). Combination treatments with norepinephrine or epinephrine with either 2-OHE2 or 4-OHE2 further increased P-UAEC proliferation versus either catecholamine or catecholestradiol treatments alone (P < 0.05). Proliferative responses for combination treatments of norepinephrine or epinephrine with 2-OHE2 were 2.22 ± 0.07-fold and 2.24 ± 0.07-fold, respectively, and with 4-OHE2 were 2.15 ± 0.07-fold and 2.23 ± 0.07-fold, respectively.

β2-ARs but Not α-ARs Mediate Catecholamine and Catecholestradiol-Induced P-UAEC Proliferation
Phentolamine and propranolol antagonism (10 μmol/L) of P-UAEC proliferation using both 0.1 (Figure 4A) and 100.0 nmol/L (not shown) doses of catecholamines yielded identical results. Neither antagonist alone altered basal P-UAEC proliferation. Increases in proliferation seen with norepinephrine or epinephrine were unaltered (P > 0.05) by nonspecific inhibition of α-ARs using phentolamine. In contrast, nonselective blockade of β-ARs using propranolol completely abrogated catecholaminemediated P-UAEC proliferation (P < 0.05).

Confirming our previous report, the magnitudes of proliferation of P-UAECs in responses to 2-OHE2 (1.89 ± 0.02-fold) and 4-OHE2 (1.88 ± 0.02-fold) were not different (Figure 4B). Increases in P-UAEC proliferation seen with 0.1 nmol/L of 2-OHE2 and 4-OHE2 were unaltered (P > 0.05) by nonspecific inhibition of α-ARs using phentolamine. In contrast, nonselective blockade of β-ARs using propranolol completely abrogated catecholestradiol-mediated P-UAEC proliferation (P < 0.05). To determine the putative role of adrenergic G-protein–coupled receptors in E2β-β-AR-induced P-UAEC proliferation, we evaluated these α-AR and β-AR antagonists on E2β-β-AR-induced proliferation of P-UAECs. The E2β-β-AR-induced rise (0.1 nmol/L) in P-UAEC proliferations was not altered (P > 0.05) by either phentolamine or propranolol.

β2-ARs and, to a Lesser Extent, β3-ARs Mediate Catecholestradiol-Induced P-UAEC Proliferation
We then evaluated subtype-specific α-AR and β-AR inhibition (10 μmol/L) on the P-UAEC proliferative responses to catecholestradiols. None of the inhibitors alone altered basal control P-UAEC proliferation (Figure 5A). Inhibition of α2-AR subtype with yohimbine did not inhibit 2-OHE2- and 4-OHE2-induced P-UAEC proliferation. In contrast, the selective antagonists of β2-AR (ICI 118 551) or β3-AR (SR 59230A), respectively, either blocked (P < 0.01) or only...
Norepinephrine with either 2-hydroxyestradiol (OHE2) or 4-OHE2 partially attenuated ($P<0.05$) the proliferation induced by 0.1 nmol/L of 2-OHE$_2$ and 4-OHE$_2$.

We further evaluated additive effects of AR subtypes and putative AR heterodimerization in regulating catecholestradiol-mediated P-UAEC proliferation (Figure 5B). Combination of ICI 118 551 with either yohimbine or SR 59230A inhibited catecholestradiol-induced proliferation of P-UAECs, demonstrating primary involvement of $\beta_2$-ARs. In contrast, combination of SR59230A and yohimbine only partially decreased catecholestradiol-induced P-UAEC proliferation, demonstrating only partial $\beta_3$-AR subtype involvement. These combination inhibitor studies support neither dimerization nor significant cross-talk between these AR subtypes.

**Stimulation of $\beta_2$- and $\beta_3$-ARs Promotes P-UAEC Proliferation**

To further evaluate $\beta_2$-ARs versus $\beta_3$-ARs, we tested the actions of specific $\beta$-AR agonists. Both of the subtype-specific $\beta_2$-AR (formoterol) and $\beta_3$-AR (BRL 37344) agonists produced concentration-dependent and similar P-UAEC proliferative responses (Figure 6A). Formoterol and BRL 37344 (100 nmol/L) produced maximal P-UAEC proliferations of $1.89 \pm 0.07$-fold and $1.90 \pm 0.07$-fold, respectively. We then validated specificities of these agonists on their respective ARs (Figure 6B). P-UAEC proliferation by formoterol was completely attenuated by $\beta_2$-AR (ICI 118 551) but not $\beta_3$-AR (SR 59230A) antagonist. $\beta_3$-AR antagonist completely abrogated responses by BRL 37344 but not by formoterol.

**Discussion**

We reported recently that, unlike their parent substrate hormone E$_2$$\beta$, 2-OHE$_2$ and 4-OHE$_2$ do not stimulate P-UAEC proliferation via classic estrogen receptors.\textsuperscript{4} Herein we hypothesized that catecholestradiols mediate P-UAEC proliferation via either $\alpha$-ARs or $\beta$-ARs and that the catecholamines will modify/interact with these proliferative effects. We describe the first report of a complete and coupled AR system in P-UAECs (not NP-UAECs) that is responsible for catecholestradiol- and catecholamine-mediated proliferation, a critical process for angiogenic-mediated uterine perfusion during gestation. These data provide a novel previously undescribed model by which estrogen metabolites function as potential circulating $\beta$-AR mimetic agonists. Therefore, modifying the phenolic A ring of estrogens to catechol moieties generates an endogenous $\beta$-AR mimetic agent with angiogenic and possibly other cardiovascular capabilities. Our key findings are as follows: (1) in NP-UAECs and P-UAECs, there are distinct AR subtypes expressed, including $\alpha_2$-ARs, $\beta_2$-ARs, and $\beta_3$-ARs, but only in P-UAECs do norepinephrine and epinephrine increase proliferation; (2) catecholamines play a complementary and a conserved role to 2-OHE$_2$ and 4-OHE$_2$ by acting as positive modulators of augmented P-UAEC proliferation responses (2-way ANOVA; group $\times$ agonist; $F_{8,45}=3.70; P=0.015$; $n=4$).

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**Figure 3.** Catecholamines stimulate pregnant (P)-uterine artery endothelial cell (UAEC) but not nonpregnant (NP)-UAEC proliferation and augment catecholestradiol-induced P-UAEC proliferation. **A.** Concentration response of NP-UAECs to 0.0, 0.1, 1.0, 10.0, and 100.0 nmol/L of norepinephrine and epinephrine (2-way ANOVA; concentration $\times$ group; $F_{8,45}=0.306; P=0.960$; $n=6$). **B.** Concentration response of P-UAECs to 0.0, 0.1, 1.0, 10.0, and 100.0 nmol/L of norepinephrine and epinephrine (2-way ANOVA; concentration $\times$ group; $F_{8,45}=10.52; P<0.001$; $n=6$). **C.** Combinations of 0.1 nmol/L of norepinephrine or epinephrine with either 2-hydroxyestradiol (OHE$_2$) or 4-OHE$_2$.
P-UAEC proliferation; (3) neither catecholestradiols nor catecholamines induce P-UAEC proliferation via α-ARs but rather solely via β-ARs; (4) 2-OHE₂ and 4-OHE₂ modulate P-UAEC proliferation primarily via β₂-ARs and, to a lesser extent, via β₁-ARs; and (5) pharmacological agonists for either β₂-ARs or β₃-ARs specifically stimulate P-UAEC proliferation, suggesting similar coupling mechanisms and/or signaling convergence.

We report, for the first time, the in vitro expressions of several specific AR subtypes, α₂-ARs, β₂-ARs, and β₃-ARs, in NP-UAECs and P-UAECs, findings consistent with reports demonstrating distinct individual AR subtypes in endothelia of aorta, choroid, placenta, femoral artery, and retina.14–19 When compared with NP-UAECs, β₂-AR and β₃-AR expressions were unaltered by pregnancy status, whereas α₂-ARs were reduced. It is unclear whether coexpression of different specific ARs within the same endothelial cells represents unappreciated signaling complexity or just simply a functional redundancy. Using high-throughput proteomic analyses of P-UAECs, we observed that β₂-ARs are abundantly localized in the P-UAEC caveolae domain, a “hub” for compartmentalizing signal transduction for regulation of multiple functions (J. Ramadoss, PhD, and R.R. Magness, PhD, unpublished data, 2011). Therefore, demonstration of specific AR expression relative to the subcellular localization of α₂, β₂, and β₃-ARs in NP-UAECs versus P-UAECs needs...
to be determined. This may fulfill distinct physiological and pathophysiologic functional significance for expression relative to localization of multiple AR subtypes in the endothelium.

Because ARs are present on the endothelium, they are undoubtedly exposed to circulating endogenous norepinephrine and epinephrine released from the adrenal medulla. Normal physiological circulating catecholamine concentrations are 1 to 2 nmol/L and increase dramatically in pathological cardiovascular conditions and during fight or flight stress responses. Hence, we demonstrated that even a low physiological concentration (0.1 nmol/L) of both norepinephrine and epinephrine significantly increases P-UAEC proliferation, but not NP-UAEC, proliferation, suggesting that catecholamines augment in vivo angiogenesis in dopamine β-hydroxylase knockout mice deficient in plasma catecholamines.23 Confirming our recent report, a low physiological concentration (0.1 nmol/L) of 2-OHE2 and 4-OHE2 stimulates P-UAEC proliferation.4 We report herein for the first time that catecholamine and catecholestradiol combinations induced significantly higher P-UAEC proliferation. We further demonstrate for the first time that both catecholamines and catecholestradiols individually elevate P-UAEC proliferation only via β-ARs, suggesting that functional β-ARs are likely important for regulating physiological and/or pathological angiogenesis during gestation. Consistent with these findings, catecholamines augment in vivo angiogenesis in dopamine β-hydroxylase knockout mice deficient in plasma catecholamines.23 Therefore, signal transduction studies are needed to further elucidate the potential differences in β-AR–mediated molecular mechanism of action of the catecholestradiol versus catecholamines in endothelial cells.

The current observation that subtype-specific β2-AR antagonist ICI 118 551 abolished P-UAEC proliferation stimulated by 2-OHE2 and 4-OHE2 suggests β2-AR coupling, whereas the partial inhibition by β3-AR blocker SR 59230A also implies potential involvement of β3-ARs. In contrast, both the specific β3-AR (formoterol) and β3-AR (BRL 37344) agonists equally induced P-UAEC proliferation, which was specifically blocked by their specific antagonists (Figure 6), suggesting that both β-ARs may regulate these proliferative effects. Thus, the partial inhibitory effects of SR 59230A on catecholestradiol responses (Figure 5A) do not point to a lack of potency or effective concentration, because a similar concentration of SR 59230A induced significant abrogation of β3-ARs in response to BRL 37344. Activation
of either β1-AR and/or β2-AR has been shown to play a role in endothelial cell proliferation from human umbilical vein, retina, and bovine aortas.14–16,18 However, ours is the first report to demonstrate that β1-AR and β2-AR mediate the catecholestradiol-induced proliferation of endothelial cells. P-UAECs express similar levels of β1-ARs and β2-ARs compared with NP-UAECs, demonstrating that the AR-mediated effects are not dependent on expression levels but rather on other gestational programming factors at the level of signaling. These data, therefore, provide a broader understanding of the mechanism of action of catecholestradiols in endothelial cell proliferation. Importantly, these results also point to the potential relevance of previously unappreciated complexities of estrogen signaling in the cardiovascular system via interactions of steroid metabolites and the endothelial AR system.

Overall, the present study indicates that actions of catecholestradiols and catecholamines via endothelial ARs represent an evolutionary conserved and highly versatile signaling mechanism for regulating endothelial proliferation. During gestation, angiogenic processes are, to a great extent, responsible for the dramatic 30- to 50-fold rises in uterine blood flow, such that, by term, the uterine vascular bed receives ~20% of the also greatly expanded cardiac output and blood volume.31,32 Furthermore, maternal uterine perfusion is maintained 1- to 2-fold in excess of the needs of the parallel, but separate, fetoplacental circulation.33 We suggested previously that, during an acute gestational flight or fight response, when catecholamines are greatly elevated, far greater expression of estrogen receptors regulate retinal endothelial cell migration and proliferation.34–35

**Perspectives**
The current study sheds new light on the existence of a previously unrecognized 2-ligand system for a single AR family representing a mechanism by which the same physiological regulators of the flight or fight responses that protect the mother during a state of acute, but repeated, physiological stress will indeed act as an angiogenic switch to subsequently induce maintenance in uterine relative to fetoplacental blood flows. This provides for a marked evolutionary advantage of maintaining delivery of oxygen and nutrients through the uteroplacental circulation, thus protecting the growing fetus from subsequent stress-induced profound reductions in uterine blood flow. These data also uncover novel complexities of estrogen signaling in the cardiovascular system via ARs and necessitate the further investigation of estrogen metabolites, such as catecholestradiols, in the vascular system, which do not signal via the classic estrogen receptors.

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**Disclosures**
None.

**References**
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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:
E2β, 2-OHE2 and 4-OHE2 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-α1-AR, rabbit anti-α2-AR, rabbit anti-β1-AR, rabbit anti-β2-AR and rabbit anti-β3-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. α1-AR, α2-AR, β1-AR, β2-AR and β3-AR proteins were detected using mouse anti-α1-AR, rabbit anti-α2-AR, rabbit anti-β1-AR, rabbit anti-β2-AR and rabbit anti-β3-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 cytotoxicity 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-OHE2 or 4-OHE2. We specifically chose to study the concentration of 0.1 nmol/L for the catecholestriadiols 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-OHE2 or 4-OHE2 (24 hours). Additional concentration studies to investigate AR activation with catecholamines alone or their interactive effects with catecholestriadiols were evaluated by combining treatments of 0.1 nmol/L 2-OHE2 or 4-OHE2 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 α2-AR inhibitor yohimbine, β2-AR antagonist ICI 118,551, and β3-AR inhibitor SR 59230A followed by catecholestriadiol 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 β2-AR agonist Formoterol as well as the β3-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 β2 and β3-AR selective agonists in P-UAECs.

**References:**


### Table S1:

<table>
<thead>
<tr>
<th>Ligand Compound</th>
<th>α-AR/β-AR</th>
<th>Agonist/Antagonist</th>
<th>Binding Selectivity Relative to other ARs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phentolamine</td>
<td>α-ARs</td>
<td>Antagonist</td>
<td>N/A</td>
<td>Meier et al, 1949.3</td>
</tr>
<tr>
<td>Propranolol</td>
<td>β-ARs</td>
<td>Antagonist</td>
<td>N/A</td>
<td>Stoschitzky et al, 1995.4</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>α₂-ARs</td>
<td>Antagonist</td>
<td>N/A</td>
<td>Doxey et al, 1984.5</td>
</tr>
<tr>
<td>ICI 118,551</td>
<td>β₂-ARs</td>
<td>Antagonist</td>
<td>≥ 100-fold</td>
<td>Bilski et al, 1983.6</td>
</tr>
<tr>
<td>SR59230A</td>
<td>β₃-ARs</td>
<td>Antagonist</td>
<td>≥ 10-fold</td>
<td>Manara et al, 1996.7</td>
</tr>
<tr>
<td>Formoterol</td>
<td>β₂-ARs</td>
<td>Agonist</td>
<td>≥ 330-fold</td>
<td>Decker et al, 1982.8</td>
</tr>
<tr>
<td>BRL37344</td>
<td>β₃-ARs</td>
<td>Agonist</td>
<td>≥ 4-fold</td>
<td>Oriowo et al, 1996.9</td>
</tr>
</tbody>
</table>

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