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 catecholestradiols, 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 (nonpregnant UAECs) and were mediated via β2-ARs and β3-ARs. We demonstrate for the first time convergence of the endothelial AR and estrogen 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.¹² Catecholestradiols improve endothelial function and alleviate hypertension in ZSF1 rats, an animal model for hypertension and metabolic syndrome.²³ We reported that E2β, 2-OHE2, and 4-OHE2 increase proliferation in ovine uterine artery endothelial cells (UAECs) derived from pregnant (P-UAECs), but not the nonpregnant (NP-UAECs) ewes.⁴ Unlike E2β, 2-OHE2- and 4-OHE2-induced P-UAEC proliferation were not blocked by ICI-182 780, demonstrating that catecholestradiol-induced P-UAEC proliferation was estrogen receptor independent.⁴

Catecholestradiols exhibit close structural similarities (Figure 1) and functional interactions with the norepinephrine and epinephrine.⁵ Because of the shared phenolic A ring “catechol” moiety, catecholestradiols interact directly with catecholamine responses, a property not shared by E2β.⁵ Catecholestradiols compete with high affinity for binding to neuroendocrine 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-
The image contains a page of a scientific document discussing the effects of catecholestradiols and adrenergic receptors on P-UAEC proliferation. The text includes references to experimental treatments involving Western analyses, proliferation experiments, and 5-Bromodeoxyuridine cell proliferation assays. The document outlines specific AR subtype-specific pharmacological activation and blockade studies, including the use of specific AR agonists and antagonists.

### Methods

- **Cell Preparation and Culture**: Protocols were approved by the University of Wisconsin-Madison Animal Care Committee. NP-UAECs and P-UAECs were isolated and cultured from nonpregnant (n=6) and late-gestation (n=6) ewes. 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 performed using rabbit anti-α-AR, anti-β-AR, and specific antibodies for ER-α/ER-β. Western analyses were performed using rabbit anti-α-AR, anti-β-AR, and specific antibodies for ER-α/ER-β. Overall group differences were determined by 1-way or 2-way ANOVA. Data means ± SEM are presented as fold of untreated control.
ANOVA (SigmaPlot 11 Statistical Software), followed by post hoc multiple pairwise comparison Student-Newman-Keuls/Bonferroni tests. Level of significance was established a priori at $P<0.05$.

**Results**

**P-UAECs Express Distinct AR Subtypes**

Western immunoblotting showed $\alpha_2$-AR, $\beta_2$-AR, and $\beta_3$-AR but not $\alpha_1$-AR or $\beta_1$-AR subtypes in P-UAECs (Figure 2A). Positive controls show protein expressions of $\alpha_1$-ARs, $\alpha_2$-ARs, $\beta_1$-ARs, $\beta_2$-ARs, and $\beta_3$-ARs in vascular smooth muscle cells, left ventricular cardiomyocytes, and adipose tissue, thus validating the absence of $\alpha_1$-ARs or $\beta_1$-ARs.

**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 ($2.08\pm0.03$-fold) observed at 100 nmol/L. At a low physiological concentration (0.1 nmol/L), norepinephrine significantly elevated proliferation (1.78$\pm0.02$-fold). The highest P-UAEC proliferative responses to epinephrine were $1.89\pm0.035$-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-OHE$_2$ or 4-OHE$_2$ (Figure 3C). At this dose, the magnitude of P-UAEC proliferative responses to catecholamines was similar to catecholestriadiols ($1.86\pm0.02$-fold versus $1.81\pm0.02$-fold, respectively). Combination treatments with norepinephrine or epinephrine with either 2-OHE$_2$ or 4-OHE$_2$ 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-OHE$_2$ were $2.22\pm0.07$-fold and $2.24\pm0.07$-fold, respectively, and with 4-OHE$_2$ were $2.15\pm0.07$-fold and $2.23\pm0.07$-fold, respectively.

**$\beta_2$-ARs but Not $\alpha_2$-ARs Mediate Catecholamine and Catecholestradiol-Induced P-UAEC Proliferation**

Phentolamine and propranolol antagonism ($10\mu$mol/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 nonselective inhibition of $\alpha_2$-ARs using phentolamine. In contrast, nonselective blockade of $\beta_2$-ARs using propranolol completely abrogated catecholamine-mediated P-UAEC proliferation ($P<0.05$).

Confirming our previous report,$^4$ the magnitudes of proliferation of P-UAECs in responses to 2-OHE$_2$ (1.89$\pm0.02$-fold) and 4-OHE$_2$ (1.88$\pm0.02$-fold) were not different (Figure 4B). Increases in P-UAEC proliferation seen with 0.1 nmol/L of 2-OHE$_2$ and 4-OHE$_2$ were unaltered ($P>0.05$) by nonselective inhibition of $\alpha_2$-ARs using phentolamine. In contrast, nonselective blockade of $\beta_2$-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 E$_2$-induced P-UAEC proliferation, we evaluated these $\alpha_2$-AR and $\beta_2$-AR antagonists on E$_2$-induced proliferation of P-UAECs. The E$_2$-induced rise (0.1 nmol/L) in P-UAEC proliferations was not altered ($P>0.05$) by either phentolamine or propranolol.$^4$

**$\beta_2$-ARs and, to a Lesser Extent, $\beta_3$-ARs Mediate Catecholestradiol-Induced P-UAEC Proliferation**

We then evaluated subtype-specific $\alpha_2$-AR and $\beta_2$-AR inhibition (10 $\mu$mol/L) on the P-UAEC proliferative responses to catecholestradiols. None of the inhibitors alone altered basal control P-UAEC proliferation (Figure 5A). Inhibition of $\alpha_2$-AR subtype with yohimbine did not inhibit 2-OHE$_2$- or 4-OHE$_2$-induced P-UAEC proliferation. In contrast, the selective antagonists of $\beta_2$-AR (ICI 118 551) or $\beta_3$-AR (SR 59230A), respectively, either blocked ($P<0.01$) or only

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**Figure 2.** Adrenergic receptor (AR) subtypes in uterine artery endothelial cells (UAECs). A, Western blots demonstrating expression of $\alpha_2$-ARs, $\beta_2$-ARs, and $\beta_3$-ARs but not $\alpha_1$-AR or $\beta_1$-AR subtypes in pregnant (P)-UAECs. Positive control lanes are vascular smooth muscle cells (VSM), left ventricle cardiomyocytes (CMC), and adipose tissue (AT). B, Expression $\alpha_2$-ARs, $\beta_2$-ARs, and $\beta_3$-ARs in nonpregnant (NP)-UAECs vs P-UAECs. Blots are representative of 2 independent experiments from individual UAEC lines.
partially attenuated (P<0.05) the proliferation induced by 0.1 nmol/L of 2-OHE2 and 4-OHE2.

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 β2-ARs. In contrast, combination of SR59230A and yohimbine only partially decreased catecholestradiol-induced P-UAEC proliferation, demonstrating only partial β3-AR subtype involvement. These combination inhibitor studies support neither dimerization nor significant cross-talk between these AR subtypes.

**Stimulation of β₂- and β₃-ARs Promotes P-UAEC Proliferation**

To further evaluate β₂-ARs versus β₁-ARs, we tested the actions of specific β-AR agonists. Both of the subtype-specific β₂-AR (formoterol) and β₁-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±0.07-fold and 1.90±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 β₂-AR (ICI 118 551) but not β₁-AR (SR 59230A) antagonist. β₁-AR antagonist completely abrogated responses by BRL 37344 but not by formoterol.

**Discussion**

We reported recently that, unlike their parent substrate hormone E₂β, 2-OHE₂ and 4-OHE₂ do not stimulate P-UAEC proliferation via classic estrogen receptors. Herein we hypothesized that catecholestradiols mediate P-UAEC proliferation via either α-ARs or β-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 β-AR mimetic agonists. Therefore, modifying the phenolic A ring of estrogens to catechol moieties generates an endogenous β-AR mimetic agonist 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 α₁-ARs, β₂-ARs, and β₁-ARs, but only in P-UAECs do norepinephrine and epinephrine increase proliferation; (2) catecholamines play a complementary and a conserved role to 2-OHE₂ and 4-OHE₂ by acting as positive modulators of augmented P-UAEC proliferation responses (2-way ANOVA; group x agonist; F₆,₄₆=3.73; P=0.015; n=4). *Increase vs control. †Increase vs 0.1 and 1.0 nmol/L norepinephrine > epinephrine. ‡Increase vs catecholestradiols or catecholamines alone.
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 caveola 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

Figure 4. β-Adrenergic receptors (ARs), but not α-ARs, mediate catecholamine and catecholestradiol-induced pregnant (P)-uterine artery endothelial cell (UAEC) proliferation. A. Effects of phenolamine or propranolol on P-UAEC proliferation to 0.1 nmol/L of norepinephrine or epinephrine. Phenolamine had no effect, whereas propranolol completely abrogated catecholamine-induced P-UAEC proliferation (2-way ANOVA; antagonist × group; F₈,₃₃=9.12; P<0.001; n=4). B. Effects of phenolamine or propranolol on P-UAEC proliferative responses to 2-hydroxyestradiol (OHE₂), 4-OHE₂, or estradiol-17β (0.1 nmol/L). Phenolamine had no effect, whereas propranolol completely abrogated 2-OHE₂- and 4-OHE₂- but not E₂-β-induced P-UAEC proliferative responses (2-way ANOVA; antagonist × group; F₆,₃₆=7.88; P<0.001; n=4). *Increase vs untreated. †Complete inhibition.

Figure 5. β₂-Adrenergic receptor (ARs) and, to a lesser extent, β₃-ARs, mediate catecholestradiol-induced pregnant (P)-uterine artery endothelial cell (UAEC) proliferation. A. Effects of yohimbine, ICI 118 551, or SR 59230A on P-UAEC proliferation to 2-hydroxyestradiol (OHE₂) or 4-OHE₂ (0.1 nmol/L). Yohimbine had no effect, whereas ICI 118 551 attenuated and SR 59230A partially inhibited catecholestradiol-mediated P-UAEC proliferation (2-way ANOVA; antagonist × group; F₈,₃₃=7.871; P<0.001; n=4). B. Effects of yohimbine, ICI 118 551, and SR 59230A combinations on P-UAEC proliferative responses to catecholestradiols. ICI 118 551, in all of the combinations, completely blocked P-UAEC proliferation responses to catecholestradiols (2-way ANOVA; antagonist combination × group; F₈,₃₃=9.551; P<0.001; n=4). *Increase vs untreated. †Complete inhibition. ‡Partial inhibition.
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/L20–22 and increase dramatically in pathological cardiovascular conditions and during flight 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, but not NP-UAEC, proliferation, suggesting that catecholamines indeed may play roles in regulating physiological angiogenesis during gestation. Consistent with these findings, 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. These data, therefore, demonstrate that catecholamines play a complementary and even an additive role to 2-OHE2 and 4-OHE2 as positive β-AR–mediated modulators of physiological angiogenesis. Our data also imply that catecholamines and catecholestradiols should exhibit similar AR subtype-specific signaling pathways to induce P-UAEC proliferation. Catecholestradiols have been shown previously to competitively bind to AR subtypes in rat cerebral cortex, striatum, and anterior pituitary, as well as to guinea pig hypothalamic, membranes.8,10 Therefore, our data show that catechol moieties of catecholestradiols and catecholamines are very important for the binding and activation of β-AR signaling.

The lack of alteration of P-UAEC proliferation when the nonspecific α-AR antagonist phenolamine and α2-AR specific blocker yohimbine were used shows that α2-ARs that were reduced by pregnancy do not play a role in catecholestradiol-induced angiogenesis in P-UAECs. There are no reports showing a role for α2-ARs in regulating endothelial cell proliferation. However, α2-ARs have been closely associated with NO signaling in endothelial cells,24 suggesting functional relevance of α2-AR expression in endothelial-mediated vasodilatation.

Consistent with our novel findings that propranolol abrogated 2-OHE2- and 4-OHE2–induced P-UAEC proliferation are reports showing that stimulation of β-ARs by various pharmacokinetic compounds stimulates proliferation of endothelial cells.15,19,25 Classically, β-ARs are prototypical G protein–coupled receptors triggering diverse signaling cascades through α-, β-, and γ-G protein subunits; adenylyl cyclase; intracellular cAMP; and protein kinase A and C.26 However, new layers of complexity in signaling suggest that β-AR activation can induce a myriad of cellular responses via p38 and p42/44 mitogen-activated protein kinases independent of adenylyl cyclase, cAMP, and protein kinase A and C.27–30 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 β1-AR blocker SR 59230A also implies potential involvement of β2-ARs. In contrast, both the specific β2-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 β2-AR and/or β3-AR has been shown to play a role in endometrial proliferation from human umbilical vein, retina, and bovine aorta.14–16 However, ours is the first report to demonstrate that β2-AR and β3-AR mediate the catecholestradiol-induced proliferation of endothelial cells. P-UAECs express similar levels of β2-ARs and β3-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 endometrial 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 in excess of the efficacious levels used herein, cardiac output redistributes away from the uterine vascular bed (α-AR mediated) to the muscles and other tissues (β-AR mediated) for survival of the mother and her fetus, thus providing a distinct short-term survival advantage for placental mammals.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.

Acknowledgments

We thank Timothy J. Morschauser, Mayra B. Pastore, Mary Y. Sun, Jason L. Austin, Gladys E. Lopez, Terrance M. Phernetton, and Cindy L. Goss.

Sources of Funding

This work was supported by National Institutes of Health grants HL49210, HD38843, and HL87144 (to R.R.M.); AA19446 (to J.R.); R25-GM083252 (to M.L. Carnes); and T32-HD041921-07 (to I.M. Bird).

Disclosures

None.

References

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Hypertension. 2011;58:874-881; originally published online September 26, 2011;
doi: 10.1161/HYPERTENSIONAHA.111.178046

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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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 catecholestradiols based on the dose-response curves from our previous study. 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 catecholestradiols 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 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 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>
<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>
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</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>
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<tr>
<td>Yohimbine</td>
<td>α₂-ARs</td>
<td>Antagonist</td>
<td>N/A</td>
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<tr>
<td>ICI 118,551</td>
<td>β₂-ARs</td>
<td>Antagonist</td>
<td>≥ 100-fold</td>
<td>Bilski et al, 1983.6</td>
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<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