Vascular Physiology and Pharmacology of Cytochrome P450- and Catechol-*O*-Methyltransferase-Derived Metabolites of Estrogen on Uterine Endothelial Adaptations during Pregnancy

By

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TABLE OF CONTENTS

DEDICATION

I dedicate this dissertation to Thaddas (Thad) Alston, Karen (Kari) Glover, the entire Alston family and Dr Karen McKinney. I am forever grateful and humbled by your generosity, belief and support.

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LIST OF ABBREVIATIONS

2-OHE_2– 2-hydroxyestradiol

4-OHE_2 – 4-hydroxyestradiol

2-OHE_1– 2-hydroxyestrone

4-OHE_1– 4-hydroxyestrone

2-ME_2– 2-methoxyestradiol

4-ME_2 – 4-methoxyestradiol

2-ME_1– 2-methoxyestrone

4-ME_1– 4-methoxyestrone

16α-OHE_1- 16-alpha-hydroxyestrone

16-epi-E_3 – 16-epi-estriol

17-epi-E_3 – 17-epi-estriol

16-keto- E_2β – 16-keto-estradiol-17β

ANGII – angiotensin II

α-AR- alpha adrenergic receptors

β-AR- beta adrenergic receptors

cAMP – cyclic adenosine monophosphate

cGMP – cyclic guanosine monophosphate

COX 1/2 – cyclooxygenase enzyme 1 and/or 2

$cPLA_2$ – Phospholipase A_2

E_1 – estrone

E_2β – estradiol-17β

E_3 – estriol

EC – endothelial cell

ECM – extra-cellular matrix

EDHF – endothelial-derived hyperpolarizing factor

eNOS – endothelial nitric oxide synthase

ER-α – estrogen receptor alpha

ER-β – estrogen receptor beta

ERK – extracellular signal-regulated kinases

GTP – guanosine triphosphate

L-NAME – N-nitro-L-arginine methyl ester

L-NMMA – NG-monomethyl-L-arginine

MAPK – mitogen-activated protein kinase

NO – nitric oxide

mPE – mild preeclampsia

sPE – severe preeclampsia

PGI_2 – prostacyclin

PGIS – prostacyclin synthase

SVR – systemic Vascular Resistance

TXA_2 – thromboxane

TXAS– thromboxane synthase

UA – uterine artery

P-UAEC- uterine artery endothelial cells derived from pregnant ewes

NP-UAEC- uterine artery endothelial cells derived from nonpregnant ewes

UBF – uterine blood flow

ABSTRACT

Accumulating experimental data provide convincing evidence that some metabolites of estrogen are biologically active and mediate multiple effects on the cardiovascular system. Estrogen is significantly elevated during pregnancy and some of the maternal vascular adaptations during pregnancy are mediated, in part, by estrogens via the classical estrogen receptors. However, it is largely unclear whether estrogen metabolites can modulate maternal vascular adaptations during. Therefore, we propose a thesis study designed to test the vascular physiology and pharmacology of estrogen metabolites on pregnancy vascular adaptation especially on endothelial-mediated angiogenic activity and vasodilator production. We hypothesize that estrogen-induced uterine endothelial vasodilatory and angiogenic responses during pregnancy are in part mediated via its biologically active metabolites, the catecholestradiols and the methoxyestradiols. Our results in these studies demonstrate that during pregnancy, plasma increases in estrogen levels are paralleled by distinct increases in estrogen metabolites including the catecholestradiols and methoxyestradiols. In addition, we show herein that estrogen metabolites induce proliferation in uterine artery endothelial cells derived from the pregnant state but not from the nonpregnant state. Estrogen metabolites also induced a higher increase in prostacyclin production by uterine artery endothelial cells derived from the pregnant state compared to the nonpregnant state. We also demonstrate that unlike the parent estrogen, estrogen metabolites-induced uterine endothelial vasodilatory and angiogenic responses during pregnancy are mediated independent of the classical estrogen receptors and occur via adrenergic or other unidentified endothelial receptors and are facilitated by mitogen-activated protein kinases. Clinically, our findings show that preeclampsia is characterized by aberrant synthesis, metabolism, and plasma accumulation of estrogens and estrogen metabolites that are likely to be associated with alterations in maternal

pregnancy-induced vascular functions. Collectively, the studies in the thesis present strong evidence that during pregnancy estrogen-induced uterine endothelial vasodilatory and angiogenic responses involve its sequential metabolic conversion to catecholestradiols and methoxyestradiols which are capable of stimulating estrogen receptor-independent endothelial vasodilatory and angiogenic endothelial adaptations during pregnancy.

CHAPTER 1:

LITERATURE REVIEW

I. Maternal Uterine Cardiovascular Adaptations of Pregnancy

Substantial increases in maternal uterine blood flow is a major hallmark of the cardiovascular adaptations seen during gestation.[1] These alterations in uterine blood flow occur in order to meet the dramatically increasing nutrient and metabolic needs of the growing fetus. The magnitude of these blood flow increases make it by far one of the most striking physiologic cardiovascular adaptations observed during pregnancy.[1] In order to allow for blood to flow increases, vasodilation, angiogenic growth and substantial remodeling of the uterine vascular beds, are responsible for the dramatic 25-30% decreases in vascular resistance that permits the increases in uterine blood perfusion with only minor decreases or alterations in blood pressure.[1] In contrast to this mild falls or no change in uterine perfusion pressure, there are dramatic falls in systemic vascular resistance during pregnancy.[1] Because there is no innervation of fetal placental vessels, active vasodilation and angiogenesis has been thought to occur primarily in the maternal uterine vasculature by the efforts of locally produced vasodilators and angiogenic factors. [2,3] The most dramatic maternal adaptation during gestation is the substantial rise in uterine blood flow, which increases, from <2% to 17-20% of cardiac output in order to maintain oxygen and nutrient delivery to the developing fetus.[2,3] Moreover these changes are also associated with reduced pressor and uterine sensitivity to the vasoconstrictor effects of Angiotensin II (ANGII) and other vasoactive agents.[1,2,3] The most important uterine vasodilators may be nitric oxide (NO) and prostacyclin (PGI_2), because they are not only constitutively produced and elevated during gestation, but are also dramatically stimulated by increases in shear stress which occurs with increased uterine blood flow. [4,5] The vascular growth for elevating blood flow and other changes in the maternal vasculature are also seen via endothelial cells associated altered proliferation and migration to induce angiogenesis and remodeling of the uterine vasulature.[6]

I.1 Vasodilatation

Vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2) have all been thought to contribute to the low vascular resistance condition during normal pregnancy. An *in vivo* physiologic role for endothelium-derived NO and PGI_2 in maternal cardiovascular adaptation during pregnancy is supported by the observations that plasma levels of nitrates/nitrites and 6-keto-PGF1α, the stable metabolites of NO and PGI_2 respectively, are increased during pregnancy.[7-10] Both NO and PGI_2 production are elevated during normal pregnancy.[1,11] In addition, NO and PGI_2 are decreased, albeit controversial, in preeclamptic pregnancy, suggesting clinical significance to this endothelial vasodilatory factor in pregnancy and dysfunction in diseased states.[12-15]Nevertheless, inhibition of NO with L-NAME or PGI_2 with indomethacin in some studies decreases uterine blood flow and enhances systemic and uterine vasoconstrictor responses to several vasoconstrictors.[10, 16, 17]

The potent vasodilator NO, is considered by many investigators to play an important role in pregnancy related vascular adaptations. NO synthesis is catalyzed by NOS, which converts L-arginine to L-Citrulline, with NO as a by-product.[18] There are 3 known NOS isoforms, designated as neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) respectively. *De novo* NO produced by the endothelium is highly labile ($t_{1/2}$ 3-5 sec) and diffuses locally to adjacent vascular smooth muscle cells where it activates soluble guanylate cyclase, which in turn converts GTP to cGMP. Inhibition of NOS using L-NAME or L-N^G methylarginine hydrochloride (L-NMMA) increases arterial pressure, uterine vascular resistance. From a systemic vasculature perspective, inhibition of NOS using L-NAME or L-N^G methylarginine hydrochloride (L-NMMA) increases systemic pressor responses to infused ANGII, norepinephrine, and arginine vasopressin , the increased pressor responsiveness was

reversed only by pretreatment with L-Arginine, but not D-Arginine, phentolamine, saralasin, or captopril.[19, 20, 21, 22, 23]

There is substantial evidence that NO produced by the uteroplacental unit plays an important role in the regulation and maintenance of vascular tone and ultimately blood flow.[2, 3, 24, 25, 26] Both NO production and eNOS expression were found to be increased in a greater amount during gestation by the uteroplacental endothelium, the level of its secondary messenger cGMP was also elevated throughout pregnancy.[27, 28, 29, 11, 30, 31] eNOS has been identified in syncytiotrophoblast and vascular endothelium in human villous tissue, as well as vascular endothelium in sheep, baboon, rat, guinea pig and rhesus monkey.[26, 32, 33,34,35, 36, 37] Indeed the levels of NOx and cGMP are elevate when the fetal growth velocity is greatest but these levels are no longer elevated at term when the fetal growth velocity slows or stops close to labor.[11, 38, 39] Observations in animal models have shown that chronic inhibition of NO production during pregnancy facilitates fetal growth restriction possibly via decreases in uteroplacental blood flow.[19], [40], [11]

Regarding vascular adaptation in the uterine vascular bed particularly in the uterine artery endothelial cells (UAECs), a guinea pig model was first used to show that only perfused "precontracted" uterine artery rings from pregnant, not nonpregnant animals' vasodilates in response to muscarinic receptor acetylcholine.[41] These findings were subsequently confirmed again in guinea pigs as well as in rats and humans using isolated precontracted vascular rings.[42, 43, 11] In support of this, Weiner et al, (1989) demonstrated that the *in vitro* release of endothelial derived relaxation factor in response to acetylcholine is greater in pregnant guinea pigs than nonpregnant. Additional studies using isolated uterine vessels have also shown that contractile responses to norepinephrine in pregnant compared with nonpregnant UA were increased by

removal of the endothelium or by blocking of NO synthesis with L-NMMA demonstrating that the endothelium is the source of endogenous vasodilator and that one of these is NO .[42, 44] Both NO and PGI_2 are implicated in the pregnancy-associated uterine vascular refractoriness of UA, because both L-NMMA, which inhibits eNOS, and indomethacin, which inhibits cyclooxygenase (COX-1 and COX-2), shifted the norepinephrine dose-response curves of pregnant guinea pigs to the left in which it resembles the curve of the nonpregnants. When both inhibitors are used the residual is considered endothelial derived hyperpolarizing factor (EDHF) which is also elevated in pregnancy.[45-47]Because EDHFs are not the focus of this thesis they will only be referred to as they impact the current body of work. Whereas these early studies have all evaluated the effect of pregnancy on UA NO release in terms of endothelium removal and NOS inhibitors on either vasoconstrictor responses or vasorelaxation responses of precontracted vessels, more recent studies of perfused isolated ovine uterine arteries are noteworthy in that they are the first to demonstrate directly both basal and agonist (ATP and the calcium ionophore A23187)-induced endothelial NOx release were significantly increased in pregnant uterine artery endothelium.[48-50] These data collectively demonstrate that eNOS plays a critical role in the adaptive processes seen in the UA in response to pregnancy.

NOS activity is found in numerous tissues including endothelium and uterine tissues. Based on its ubiquity, NOS is considered as a signal transduction system for paracrine/autocrine functions of many tissues.[1, 16, 29, 51-53] Acute NO biosynthesis regulation has been shown to be partly activated by Ca^{++} independent mechanisms such as phosphorylation involving activated MAPK or PI3K-AKT.[54-56, 57] We have shown that there is both acute (1-30 min) regulation of NO production during elevations in shear stress and ERK1/2 (MAPK p22/24) which is followed by

increases in chronic regulation of NO biosynthesis in association with rises in eNOS expression.[57-59]

PGI_2 is produced from the sequential actions of $cPLA_2$, COX-1 and/or COX-2, and PGIS.[60] $cPLA_2$ protein expression is only regulated in the endothelium and not the vascular smooth muscle of uterine arteries and is increased during pregnancy but not during the ovarian cycle.[61] COX-1 protein expression is substantially greater in the endothelium than in the vascular smooth muscle of uterine arteries.[62] COX-1 protein, a key enzyme in the production of PGI_2 from arachidonate, exhibits a marked increase in expression during pregnancy and is elevated, but to a lesser extent, during the follicular phase of the ovarian cycle in uterine artery endothelium.[63, 64] COX-1 mRNA also is increased in uterine artery endothelium during pregnancy compared with nonpregnancy and in the follicular and luteal phases of the ovarian cycle compared with levels detected in ovariectomized controls. [62] However, COX-1 protein levels in the omental artery endothelium were unaltered by ovariectomy, phase of the ovarian cycle, or pregnancy suggesting local regulation in the uterine compartment.[62] In a previous study, PGIS expression was slightly greater in the vascular smooth muscle than in the endothelium during pregnancy in the uterine but not systemic (omental) vasculature.[64] Furthermore, PGIS in the endothelium of uterine arteries was increased during pregnancy compared with levels detected in ovariectomized and luteal phase sheep, whereas omental artery endothelial PGIS was unaltered by the ovarian cycle but was elevated during pregnancy. PGIS expression in the VSM was specific to the uterine arteries and only increased during pregnancy.[64]

PGI_2 is also elevated in systemic arterial and uterine venous plasma during pregnancy.[65] Dramatic rises in uterine blood flow in pregnancy are associated with increases in *de novo* uterine vascular PGI_2 secretion by the uteroplacental unit and accompanied by augmented

expression of uterine artery endothelial cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase 1 (COX-1) and prostacyclin synthase (PGIS).[64, 65, 52] Studies have shown that infusion of estradiol-17β ($E_2\beta$) in sheep markedly causes rises in uterine blood flow, increases the uterine arterial expression of $cPLA_2$, COX-1 and PGIS which stimulate production of the stable PGI_2 metabolite, 6-keto-PGF1α.[65, 66]It is important to note that in nonpregnant ewes, intra-arterial infusion of PGI_2 resulted immediately in increased uterine blood flow, which returned rapidly to baseline after termination of the infusion demonstrating that PGI_2 has a vasodilatory effect on the uterine arteries and suggest that this substance has a role in the regulation of uterine blood flow.[67] In addition, in human umbilical vein endothelial cells (HUVECs), $E_2\beta$ has also been shown to selectively stimulate PGI_2 production *in vitro* primarily via ER-α.[68]

It is important to note that a physiological cause and effect relationship has not been established between increases in uterine blood flow and PGI_2. Magness et al (1992) have shown that local infusion of the nonspecific COX-1 and COX-2 inhibitor indomethacin does not significantly reduce basal uterine blood flow in pregnant sheep.[69, 70] In this study, uterine perfusion remained unchanged following indomethacin infusion even though ipsilateral prostanoid production (6-keto-PGF1a and PGEs) was depressed 78% throughout the study. These observations support the conclusion that during late ovine gestation, basal uterine blood flow may not be primarily dependent on maintaining basal production of prostanoids such as PGI_2. [69] Nevertheless, the importance of PGI_2 in the maintenance and/or rises in uterine blood flow throughout pregnancy cannot be understated. Because as outlined my Magness et al (1996), it maybe that PGI_2 plays a role in mediating the attenuated ANG II vasoconstrictor responses observed during pregnancy because infusion of ANGII stimulates PGI_2 production by intact uterine but not omental arteries from pregnant sheep.[16]

Compared to other molecular mediators of vascular tone, PGI_2 is made by PGIS at the end of a sequential enzymatic process that is also the same process used by thromboxane synthase (TXAS) for precursors to makes thromboxane A_2 (TXA_2), potent vasoconstrictor.[71] Therefore, PGIS and TXAS enzymes act downstream of $cPLA_2$ and COX-1-signaling respectively to catalyze the formation of PGI_2 and TXA_2, two major prostanoids exerting directly opposing effects on the vasculature.[60] Since both PGI_2 and TXA_2 are end products of the sequential reactions catalyzed by the same serial enzymes and precursors with the exception of PGI_2 synthase or TXA_2 synthase enzymes respectively, the PGI_2/TXA_2 ratio is considered of relevance in the regulation of physiologic and clinical vascular tone.[10, 60, 62] Several studies have shown that an imbalance in the generation of PGI_2 relative to TXA_2 is associated with hypertension, atherosclerosis and gestational vascular diseases such as preeclampsia.[72-74] Classically, PGI_2, is known to be produced mainly by vascular endothelial cells, whereas TXA_2, a potent vasoconstrictor is principally produced by platelets. In this regard, little is known about whether endothelial cells including the uterine endothelium can produce TXA_2 and if this production plays a role in regulating the ratio of PGI_2 and TXA_2 during pregnancy. Since both PGI_2 and TXA_2 are labile and convert rapidly into the stable metabolites 6-keto-PGF1α and thromboxane B_2 (TxB_2), respectively, the PGI_2 and TXA_2 production are evaluated measuring these two stable metabolite levels in biological samples.[60]

I.2 Angiogenesis

Angiogenesis represents the development of new blood vessels from pre-existing blood vessels. Classically, the angiogenic paradigm is initiated by growth factors that increase the permeability of the vessels, stimulate proteolytic degradation of the extracellular matrix (basal and reticular lamina) by specific proteases (collagenases and plasminogen activators), and cause proliferation

of endothelial cells. [75]The process is completed by chemotactic migration of the endothelial cells, formation of a lumen, and the functional maturation of the endothelium as well as recruitment of pericytes and smooth muscle cells.[75-77] The angiogenic phenotype of proliferating and migrating endothelial cells can also be stimulated by other factors including steroids such as estrogens.[75] Each single element of the angiogenesis paradigm, basement membrane disruption, cell migration, cell proliferation, and tube formation, can be accurately tested *in vitro* using a myriad of techniques including and not limited to 5-Bromodeoxyuridine (BrdU) incorporation cell proliferation assay, direct cell counting assays and tube formation assays.

"The [umbilical] vessels join on the uterus like roots of plants and through them the embryo receives its nourishment" Aristotle, On the Generation of Animals (ca 340 BC).

Reynolds and Redmer brought historical perspective to uterine angiogenesis with this above quotation, and reported that Aristotle made it perfectly clear the importance of vessel sprouting during pregnancy and its overall impact on fetal well-being.[57] Substantial remodeling and angiogenic growth of the uteroplacental vascular beds play an important role in uterine cardiovascular adaptations seen during pregnancy as well as ultimate fetal well-being. [78, 79] Since the uterus and the growing fetus require an increased supply of blood during pregnancy, the uteroplacental vasculature undergoes the process of extensive blood vessel formation and remodeling.[79] Meschia proposed that "the large increase of blood flow to the uterus during pregnancy …results primarily from the formation and growth of the placental vascular bed. [80] Numerous studies have subsequently confirmed that angiogenesis is indeed a major component of the increase in uterine blood flow throughout gestation, and establishment of functional

maternal/fetal/placental vascular beds is one of the critical events during fetal development.[75, 81]

In human pregnancy, intact capillaries grow and surround the syncytiotrophoblast layer and form a capillary plexus connected to the syncytiotrophoblast lacunae and constitute the first very simple vascular system supplying blood to the embryo.[79, 82] Concurrent with the growth of the uterus and vascular adaptation at the maternal/fetal interface, there are also profound changes in the vascular system of the deeper part of the uterus supplied by basal arteries during human pregnancy.[82] Perhaps one of the first data sets to demonstrate uterine angiogenic changes during pregnancy published in studies by Horn in 1918, who compared the uterine vasculature of nulliparous, multiparous nonpregnant and multiparous pregnant uteri of women and clearly demonstrated permanent angiogenic changes of the uterine vasculature in response to pregnancy.[83] Reynolds et al 2010 have shown that total capillary volume of the maternal placenta in sheep increases dramatically during pregnancy, in conjunction with dramatic capillary growth and continues throughout pregnancy.[77] In their model, the caruncular (maternal placental) capillary beds grow primarily via an increase in capillary size (area per capillary) and increases in capillary number or surface densities, resulting in 3-fold increase in capillary area density and/or branching pattern compared to the nonpregnant state.[77]

A broad spectrum of factors plays a crucial role in the complex initiation and regulation of angiogenesis during pregnancy, and the switching on of angiogenesis is regulated by a change in the local production of angiogenic factors such as growth factors and steroids including estrogens. However, the role of endogenous estrogens in the direct or indirect regulation of the normal process of angiogenesis during pregnancy is still unclear. Based on the patterns of the systemic and local levels of primarily placental (and possibly ovarian) derived estrogens (reviewed in detail below), it is likely that these steroids work in a temporal fashion to alter

endothelial signaling factors and cell phenotypes to induce vasuclar endothelial cell proliferation and/or migration and alter vascular development. Thus it is important to investigate the ability of estradiol-17β and other estrogens to alter these angiogenic aspects of endothelial cell biology and whether this plays a role in pregnancy-induced angiogenesis as well as in the pathophysiologic states such as in preeclampsia.

II. Cardiovascular Physiology and Pharmacology of Estradiol-17β during Pregnancy

II.1 Endogenous versus Exogenous

We have established that during normal gestation, uterine blood flow (UBF) increases substantially by as much as 50 fold in order to provide sufficient nutrient and oxygen supply for growth of the developing placenta and fetus.[84,85] An insufficient elevation in UBF in pathological pregnancies leads to a shortage of nutrient and oxygen delivery/extraction and is associated with fetal intrauterine growth restriction (IUGR) and higher prenatal and neonatal morbidity, or even mortality.[86, 87] A fundamental role for estrogen in elevating UBF has been definitively established using both *in vivo* and *in vitro* models. These include a concomitant increase in circulating estrogen levels with UBF during the follicular phase of the ovarian cycle and during normal pregnancy [88-92] as well as the very dramatic increase in UBF in pregnant and nonpregnant sheep following a bolus injection of exogenous estrogen. [87, 93-96] There also is a substantial body of evidence showing that estrogen-induced and pregnancy-associated rises in UBF are, to a great extent, mediated by an up regulation of the endothelial production of the potent vasodilator nitric oxide (NO) [97-99] via increasing endothelial nitric oxide synthase (eNOS) protein expression [100-102], and by increasing eNOS activity. [103-108] However, additional mechanisms which underline the estrogen and pregnancy associated rises in of UBF also include stimulation of angiogenesis processes. Both the estrogen mediated vasodilatory and angiogenic responses of the uterine

vasculature are driven to a large extent by ER-mediated processes. Estrogen-induced rises in UBF has been postulated to be mediated by one or both of the specific ERs.[87, 109] In ovariectomized sheep, we reported that exogenous estrogen-induced rises in UBF are to a great extent (~ 70%) inhibited by the non-subtype specific estrogen receptor (ER) antagonist ICI 182,780 [110] thus demonstrating that the ER-induced rises in UBF are partly mediated by one or more ER-dependent mechanisms. In these studies, Magness and co-workers also demonstrated that local uterine artery infusions of ICI 182,780 unilaterally reduced UBF from its maximum during the follicular phase of the ovarian cycle and pregnancy, two physiological states of high endogenous estrogen levels. To our knowledge there is only one reported preliminary physiologic study in the ovine model showing that selective ER-α ligands are more potent than ER-β ligands for increasing UBF thus supporting the premise that estrogen mediated vasodilatation may be more ER-α than ER-β mediated. [111]

II.2 Estradiol-17β Signaling via the Classic Estrogen Receptors

Classically, the biological functions of estrogen in target tissues are believed to be mediated by specific high-affinity nuclear ERs that function as ligand-activated transcription factors to regulate gene expression.[112] However rapid activation of signaling mechanisms such as extracellular signal-regulated kinases might be a mechanism responsible for acute activation of eNOS by estrogen to produce NO in uterine artery endothelial cells (UAECs) *in vitro*. A myriad of cell type-specific signaling pathways are activated upon E_2β binding to ER-α and ER-β. These signaling events include cascades involving: phospholipase C (PLC)/protein kinase C (PKCs)[113], Ras/Raf/MAPKs[114], phosphatidyl inositol 3 kinase (PI3K)/AKT,[115] and cAMP/ protein kinase A (PKA).[116] For example, in vascular endothelial cells the Src/PI3K/AKT pathway mediates rapid E_2β-dependent activation of eNOS and the release of nitric oxide.[117, 118]

Indeed, we previously demonstrated that the rapid activation of the eNOS-NO pathway by estrogen in UAECs is mediated by ER localized on the plasma membrane.[103] Regardless of the cellular location, two types of ERs have been identified so far, including the originally described ER-α as well as the subsequently discovered ER-β.[119, 120]

II.3 Expression of ERs in Uterine Endothelium: *in vivo* and *in vitro*

We have established several informative assays to study expression profiles of both ER-α and ER-β in UA during the estrous cycle and pregnancy. Because the expression levels of ER-α and ER-β may vary under different physiologic conditions and clinical settings[121, 122] understanding their regulations are important as it relate to the functional settings. The presence of ERs in uterine and other arteries has been documented by several groups including our own laboratories. [106, 123-128] We have shown that ER-α and ER-β receptors are present in ovine UAendo and VSM, are regulated by the reproductive state via alterations in ovarian/placental steroid (estrogen/progesterone) levels.

We have also demonstrated the cellular (endothelium *versus* VSM) localization of ER-α and ER-β mRNAs in UAs, by performing *in situ* hybridization using ^{35}S-labelled sense (control) or antisense riboprobes synthesized from specific ovine ER-α and bovine ER-β cDNAs. Hybridization with antisense probes revealed that ER-α and ER-β mRNA are both strongly expressed in the ovine UA endothelium along the lumen of the vessels. Expression of the ERs was also observed throughout the tunica media. Tissue sections subjected to hybridization with sense probes showed minimal background levels of ER-α and ER-β mRNAs.

We also reported[106, 126], that UA tissue sections from late gestation (Days 120–130 of pregnancy) sheep show intensive immunoreactive signals for both ER-α and ER-β proteins in the nuclei and to a lesser extent the cytoplasm, of the endothelial and smooth muscle cells. However,

the cytoplasm immunostaining of ER-α and ER-β may be derived from plasma membrane staining since the immunohistochemical technique used in this experiment does not give a clear subcellular structure of cytoplasm and plasma membrane. In subsequent studies we showed ER-α protein was expressed uniformly throughout the UA vascular cells (non-nuclear and nuclear staining), whereas ER-β exhibited heavy, preferential nuclear staining[127] an observation consistent with reports in ovine fetal pulmonary endothelial cells and UAECs.[106, 129, 130]

Using semi-quantitative Western blot analysis, we have determined that ER-α and/or ER-β protein expression is modified in the reproductive vs. non-reproductive endothelia during physiological states of elevated estrogen (follicular phase and pregnancy) or progesterone (luteal phase and pregnancy). Changes in UAendo ER-α and ER-β protein were compared to the endothelium derived from 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 ER-α in follicular *versus* luteal UAendo there was no significant ER-α change in any other endothelial preparation from follicular or pregnant sheep. Besides UAendo, when compared to luteal endothelium, ER-β was not significantly changed in any of the follicular endothelial preparations that were surveyed. By contrast, during pregnancy, all three reproductive endothelial isolated proteins displayed significantly increased ER-β expression. Specifically, UAendo ER-β from the pregnant group was increased 1.6-fold and MAendo 1.5-fold over luteal controls. Placental endothelial ER-β was 2.3-fold higher than luteal UAendo and clearly higher than pregnant UAendo. Since PAendo ER-α was lower than luteal UAendo it appears that ER-β may be the primary ER in the ovine placental vasculature. These observations also point to the potential for a unique role of ER-β to regulate reproductive vascular functions during pregnancy. The only non-reproductive tissue in

which ER-β changed significantly was CAendo from pregnant sheep, which rose 1.5-fold over luteal. In MAendo and CAendo follicular phase levels of ER-β were intermediate and thus not significantly different from either luteal or pregnant levels. Others have shown multiple tissue comparisons or partial surveys of vascular ER expression.[131-140] In our study we reported on endothelial isolated proteins extensively studied with regard to endocrine status of the animal. Although ER-α was readily detectable in the vessels tested, none of the other reproductive or non-reproductive endothelia surveyed except UAendo and PAendo had ER-α levels that were different relative to luteal phase expression. ER-β 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, ovariectomy with or without hormone replacement (not shown) may have functional significance in that we have demonstrated estrogen profoundly increases UBF 10- to 20-fold in as little as 2 h, omental blood flow was unaltered.[141, 142] It is of note that ER-β was indeed elevated by pregnancy in the three reproductive endothelia (UA, MA and PA), but also CAendo and that follicular ER-β levels were similar to pregnancy levels in UA, MA and CA. Thus CA was the only non-reproductive endothelium to show ER-β regulation and suggests a more ubiquitous role for this receptor subtype in the mammalian vasculature especially in lieu of the observation that ER-β KO mice develop age-dependent hypertensive cardiovascular disease.[143] The finding that ER-β 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.[85, 144] Furthermore, blood flows to the vascular beds we evaluated are differentially affected by exogenous $E_2\beta$ treatment, i.e. elevated in the uterine, mammary, and coronary, but not omental or renal. [95, 145]Moreover, estrogen-induced rises in UBF [21, 31, 90], coronary blood flow[146]and mammary blood

flow[147] 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 [29, 127, 148, 149], suggesting elevations in eNOS activation and thus NO production rather than 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 estrogen.

We also have shown the presence of ERs in cultured UAECs in order to establish an *in vitro* model of estrogen actions. We measured ER-α protein in isolated UAEC plasma membranes and nuclear extracts by Western blotting and performed binding studies using E_2β-BSA-FITC as a ligand. We observed the classical 67-kDa ER-α protein was detectable in both plasma membrane and nuclear extracts, whereas higher ER-β protein levels were found in the nucleus compared to plasma membranes. In addition, binding studies using E_2β-BSA-FITC as a ligand showed that fluorescence labeling was primarily localized on UAEC plasma membrane.[126] These data were confirmed using the immunocytochemical localization of both ER-α and ER-β in cultured UAECs from pregnant. Subsequently we determined using western analysis and RT/PCR that both ER-α and ER-β are expressed in cultured UAECs from pregnant sheep concluding that these UAECs may provide a useful cell culture model for the study of estrogen actions on uterine vascular function.

Moreover, the expression of several ER-β variants has been identified in human reproductive tissues with limited functional data currently available. However, the ER-α_2 variant has been postulated to act as putative dominant negative regulators of estrogen action.[150] This truncated variant was detected in the UAE and in cultured UAECs during the course of our experiments. ER-α_2 is the result of the splicing deletion at exon 5 and it is missing all the C-

terminal amino acids and lacks most of the ligand-binding domain of ER-β.[151] A previous functional study showed that it may serve as a dominant negative receptor capable of block both ER-α and ER-β signaling pathways.[152] Its potential role in estrogen actions and pregnancy are largely unknown.

II.4 Estrogen Receptor-α versus Estrogen Receptor-β Selectivity

While both ER-α and ER-β are bound by $E_2\beta$ as well as to other pharmacological agonists and antagonists (e.g. SERMs), the molecular and structural difference of these receptors allow for a wide range of functional heterogeneity. These differences may partly explain selective estrogenic actions of $E_2\beta$ in the same or diverse target cells and tissues. ER-α and ER-β are members of nuclear receptor super-family sharing conserved regions, named A/B, C, D, E, and F domains as well as the N-terminal transactivation domain, the DBD, the dimerization domain, the nuclear localization sequence (NLS), and the ligand-binding domain (LBD).[153, 154] However, ER-α and ER-β are the products of distinct and separate genes (ESR1 and ESR2, respectively) found on different chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively).[155, 156] ER-α and ER-β only share a 12% homology in their A/B regions, a 16% homolog in their D region, a 59% homology in their E domain and a 9% homology in their F regions.[157, 158] In this regard, ER-α and ER-β only share 56% amino acid homology in their LBDs, and differ greatly in their N-terminal and C-terminal/DNA binding regions.[153, 154, 159, 160] In terms of the differences in their A/B regions, they differ in both length and amino acid sequence of this region which contains the solvent exposed and negatively charged activation function-1 (AF-1) domain which also has numerous phosphorylation sites for signal transduction.[154] ER-α and ER-β additionally share very low amino acid sequence homology in the α-helices of their DNA binding domains.[154] Investigation of the dynamic plasticity of the ligand-binding pocket of ERs has shown that in this

specific region ER-β differs from ER-α based on a substitution of Leu384/Met421in ER-α corresponding to Met336/Ile373 in ER-β.

Structural differences between ER-α and ER-β specifically suggest a structural basis for selective ligand-dependent receptor activation and differential ER activation.[161] These differences in structures has led to the development of several pharmacological ligands that discriminate between the two ER subtypes on the basis of affinity, efficacy and inherent differences in coupling to other signaling components. As shown in Table 1, for agonist ligands, the diarylethanes such as diarylpropionitrile (DPN) have been demonstrated to possess selectivity and potency for ER-β over ER-α owing to preferential interaction with a key methionine residue in the ligand-binding.[161] Consequently, DPN demonstrates a 72-fold higher binding affinity towards ER-β over ER-α.[162, 163] On the other hand, compounds such as propylpyrazole triol (PPT) demonstrate more than a 410-fold agonistic potency for ER-α over ER-β exhibiting high level of ER-α selectivity.[161] With regards to antagonist ligands, the phenol containing compounds such as 4-[2-phenyl-5, 7-bis(trifluoromethyl) pyrazolo [1, 5-a] pyrimidin-3-yl] phenol (PHTPP) show considerable affinity and selectivity towards ER-β over ER-α.[164] Likewise, 1,3-bis(4-hydroxy-phenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1Hpyrazoledi hydrochloride (MPP) demonstrates up to a 200-fold antagonistic selectivity for ER-α over ER-β.[165, 166] These ER-subtype selective compounds provide a specific utility with pharmacological approaches to elucidating the differential and or/distinct roles of ER-α and ER-β in the physiologic functions of estrogen. Importantly, these and other selective compounds (Table 1) also point to the relevance for the appreciation of the structural differences between ER-α and ER-β that may help explain why estrogen-induced activation of one of the ER subtypes may fulfill a complete and distinct physiologic response independent of the other. Using these specific

pharmacologic agents coupled with additional molecular approaches such as siRNA or adenoviral over expression of ER-α and ER-β in cell lines provides quite convincing evidence of the individual and complementary roles of the functions of these two ER subtypes.

Table 1: Selectivity of pharmacologic ligand compounds to ER-α and ER-β

Ligand Compound	ER-α/ER-β Selectivity	Agonist/Antagonist	Relative Binding Selectivity	Reference
Estradiol-17β	ER-α/ER-β	Agonist	N/A	Kuiper et al., 1997
ICI 182,780	ER-α/ER-β	Antagonist	N/A	Wakeling et al., 1991
*4-OH Tamoxifen	ER-α/ER-β	Antagonist*	N/A	Wakeling et al., 1984
ZK 164015	ER-α/ER-β	Antagonist	N/A	Walter et al., 2004
PPT	ER-α	Agonist	~ 410-fold	Kraichely et al., 2000
Y 134	ER-α	Agonist	~ 125-fold	Yang et al, 2004
*(*R*,*R*)-THC	ER-α	Agonist*	~ 120-fold	Meyers et al., 1999
Raloxifene	ER-α	Agonist	~ 15-fold	Fitzpatrick et al., 1999
MPP	ER-α	Antagonist	~ 200-fold	Sun et al., 2002
Genistein	ER-β	Agonist	~ 26-fold	Manas et al., 2004b
DPN	ER-β	Agonist	~ 70-fold	Meyers et al., 2001
FERb 033	ER-β	Agonist	~ 62-fold	Minutolo et al., 2009
WAY 200070	ER-β	Agonist	~ 68-fold	Malamas et al., 2004
PHTPP	ER-β	Antagonist	~ 36-fold	Compton et al., 2004
*(*R*,*R*)-THC	ER-β	Antagonist*	~ 4-fold	Sun et al., 1999
*RU486	ER-β	Antagonist*	~ 5-fold	Fang et al., 1997

*Denotes ligand compounds with both know partial ER agonistic and antagonistic activities. N/A means unknown or negligible binding selectivity or equal binding affinity for both ER-α and ER-β.

The molecular mechanism(s) responsible for the physiological selectivity of endogenous estradiol-17β towards the classical ER-α and/or ER-β remain unknown and are the focus of significant fundamental research into this area. For example, three dimensional crystallography binding studies have demonstrated that estradiol-17β indeed binds 'upside-down' to ER-β compared to its binding to ER-α implying differential conformation of the receptor complex and perhaps this accounts for the differential signaling activation described for these two receptors when exposed to the same ligand.[167] For the purposes of this thesis, it is thus conceivable that this differential rotated binding orientation enables estrogen to induce diverse and differential genomic and/or non-genomic biological outcomes based on selective structural and conformational changes of the ER-α and/or ER-β receptor complex.

The emerging evidence for the considerable functional diversity between ER-α and ER-β derives from the fact that there are distinct endothelium-dependent genomic and/or non-genomic processes showing impaired in estrogen-receptor null mice. For example, in ER-β knockout mice compared to wild type or ER-α deficient mice, long-term genomic mechanism such as proliferation and migration of several endothelial cell types are severely impaired.[168, 169] On the other hand, compared to wild type or ER-β knockout the rapid endothelium-dependent nitric oxide-mediated vasodilatation is significantly reduced in ER-α deficient mice.[170, 171] In this regard, it is possible that whilst both ERs are important for proper vascular homeostasis, functional heterogeneity allows for more diverse mediation of selective actions. Additionally, these possibilities also suggest that the spatial and subcellular localization of membrane versus nuclear ER-α or ER-β also confer other additional and distinct functions attributed to the acute or prolonged ER actions. However, little is known whether there are possible structural differences

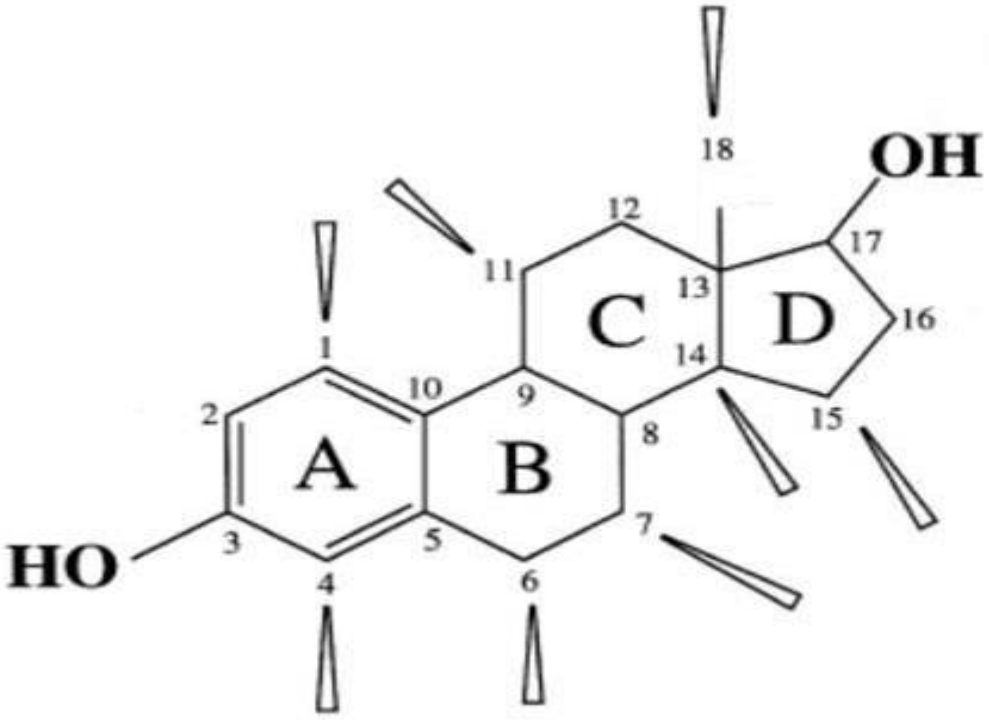
in these receptors subpopulations in the plasma membrane or whether this may represent a more complex diversity of ER-α or ER-β on estrogen-mediated physiologic effects respectively.

III CYP450s, COMT and the Sequential Metabolism of Estrogens

III.1 Cytochrome P450 Subtypes and Catechol-*O*-Methyltransferase

Diverse pathways extensively convert estrogens to multiple biologically active metabolites. Members of the cytochrome P450 (CYP450s) family are the major enzymes catalyzing β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH*)-dependent oxidative metabolism of estrogens to multiple hydroxylated metabolites (see diagram below). [172] Estrogens contain multiple hydroxylation sites, and the type of hydroxylated estradiol metabolite formed is defined by the position of hydroxylation (C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16, C-17and C-18; see figure and table 1). For example, hydroxylation of estradiol at C-2 and C-4, by CYP1A1/1A2, CYP3A4 and CYP1B1, respectively, results in the formation of catecholestradiols, 2-hydroxyestradiol and 4-hydroxyestradiol.[173, 174]Although most of the oxidative metabolism of estrogens takes place in liver, some estrogen-metabolizing isoforms of the CYP450s that are usually expressed at low or undetectable levels in liver are selectively expressed significantly in certain extrahepatic tissues.[172] "Cytochrome P450" was proposed as a temporary name for colored pigment in the cell, having a 450 nm Soret Peak when reduced with carbon monoxide saturation; but unfortunately the name stuck around for varied use.[175] In reality, these enzymatic proteins are not "true" cytochromes but "heme monooxygenases". [172] CYP450s proteins are arranged into families and subfamilies, which are derived from percent amino-acid sequence identity. Proteins that have roughly >40% sequence similarity are members of the same gene family, whereas those with >70% similarity are members of the same subfamily.[176] Enzymes in the CYP450 gene families show some redundancy and overlapping

substrate specificity and control the levels of sex steroids such as estrogens, corticosteroids, cholesterol, bile acids and retinoic acid.[172, 177] Several extrahepatic target tissues or cultured cells including the uterus, ovary, brain, mammary, placenta, endothelial cells and smooth muscle cells express estrogen CYP450 metabolizing enzymes such as CYP1A1, CYP1A2, CYP1B1, CYP3A4 and CYP1B1.[172, 177] CYP1A1, CYP1A2, CYP1B1, CYP3A4 and CYP1B1 enzymes primarily hydroxylate estrogens at the carbon 2 and 4 (C-2 and C-4) positions, respectively, and play a role in estrogen levels in certain tissues.[172]



Estrogens contain multiple hydroxylation sites, and the type of hydroxylated estradiol metabolite formed is defined by the position of hydroxylation (C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16, C-17and C-18

Following hydroxylation, estrogen metabolites such as catecholestradiols (hydroxylated estrogens at the carbon 2 and 4 (C-2 and C-4) positions) are involved in several other pathways including enzymatic *O*-methylation to methoxyestradiols by catechol-*O*-methyltransferase (COMT),[172] an important enzyme in the metabolism of compounds which have a catechol structure. COMT is a ubiquitous cytosolic enzyme that is expressed in most tissues and present in a highly active form in vascular endothelial cells, smooth muscle cells and cardiac cells.[178-180] COMT was first characterized by Axelrod and Tomchick in 1958 when they first described that the enzyme-catalyzed *O*-methylation of catecholamines and other catechols.[181] The interest in COMT was rekindled in the late 1980s when the potent and selective second-generation COMT inhibitors were developed and available for use.[178]

The ultimate importance of COMT is being clarified in a new strain of animals lacking the COMT gene (COMT-KO mice).[178] Interestingly, COMT-KO mice exhibit very low levels of 2-methoxyetradiol and exhibit preeclampsia-like phenotype including fetal wastage, low uteroplacental blood flow, proteinuria. [182] Treatment of COMT-KO mice with 2-methoxyestradiol alleviates all preeclampsia-like symptoms without toxicity.[182]

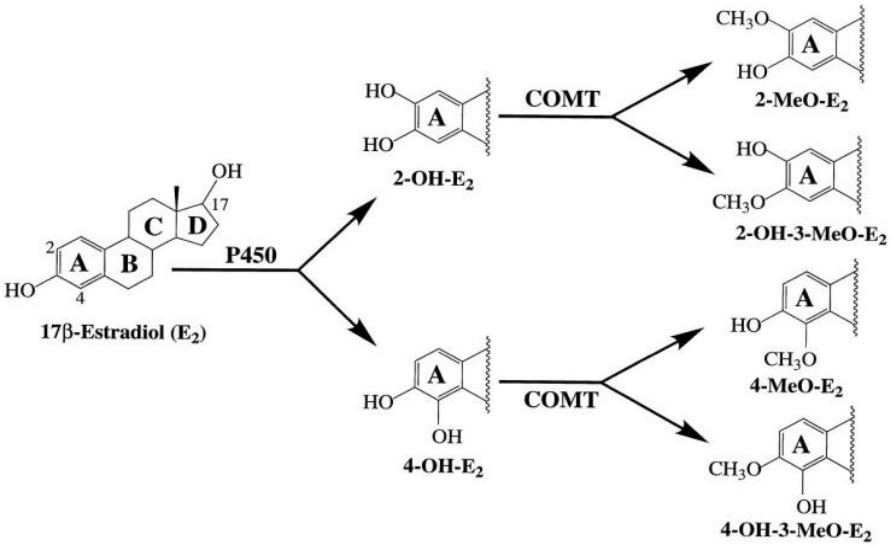


Table 2: Metabolites of Estrogens. All estrogen metabolites listed were found in biological samples (tissues, blood, and urine) or formed during *in vitro* incubations.

Position of Oxidation	Estrogen Metabolite Systemic Chemical Name	Estrogen Metabolite Common Name
C-1	1,3,5(10)-Estratrien-1,3-diol-17-one	1-Hydroxyestrone
C-2	1,3,5(10)-Estratrien-2,3-diol-17-one 1,3,5(10)-Estratrien-2,3,17β-triol 1,3,5(10)-Estratrien-2,3,16α,17β-tetrol	2-Hydroxyestrone 2-Hydroxyestradiol 2-Hydroxyestriol
C-4	1,3,5(10)-Estratrien-3,4-diol-17-one 1,3,5(10)-Estratrien-3,4,17β-triol 1,3,5(10)-Estratrien-3,4,16α,17β-tetrol	4-Hydroxyestrone 4-Hydroxyestradiol 4-Hydroxyestriol
C-6	1,3,5(10)-Estratrien-3,6α-diol-17-one 1,3,5(10)-Estratrien-3,6β-diol-17-one 1,3,5(10)-Estratrien-3-ol-6,17-dione 1,3,5(10)-Estratrien-3,6α,17β-triol 1,3,5(10)-Estratrien-3,6β,17β-triol 1,3,5(10)-Estratrien-3,17β-diol-6-one 1,3,5(10)-Estratrien-3,16α,17β-triol-6-one 1,3,5(10)-Estratrien-3,6α,16α,17β-tetrol	6α-Hydroxyestrone 6β-Hydroxyestrone 6-Ketoestrone 6α-Hydroxyestradiol 6β-Hydroxyestradiol 6-Ketoestradiol 6-Ketoestriol 6α-Hydroxyestriol
C-7	1,3,5(10)-Estratrien-3,7α-diol-17-one 1,3,5(10)-Estratrien-3,7β-diol-17-one 1,3,5(10)-Estratrien-3,7α,17β-trio 1,3,5(10)-Estratrien-3,7β,17β-triol 1,3,5(10)-Estratrien-3,17β-diol-7-one 1,3,5(10)-Estratrien-3,7α,16α,17β-tetrol	7α-Hydroxyestrone 7β-Hydroxyestrone 7α-Hydroxyestradiol 7β-Hydroxyestradiol 7-Ketoestradiol 7α-Hydroxyestriol
C-11	1,3,5(10)-Estratrien-3,11β-diol-17-one 1,3,5(10)-Estratrien-3-ol-11,17-dione 1,3,5(10)-Estratrien-3,11β,17β-triol 1,3,5(10)-Estratrien-3,17β-diol-11-one 1,3,5(10),11-Estratrien-3,17α-diol 1,3,5(10),9,11-Estratrien-3-ol-17-one	11β-Hydroxyestrone 11-Ketoestrone 11β-Hydroxyestradiol 11-Ketoestradiol (11)-Dehydroestradiol-17α D(9,11)-Dehydroestrone
C-14	1,3,5(10)-Estratrien-3,14α-diol-17-one 1,3,5(10)-Estratrien-3,14α,17β-triol	14α-Hydroxyestrone 14α-Hydroxyestradiol
C-15	1,3,5(10)-Estratrien-3,15α-diol-17-one 1,3,5(10)-Estratrien-3,15β-diol-17-one 1,3,5(10)-Estratrien-3,15α,17β-triol 1,3,5(10)-Estratrien-3,15α,16α,17β-tetrol	15α-Hydroxyestrone 15β-Hydroxyestrone 15α-Hydroxyestradiol 15α-Hydroxyestriol (estetrol)
C-16	1,3,5(10)-Estratrien-3,16α-diol-17-one 1,3,5(10)-Estratrien-3,16β-diol-17-one 1,3,5(10)-Estratrien-3-ol-16,17-dione 1,3,5(10)-Estratrien-3,16α,17β-triol 1,3,5(10)-Estratrien-3,16β,17β-triol 1,3,5(10)-Estratrien-3,17β-diol-16-one 1,3,5(10)-Estratrien-3,16β,17α-triol	16α-Hydroxyestrone 16β-Hydroxyestrone 16-Ketoestrone 16α-Hydroxyestradiol (estriol) 16-Epiestriol 16-Ketoestradiol 16,17-Epiestriol
C-17	1,3,5(10)-Estratrien-3,17α-diol 1,3,5(10)-Estratrien-3,16α,17α-triol	17α-Estradiol 17-Epiestriol
C-18	1,3,5(10)-Estratrien-3,18-diol-17-one	18-Hydroxyestrone

III.2 Metabolism of Estrogens

2-hydroxylation of $E_2\beta$ or estrone to "catechol" estrogens is a major metabolic pathway in the liver.[177] Many different isoforms of CYP450s including CYP1A1, CYP1A2, CYP3A4 contribute to the 2-hydroxylation of $E_2\beta$ in the liver.[177] CYP1A1 metabolizes $E_2\beta$ largely to 2-hydroxyestradiol, whereas 4-hydroxyestradiol is a minor product. *In vivo* metabolism of $E_2\beta$ to 2-hydroxyestradiol accounts for 50% of the estradiol metabolites formed and the levels of catecholestradiols range from 0.12-0.3 μmol/L in peripheral blood.[183] Thus, substantial amounts of 2-hydroxyestradiol are available to be converted to 2-methoxyestradiol as well as for biological/physiological activity. Due to the rapid conversion of 2-hydroxyestradiol to 2-methoxyestradiol, accurate data on the physiological plasma levels of 2-hydroxyestradiol in may biological systems has been difficult to assertain.[172]NADPH-dependent 2-hydroxylation of $E_2\beta$ and/or estrone has been observed with microsomes prepared from various extrahepatic tissues such as uterus, breast, placenta, kidney, brain and pituitary.[172, 177] Several important consequences of locally-formed 2-hydroxyestrogens have been suggested. 2-hydroxystrogens can bind to the classical estrogen receptors (ERs), but with a markedly reduced binding affinity compared to $E_2\beta$.[176, 184] 2-hydroxyestrogens bind with high affinity to steroid hormone binding globulin and in this regard possess a higher affinity compared to $E_2\beta$.[173] 2-hydroxyestrogens inhibit COMT-catalyzed *O*-methylation of catecholamines, which may exert a modulatory effect on the neurophysiological/pharmacological effects of catecholamines in the vascular system. [185]

Although 2-hydroxylation of estrogens is the dominant pathway for catecholestrogen formation in the liver, 4-hydroxylated estrogens are also formed.[177]4-hydroxylation of estrogens is catalyzed mainly by CYP1B1 and CYP3A4 and to a lesser-extent by CYP1A1 and CYP1A2. [172] In human liver microsomes, the CYP1B1 is believed to play a major role in the 4-

hydroxylation of $E_2\beta$.[177] In contrast to the observations indicating that 4-hydroxylation of estrogens is a minor pathway for catecholestrogen formation in liver, recent studies showed that 4-hydroxylation of estrogens is a dominant pathway for catecholestrogen formation in several extrahepatic tissues including the uterine compartment.[172] For instance, in human uterine myometrial and myoma tissues, more 4-hydroxylated metabolites of $E_2\beta$ are formed compared to 2-hydroxylated forms.[177] 4-hydroxyestrogens also possess very low binding affinity to the classical ERs compared to $E_2\beta$ (one-fourth the binding affinity of $E_2\beta$).[176] Interestingly, the interaction of 4-hydroxylated estrogen metabolites with the classical ERs appears to occur with a reduced dissociation rate compared with $E_2\beta$, suggesting that the association of 4-hydroxyestrogens with the ERs may last longer than that for its parent hormone or 2-hydroxylated forms.[176]

Following hydroxylation by CYP450s, hydroxylated estrogens with a "catechol" moiety (catecholestrogens) are methylated via enzymatic *O*-methylation to methoxyestogens, i.e., 2-methoxyestradiol and 4-methoxyestradiol, by COMT.[172] Because COMT activity is very high in liver and kidney, red blood cells, uterine endometrium, the mammary gland and many other tissues, methoxyestrogens have been shown to be the most abundant estrogen metabolites in human plasma and urine.[177] For example, in pregnant women, the mean plasma concentration of unconjugated 2-methoxyestrogens has been estimated to be in the high pg/ml range[186] however, these data have yet to be shown. Interestingly, methoxyestrogens such as 2-methoxyestadiol have higher binding affinity for sex hormone-binding globulin than $E_2\beta$ or the catecholestrogens, and these high binding affinities of these methoxyestrogens may contribute to their high plasma and urine levels.[172, 186, 187] Another reason for their high plasma levels may also be the fact that methoxyestrogens have very long half-life in the systemic circulation.[188] Methoxyestrogens have

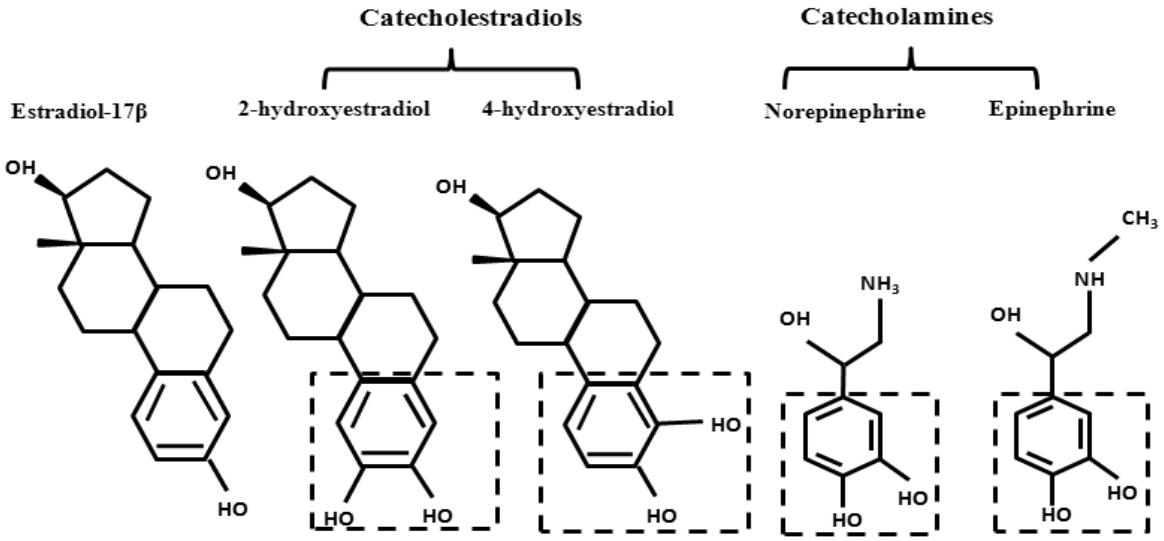
little or no binding affinity for the classical estrogen receptors when compared to $E_2\beta$.[176] High concentrations of catecholamines in target tissues may inhibit COMT-catalyzed *O*-methylation of catecholestrogens, which will result in decreased formation of methoxyestrogens.[186]

Apart from CYP450- and COMT-mediated hydroxylation and methylation respectively, $E_2\beta$ or estrone can also be conjugated to glucuronides and sulfates by conjugating enzymes in liver and target cells to make conjugated estrogens (see schematic diagram below).[189, 190] It has been well documented that conjugated estrogens may also be important precursors for metabolic formation of hormonally active estrogens in target cells and tissues. Glucuronidated and sulfonated estrogens themselves have no estrogen receptor binding affinity and their estrogenic activity may results from the release of unconjugated estrogens by enzymatic deconjugation in liver as well as in target tissues or cells.[190] It is noteworthy that high levels of estrogen glucuronosyltransferase and sulfotransferase, which converts $E_2\beta$ and estrone to their corresponding conjugated hormones, are expressed in uterine compartment and their enzyme activity is stimulated in the following chronic administration of estrogens. [172] Equally importantly, it should also be noted that estrogen sulfates or glucuronides can also be hydroxylated at multiple positions by NADPH-dependent CYP450 enzymes in target cells and tissues suggesting that these conjugated metabolites may be an additional source of hydroxylated estrogen metabolites when deconjugated (see schematic diagram below).[191] Conjugated estrogens and estrogen metabolites also point to an important fact that in consideration of the systemic and local levels of estrogens, both conjugated and unconjugated levels have to be taken into account. Only measurements of both types of estrogens (conjugated and unconjugated) can give a clear and accurate picture of the levels of estrogens within the circulation as well as local cell and tissue levels.

III.2 Interaction with Catecholamine Synthesis and Metabolism

Since COMT catalyzes the formation of methoxylated products in the presence of Mg^{2+} and S-adenosyl-l-methionine from the substrate which must contain a "catechol" moiety[178], endogenous substrates of COMT include the catecholamine neurotransmitters, i.e. L-DOPA, dopamine, norepinephrine and epinephrine, and of course catecholestrogens.[178] Several exogenous substances and drugs such as benserazide, carbidopa, dobutamine, fenoldopam, isoprenaline, α-methyldopa, rimiterol and vitamin C are also metabolized by COMT. [178] This property of COMT also point to the potential relevance of the convergence of the sympathomimetic system and estrogen metabolism system in the regulation of pregnancy-induced vascular adaptations. This is because catecholestrogens share very close structural similarities with catecholamines (see diagram below).[177] Moreover, both families of compounds have affinities for COMT and tyrosine hydroxylase. Owing to their structural similarities with catecholamines (see diagram below), catecholestrogens have the ability to directly affect several neurotransmission and neuroendocrine pathways, a property not shared by the parent estrogen. Functionally, catecholestrogens (specifically catecholestradiols) alter uptake of norepinephrine, dopamine, GABA and serotonin in the brain.[192, 193] In addition, catecholestrogens directly compete tyrosine for binding to tyrosine hydroxylase, considered the rate limiting enzyme in catecholamine synthesis.[194] Therefore, the metabolism of estrogens interacts directly with the synthesis of catehcolamines. Catecholestrogens can also strongly compete catecholamines for direct binding to COMT, which methylates catecholamines into the so called "inactive" catecholamines. [186] In this regard, the metabolism of estrogens to the catecholestrogens interacts with catecholamine metabolism. Collectively, these observations suggest that estrogen

metabolism interacts with catecholamines synthesis and metabolism and in all likelihood directly impact endogenous levels of these catehcolamines as well as their vascular physiology



IV. Cardiovascular Physiology and Pharmacology of Estrogen Metabolites

Numerous evidences have shown that the sequential conversion of estrogens especially $E_2\beta$ to catecholestrogens and methoxyestrogens by CYP450s and COMT, respectively, contributes importantly to the cardiovascular effects of estrogens (e.g. $E_2\beta$) in many systems. These findings have indeed challenged the prevailing orthodox view that all biological effects of estrogens especially $E_2\beta$ are initiated by binding of estrogens to the classical estrogen receptors and that estrogen metabolites are less potent, irrelevant, hormonally inactive and destined for excretion. Over the years, numerous investigators have shown that estrogen metabolites especially the $E_2\beta$ metabolites (catecholestradiols and methoxyestradiols) induce multiple effects on the vascular system which may or may not be associated with the actions of the parent substrate. These positive effects of estrogen metabolites are mediated in part by selective and differential actions on the uterine and other vasculatures as well as on vascular smooth muscle cells, cardiac fibroblasts and vascular endothelial cells from a myriad of vasculatures.

IV.1 Effects of Estrogen Metabolites on the Uterine Vasculature

Studies by Rosenfeld and Jackson demonstrated that infusion of catecholestrogens directly into the uterine artery of oophorectomized and nonpregnant sheep significantly increased uterine blood flow.[195] In this study, there was a delay in the response of about 30 minutes following infusion of catecholestrogens, followed by a gradual but steady increase in uterine blood flow, with maximum levels attained between 90-120 minutes, and then a gradual decrease in uterine blood flow, returning to baseline within 6-12 hours.[195] Subsequently, Stice et al 1987 showed that catecholestrogens, particularly 4-hydroxyestradiol, directly increased uterine blood flow in pregnant gilts.[196] These studies by Stice and co-workers also demonstrated that these actions resulted from direct interactions of catecholestrogens with calcium channels also pointing to an indirect relationship with endothelial vasodilators which require calcium-mediated signaling for maximal functionality.[196] However, these studies did not investigate the direct relationships between estrogen metabolites and endothelial-mediated vasodilators that may be responsible for these responses. Nevertheless, it would appear that estrogen metabolites especially the catecholestrogens play important roles in the regulation of uterine vascular tone during pregnancy.

IV.2 Effects of Estrogen Metabolites on Key Endothelial Vascular Cell Types

In vascular endothelial cells, estrogen metabolites stimulate proliferation as well as the generation of the potent vasodilatory PGI_2.[197] In this regard, 2-methoxyestrone, 2-methoxyestradiol and 16α-hydroxyestrone stimulated significant proliferation and production of PGI_2 in cultured human umbilical vein endothelial cells.[197, 198] It is important to note that the induction of proliferation by catecholestradiols and methoxyestradiols on endothelial cells are concentration-dependent. In this regard, low concentrations of 2-hydroxyestrone, 2-

hydroxyestradiol, 2-hydroxyestriol, 4-hydroxyestrone and 4-hydroxyestradiol significantly induced proliferation of cultured human umbilical vein endothelial cells,[198] whereas, higher pharmacological concentrations of these metabolites inhibited endothelial cell proliferation and are thus anti-angiogenic.

Studies have also shown that estrogen metabolites are potent antioxidants in vascular endothelial cells. Data from these studies showed that 2-hydroxyestrone and 2-hydroxyestradiol are potent antioxidative substances with respect to oxidation of LDL, considerably exceeding the effect of their respective parent substrates.[199] It is possible that the effects of these metabolites on preventing the oxidation of LDL plays a role in the synthesis of PGI_2 since oxidized LDL has been shown to decrease PGI_2 synthesis in vascular endothelial cells in several vasculatures. Furthermore, compared to vitamin E, a potent antioxidant, the effect of the two catecholestrogens was even significantly higher. 16α-hydroxyestrone also induced significant antioxidative properties.[199] These findings also demonstrate the functional vasodilatory properties of estrogen metabolites by potentiating the vasodilatory activity of NO release under basal conditions by preventing oxidation of NO. Indeed, $E_2\beta$ induced endothelium-mediated vasodilatory actions has been shown to be associated with decreased levels of superoxide anion, and this may account for the enhanced NO bioactivity and decreased peroxynitrite release.[200] 2-Hydroxyestradiol and 2-methoxyestradiol also inhibit endothelin-1 synthesis, a potent vasoconstrictor, by coronary artery endothelial cells, and this may contribute to their ability to improve endothelial vasodilatory function.[200]

The maternal uterine vascular endothelial cells determine in part the ability of the healthy uterine arteries to support pregnancy and to resist pathological processes such as during preeclampsia. The effects of estrogen metabolites on the uterine vasculatures have not been

evaluated since the studies of Rosenfeld and Jackson (1982) as well as Stice and coworkers (1987). Consequently, it is critical to examine the effects of estrogen metabolites on this pivotal endothelial cell type during pregnancy to bring perspective to the physiological effects of estrogen metabolites in pregnancy as well as in pregnancy-related diseases such as preeclampsia.

IV.3 Interactions with Catecholamines and Adrenergic Receptors

Catecholestradiols exhibit close structural similarities and functional interactions with the catecholamines, norepinephrine and epinephrine as well as dopamine.[186] Because of the shared phenolic "catechol" moiety, catecholestradiols interact directly with catecholamine responses, a property not shared by the parent estrogens.[186]For example, in the rat cerebral cortex, striatum, and anterior pituitary, the catecholestrogens strongly compete norepinephrine and epinephrine for binding to the α- and β-adrenergic receptors (α- and β-ARs).[201] In this regard, the binding of catecholestrogens to α- and β-ARs was highly comparable and similar to binding of the catecholamines to the same receptors.[201] In interestingly, data in these studies also showed that the catecholestrogens bound more tightly to the α- and β-ARs compared to the catecholamines at the same concentrations of substrate.[201]In addition, findings by Etchegoyen et al [192]showed that in the guinea pig hypothalamus, catecholestrogens competed norepinephrine and epinephrine for binding to the α- and β-ARs and impaired catecholamines-induced adenylate cyclase activity and cAMP production. Other studies have also shown that catecholestrogens inhibit glucose-induced insulin secretion in pancreatic islets isolated from normal rats via α-AR receptors suggesting that catecholestrogens may induce effects via the adrenergic receptors.[202]Moreover, 3D structural and functional analyses demonstrate that the "catechol" moiety of catecholamines and other compounds is functionally important in the activation specific transmembrane domains of α- and β-ARs for optimal activities.

V. Implications for Preeclampsia

Preeclampsia is a multisystemic hypertensive disorder of pregnancy estimated to affect 8% to 10% of all pregnancies in the United States.[203] Although preeclampsia is one of the leading causes of maternal death and the main contributor of prenatal morbidity and low birth weight, the etiology of this disorder is unknown.[204] Preeclampsia develops during pregnancy and ceases after delivery, implicating the uteroplacental compartment as a primary culprit.[204] Manifestations generally associated with preeclampsia include an increased responsiveness to vasoconstrictors, decreased vasodilatory responses, increases in arterial pressure, proteinuria, decreased angiogenic factors, and vascular endothelial damage.[205]Therefore, preeclampsia is associated with impairments in several normal maternal vascular adaptations seen during pregnancy including insufficient vasodilatation and angiogenesis.

The initiating event in preeclampsia is suggested to involve reduced placental perfusion, maternal endothelial cell dysfunction and placental insufficiency.[205] The factors involved in mediating the hypertension during preeclampsia are unknown and may involve a delicate balance of vasoconstrictors and vasodilators as well as angiogenic factors.[206] Evidence indicates that NO, PGI_2 and angiogenic factors plays an important role in mediating physiological changes during normal pregnancy including rises and maintenance of uterine blood flow.[207] Therefore it is feasible that there is defective vasodilatation and angiogenesis during the pathophysiology of preeclampsia because reduction in the blood flow to organs and the development of endothelial dysfunction precedes the clinical manifestation of this disease. Evidence also supports a role for primary estrogens in preeclampsia since primary estrogens rescue several endothelial mediated processes in cells derived from the preeclamptic state.[208-210] However, whether primary estrogens can be useful biomarkers for maternal and fetal well-being in adverse pregnancies including

preeclampsia has been a subject of controversy. Several studies support evidence that serum and urinary primary estrogens maybe useful for screening for adverse pregnancy outcomes[208], whereas others contend that measurement of these estrogens is of little value.[211] Nevertheless, the levels and plasma profile of circulating primary estrogens in preeclampsia are, at best, unclear and this is further complicated by the lack of information on specific functional estrogen metabolites thus hindering our comprehensive understanding of their role(s) in its pathophysiology. There have also discussed increasing strong support for the concept that regulation of maternal cardiovascular adaptation during pregnancy is partly mediated by estrogen metabolites.[212, 213] Estrogen metabolites may also play a role in preeclampsia because pregnant mice deficient in COMT, an enzyme that catalyzes the methylation of catecholestrogens to methoxyestrogens, exhibit a preeclampsia-like phenotype.[182] In addition, treatment of these mice with exogenous 2-ME_2 ameliorates all the preeclampsia-like phenotype.[167] However, the metabolism of estrogens is very complex and necessitates that a plethora of other functional metabolites of estrogens other than 2-ME_2 be properly accounted for to achieve a more comprehensive knowledge of their potential collective contributions to the pathogenesis of preeclampsia.

VI. Statement of Hypothesis

In light of these above described observations, we **hypothesize** that during pregnancy, changes in uterine blood flow associated with estrogen-induced uterine endothelial vasodilatory and angiogenic responses are in part mediated via its biologically active metabolites, the catecholestradiols 2-OHE_2 and 4-OHE_2 and the methoxyestradiols 2-ME_2 and 4-ME_2.

CHAPTER 2:

SYSTEMIC AND LOCAL UTERINE PLASMA CONCENTRATIONS OF ESTROGEN AND ESTROGEN METABOLITES DURING PREGNANCY

Concentrations of Total (Conjugated + Unconjugated) Estrogens and Estrogen Metabolites in Systemic and Local Plasma of Late Pregnant Ovine: Implications for Vascular Function

[Manuscript in Preparation, *JPET, 2013*]

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ABSTRACT

Endogenous estrogens and estrogen metabolites play key roles in gestational vascular adaptations during pregnancy, yet the plasma levels of specific estrogens and/or estrogen metabolites and patterns of estrogen metabolism during pregnancy remain unclear. We performed liquid chromatography mass spectrometry to compare the systemic versus local plasma levels of total (conjugated + unconjugated) primary estrogens and estrogen metabolites in late pregnant ovine compared to the nonpregnant (luteal and follicular) states. Compared with the luteal or follicular states, estrone was higher in late pregnant ovine, and these levels were higher in the uterine compared to systemic plasma. The level of estradiol-17β was significantly higher in late pregnant ovine versus the luteal and/or follicular states, however, these levels were lower in the uterine circulation versus the systemic circulation. Compared with the luteal and follicular states, 16-keto-estradiol-17β levels were higher in late pregnancy and not different between vasculatures. Estriol was higher in late pregnant ewes and not different between vasculatures. 2-hydroxyestrone and 4 hydroxyestrone were lower in late pregnancy and elevated during the follicular phase. 16-α-hydroxyestrone and 2-hydroxyestradiol were higher in late pregnant ovine compared to nonpregnant states. 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol were higher in late pregnant ovine compared to nonpregnant ewes. 2-methoxyestrone was unchanged. 16-epi-estriol and 17-epi-estriol were higher in late pregnancy. Our findings show that pregnancy is characterized by distinct synthesis, metabolism, and accumulation of estrogens and estrogen metabolites that are likely to be associated with vascular function. These results underscore the need to further investigate the functional vascular and other physiology of estrogens and estrogen metabolites in pregnancy.

INTRODUCTION

Primary estrogens, estrone, estradiol-17β, and estriol, acting directly, indirectly or both, play major roles in the regulation of the maternal cardiovascular adaptations to pregnancy.[1] We and others have demonstrated that estrogens play a key role in the modulation of local and systemic vascular resistance and uterine blood flow as well as several other aspects of pregnancy vascular adaptations including regulation of vasodilatation and angiogenesis.[10,27, 29,31,85] Although consensus conclusions demonstrate that estrogens play key roles in the regulation of these adaptations and are markedly elevated, the true concentrations of these estrogens during pregnancy remains controversial, perhaps reflecting limitations of different analytical techniques used. However, another plausible explanation for these apparently contradictory findings may relate to the studies reporting levels of conjugated versus unconjugated levels of these estrogens as well as systemic versus local uterine concentrations during pregnancy. Nevertheless, the systemic versus local plasma levels of primary estrogens in pregnancy are, at best, unclear, and necessitate further investigation to increase our understanding of their relative roles in pregnancy.

Our understanding of the roles and levels of primary estrogens in pregnancy is further complicated by the lack of information on estrogen metabolites. Primary estrogens are converted by cytochrome P450s into multiple hydroxylated metabolites including 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone, 2-hydroxyestradiol, and 4-hydroxyestradiol.[172,173] Hydroxylated primary estrogens undergo enzymatic *O*-methylation by catechol-*O*-methyltransferase to form the methoxyestrogens, such as 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol, and 4-methoxyestradiol. [172,173,174] Primary estrogens can also be enzymatically converted to 16-keto-estradiol-17β, 16-epi-estriol, and 17-epi-estriol. [172,174]

Indeed, accumulating experimental data provide convincing evidence that regulation of maternal cardiovascular adaptations during pregnancy are, in part, mediated by many of the estrogen metabolites and that these effects are largely independent of the classical estrogen receptors.[196,197,198,199] However, the systemic or local concentrations of estrogen metabolites during pregnancy are essentially nonexistent and no doubt hinder our comprehensive understanding of their critical roles in pregnancy.

In this report, we compared the systemic versus local plasma levels of total (conjugated + unconjugated) primary estrogens and estrogen metabolites in late pregnant ovine compared to the nonpregnant (luteal and follicular) states. We hypothesized that compared to the nonpregnant states, pregnancy is characterized by distinct elevated levels of estrogens and estrogen metabolites in the systemic compared to the local uterine circulation. Our results indicate that increases in primary estrogens during pregnancy are accompanied by parallel elevations of some biologically active estrogen metabolites that should be taken into account when measuring and/or investigating the roles of estrogens or estrogen metabolites in pregnancy.

MATERIALS AND METHODS

Animals and Sample Collection

Animal protocols and procedures were approved by University of Wisconsin–Madison Research Animal Care and Use Committee of the School of Medicine and Public Health and the College of Agriculture and Life Sciences. Plasma samples from 4 nonpregnant luteal, 4 nonpregnant follicular and 4 late pregnant ewes (120-130 days; term= 147 days) were obtained from the uterine veins or maternal artery via the superficial saphenous branch of the femoral artery. Blood was centrifuged for 10 min at 3000 rpm at 4°C and plasma was aspirated and stored at −80°C until measurement.

Liquid chromatography-tandem mass spectrometry

Plasma levels of estrogens and estrogen metabolites were measured by a sensitive, specific and precise high performance liquid chromatography-tandem mass spectrometry method utilizing selected reaction monitoring for measuring the absolute quantities of 15 total (conjugated and unconjugated) estrogens and estrogen metabolites and performed at Primera Analytical Solutions Corp., Princeton, NJ. Methodology is briefly described below.

Chemicals and Reagents

Sodium acetate, Spectrum; Sodium bicarbonate, J.T.Baker; L-ascorbic acid, Lot J.T.Baker; β-glucuronide/sulfatase, Sigma; Dansyl chloride, Fluka; Acetone, Acetonitrile and Methanol, Pharmco-AAPER and Formic Acid, Spectrum. All other chemicals and reagents utilized were HPLC or ACS grade.

Calibration Standards

Estrone, Estradiol-17β, Estriol, 2-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol, 16α-hydroxyestrone, 2-methoxyestrone, 4-methoxyestrone, 3-hydroxyestrone, 2-methoxyestradiol, 4-methoxyestradiol, 16-ketoestradiol-17β, 16-epiestriol and 17-epiestriol were bought from Steraloids Inc, Newport, RI.

Preparation of Calibration Curve

Preparation of Stock standard solution:

Stock solutions of estrogen M=metabolites were prepared at 100 μg/ml by dissolving accurately weighed estrogen powders in methanol containing 0.1% L-ascorbic acid in a volumetric flask and stored at -20 °C. The 200 ng/ml EM working standards were prepared by dilution of the stock solutions using methanol with 0.1% L-ascorbic acid.

Preparation of spiked standard solutions:

Calibration standard spiked solutions were prepared by dilution of the stock standard solution with methanol containing 0.1% L-ascorbic acid.

Sample Preparation Procedure

Hydrolysis: 0.5 mL of freshly prepared enzymatic hydrolysis buffer was added to the study samples and incubated for 20 h at 37 ˚C.

Extraction: 8 mL of MTBE was added into the samples (including calibration curve samples) and then tumbled for 30 minutes. After freezing the aqueous layer in the freezer at -70˚C for 30 minutes, the organic layer was transferred to another labeled clean tube and dried down at 60˚C under a nitrogen stream.

Derivatization: 100 µL of 0.1 M sodium bicarbonate buffer (pH 9.0) and 100 µl of dansyl chloride solution (1 mg/mL in acetone) was added to the samples. The samples were incubated at 60 ˚C for 5 minutes prior to analysis on the LC-MS/MS.

The samples were analyzed in two separate batches, with a calibration curve included in each run before and after the samples in each batch. For each batch, from the calibration curve data, the LLOQ was determined by having at least one of the lowest calibrators within ±20% of the nominal value. The unit for the calculated concentration of estrogens and their metabolites is pg/ml. Ten point calibration curves were used with nominal values at 8 pg/ml, 20 pg/ml, 40 pg/ml, 80 pg/ml, 200 pg/ml, 400 pg/ml, 800 pg/ml, 1600 pg/ml, 2400 pg/ml, and 3200 pg/ml. Because of the indeterminant LLOQ for each run, the criteria for acceptance were that 2/3 of the samples in the calibration curves were within ±20% of the nominal concentrations above the LLOQ. Points below the final LLOQ were not included as points in the curve. The acceptance criteria for the determination of the LLOQ was that at least one of the standards at the value determined for the LLOQ needs to be within ±20% of the nominal value.

Statistical Analysis

Data (Mean±SEM) were analyzed using a 2-way ANOVA with "pregnancy status" and "local/systemic levels" as "between" factors. Multiple pairwise comparisons were performed using Student-Newman-Keuls test. Level of significance was established a *priori* at $P<0.05$.

RESULTS:

Plasma Profile of Primary/Classical Estrogens in Pregnancy

Plasma levels of estrone were significantly increased in late pregnant ovine compared to either the respective luteal and follicular group. In addition, the uterine concentration of estrone in late pregnant ovine at 1462 ±128 pg/mL was significantly higher than in the systemic circulation at 914±128 pg/mL. Levels of estrone in the uterine and systemic circulation in the luteal group (28±10 and 55±10 pg/mL respectively) were similar to levels in the follicular group (50±25 and 60±25 pg/mL respectively).

Estradiol-17β levels were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of estradiol-17β in the follicular group were also significantly increased compared to levels in the luteal group. The uterine levels of estradiol-17β (1106±129 pg/mL) in late pregnant ovine were significantly lower than systemic plasma levels (1575±129 pg/mL). The levels of estradiol-17β in uterine and systemic plasma of follicular ovine were similar (411±26 and 382±26 pg/mL respectively).

Plasma levels of 16-keto-estradiol-17β were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of this estrogen were also higher in the follicular group compared to levels in the luteal group. The uterine levels of 16-keto-estradiol-17β (751±138 pg/mL) were similar to the systemic levels (785±138 pg/mL).

Estriol levels were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of estriol in the follicular group were also significantly increased compared to levels in the luteal group. The uterine levels of estriol (35±2 pg/ml) in late pregnant ovine were not different from the systemic plasma levels (34±2 pg/mL).

Levels of 2-hydroxyestrone and 4-hydroxyestrone were lower in the late pregnant group compared to the follicular group although higher than the luteal group. On the other hand, plasma levels of 16-α-hydroxyestrone were higher in the late pregnant group compared to the nonpregnant luteal and follicular group. The uterine levels of 16-α-hydroxyestrone (885±98 pg/mL) in late pregnant ovine were significantly higher than systemic plasma levels (637±99 pg/mL).

Plasma levels of 2-hydroxyestradiol levels were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of 2-hydroxyestradiol in the follicular group were also significantly increased compared to levels in the luteal group. The uterine levels of estriol (1009±87 pg/ml) in late pregnant ovine were not different from the systemic plasma levels (994±87 pg/mL).

Levels of 2-methoxyestrone were not different among the study groups. However, plasma levels of 3-methoxyestrone and 4-methoxyestrone were both elevated in the late pregnant group compared to the nonpregnant luteal and follicular group; but only 3-methoxyestrone was different between the uterine versus the systemic vasculature.

2-methoxyestradiol levels were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of 2-methoxyestradiol in the follicular group were also significantly increased compared to levels in the luteal group. The uterine levels of 2-methoxyestradiol (1301±109 pg/mL) in late pregnant ovine were significantly lower than

systemic plasma levels (1554±110 pg/mL). On the other hand, 4-methoxyestradiol levels were significantly higher in late pregnant ovine compared to the luteal and follicular group. The levels of 4-methoxyestradiol in the follicular group were also significantly increased compared to levels in the luteal group. The uterine levels of estriol in late pregnant ovine were not different from the systemic plasma levels.

The plasma levels of 16-epi-estriol and -17-epi-estriol were both significantly elevated in late pregnant ovine compared to the nonpregnant groups. Levels of these estriol metabolites were also elevated in the follicular group compared to the luteal group. However, levels of these metabolites were not different between the uterine versus the systemic levels.

DISCUSSION

To study the role of endogenous estrogens and estrogen metabolites in pregnancy vascular adaptations, including in vasodilatation and angiogenesis, evaluating the individual plasma patterns profile levels in plasma across the estrous cycle and pregnancy may be advantageous because it provides the precise endogenous levels available in the circulation for utilization the specific physiological state being studied. In our study, we evaluated the local and systemic concentrations of total (conjugated + unconjugated) estrone, estradiol-17β, 16-keto-estradiol, estriol, 2-hydroxyestrone, 4-hydroxyesrone, 16α-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol, 4-methoxyestradiol, 16-epi-estriol and 17-epi-estriol late pregnant ovine versus nonpregnant (luteal and follicular) states.

During pregnancy, the placenta is the main source of these estrogens using circulating steroid precursors from the maternal uterine compartment, adrenal glands, as well as fetal adrenal glands.[1,214] The placental biosynthesis of primary estrogens during pregnancy is complex

and results from an interdependence of separate maternal, placental, and fetal systems that individually do not possess the necessary enzymatic capabilities to make these critical estrogens.[214] Because the placenta does not express 17α-hydroxylase, the obligatory synthesis of C19-steroid precursors is not possible for estrogen synthesis.[214] Thus, the primary estrogens are synthesized from C-19 precursors synthesized from the maternal and fetal adrenal glands.[214] Our findings are consistent with studies by other investigators showing that estrone, estradiol-17β, and estriol are increased during pregnancy and parallel the formation of an extensive vascular bed to support fetal growth.[1,21,214] In addition, estrone and estradiol-17β but not estriol have been shown to act as potent vasodilators in the uterine vascular bed.[1,27,28,29] Therefore, since pregnancy is characterized partly by uterine and systemic vascular vasodilatory responsiveness, our findings suggest that the activities of uteroplacental aromatase, 17β-hydroxysteroid dehydrogenase, placental sulfatase, and fetal 16α- and 17α-hydroxylase may be responsible for the levels and thus actions of these steroids. We also report, for the first time, that estriol is synthesize by the ovine and is detectable during pregnancy necessitating the importance of investigating the role of this primary steroid during pregnancy.

We report herein, for the first time, that the plasma level of 16-keto-estradiol-17β is significantly increased during pregnancy. The concomitant increased levels of 16-keto-estradiol-17β, with the levels of estradiol-17β and estriol, as well as structural and kinetic evaluations led us to hypothesize that this metabolite may be an interconversion metabolite between estradiol-17β and estriol. Thus, collectively, our observations suggest that elevated levels of 16-keto-estradiol-17β may be important during pregnancy and should be further investigated.

The present findings also show, for the first time, that plasma level of 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone, and 2-hydroxyestradiol levels are altered during

pregnancy. The increased levels of both 2-hydroxyestrone and 2-hydroxyestradiol during late pregnancy compared to the luteal phase suggest increased activities of CYP1A1, CYP1A2, and CYP3A4 that primarily hydroxylate estrogens in the C-2 position to form catecholestrogens. However, it is important to point that, the levels of these catecholestrogens were higher in the follicular phase compared to late pregnancy perhaps pointing to an important role for these metabolites during that time of the estrous cycle. We report that levels of 4-hydroxyestrone and 16-α-hydroxyestrone are increased during late pregnancy perhaps pointing to physiologic importance of these metabolites during pregnancy. Therefore, these data suggest that formations of hydroxylated estrogens, which have been demonstrated to possess several uteroplacental vascular effects, including vasodilatory activities, induction of endothelial cell proliferation, generation of prostacyclin, and synergistic effects with nitric oxide, are increased during pregnancy and may contribute to the regulation uterine perfusion and blood flow.[197,199,223]

The enzymatic *O*-methylation of catecholestrogens by COMT forms several methoxyestrogens. To the best of our knowledge, this is the first report showing the plasma levels of 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol methoxyestrogens in the local and systemic circulations. Indeed, low activity of COMT and leading to low levels of 2-methoxyestradiol in human placentas have been associated with preeclampsia and pregnant COMT-deficient mice exhibit a preeclampsia-like phenotype.[182,231,292] Therefore, our finding suggests that the methoxyestrogens which have been shown to induce various positive vascular effects via vasoactive and intracellular molecules such as nitric oxide, prostacyclin, endothelin-1, cyclic nucleotides, hypoxia-inducible factor 1, and adhesion molecules, are increased during pregnancy necessitate further investigation. [182,197,199]

We noted, for the first time, that 16-epi-estriol and 17-epi-estriol are both significantly increased during pregnancy, suggesting that in addition to increased estriol levels, epimerization metabolism of estriol is also increased during pregnancy. 16-epi-estriol has been demonstrated to possess strong anti-inflammatory effects without profound immunosuppressive or glycogenic activities, whereas, 17-epi-estriol possesses negative effects on adhesion by suppressing tumor necrosis factor-α–induced and nitric oxide–mediated vascular cell adhesion molecule 1 expression.[277,278,279,280] Thus, since pregnancy is associated with increased expression of adhesion molecules and increased levels of proinflammatory cytokines that induce alterations in vascular vasodilatory responsiveness, our findings suggest the first possibility that low 16-epi-estriol and 17-epi-estriol may potentially contribute to inflammatory and adhesion responses during pregnancy.

In summary, our findings provide the first complete evidence that pregnancy is associated with increased and distinct synthesis, metabolism, and plasma accumulation of 15 estrogens and estrogen metabolites, therefore suggesting important roles for a plethora of estrogens and estrogen metabolites during pregnancy. In addition, the specific increases in these estrogens and estrogen metabolites are significantly higher during pregnancy compared to the nonpregnant states pointing to perhaps the physiologic importance of these steroids during pregnancy. These observations have also led us to investigate the plasma levels of these steroids in intra uterine growth restriction (see Appendix A.4-A.7). Intrauterine growth restriction (IUGR) accounts for a large incidence of infant mortality and morbidity worldwide. We had previously developed an ovine model of uterine space restriction (USR) which is associated with reduced placental weight and efficiency.[219] The placental is the main source of estrogens and estrogen metabolites which play key roles in regulating pregnancy-related vascular functions that modulate normal fetal

growth. We thus investigated if USR associated IUGR demonstrates alterations in the synthesis and metabolism of these estrogens and estrogen metabolites (see Appendix A.4-A.7). Those studies show that there is a distinct decrease in plasma concentration of products of primary synthesis including estrone, estradiol-17β and 16-keto-estradiol in the uterine space-restricted pregnancies, suggesting alterations in aromatase, 17β-HSD and other associated enzymes. In addition, there is also a decrease in plasma concentrations of products of hydroxylation including 16-alpha-hydroxyestrone and 2-hydroxyestradiol estriol in the uterine space-restricted pregnancies that are likely to be associated with alterations in the functions of cytochrome P450. There is a decrease in the plasma concentrations of the catechol-*O*-methyltransferase-derived metabolites of estradiol-17β, 2-methoxyestradiol and 4-methoxyestradiol, in in the uterine space-restricted pregnancies, suggesting aberrant metabolism of the products of *O*-methylation. We report, for the first time, plasma accumulation of estriol and estriol metabolites in pregnant ovine, suggesting that the ovine specie is capable of synthesizing estriol during pregnancy and this necessitates the investigation of the role of these steroids during pregnancy in sheep. Our findings show that uterine space restriction−induced placental inefficiency is characterized by aberrant synthesis, metabolism, and accumulation of estrogens and estrogen metabolites that are likely to be associated with alterations in vascular adaptations to pregnancy including uteroplacental blood flow. Therefore, this ovine model allows for examination of the interactive effects of uterine space restriction-induced IUGR on placental estrogen biosynthesis and metabolism which may help uncover relationships between placental function, intrauterine growth restriction and decrease in uteroplacental blood flows.

TABLES AND FIGURES

Figure 2.1

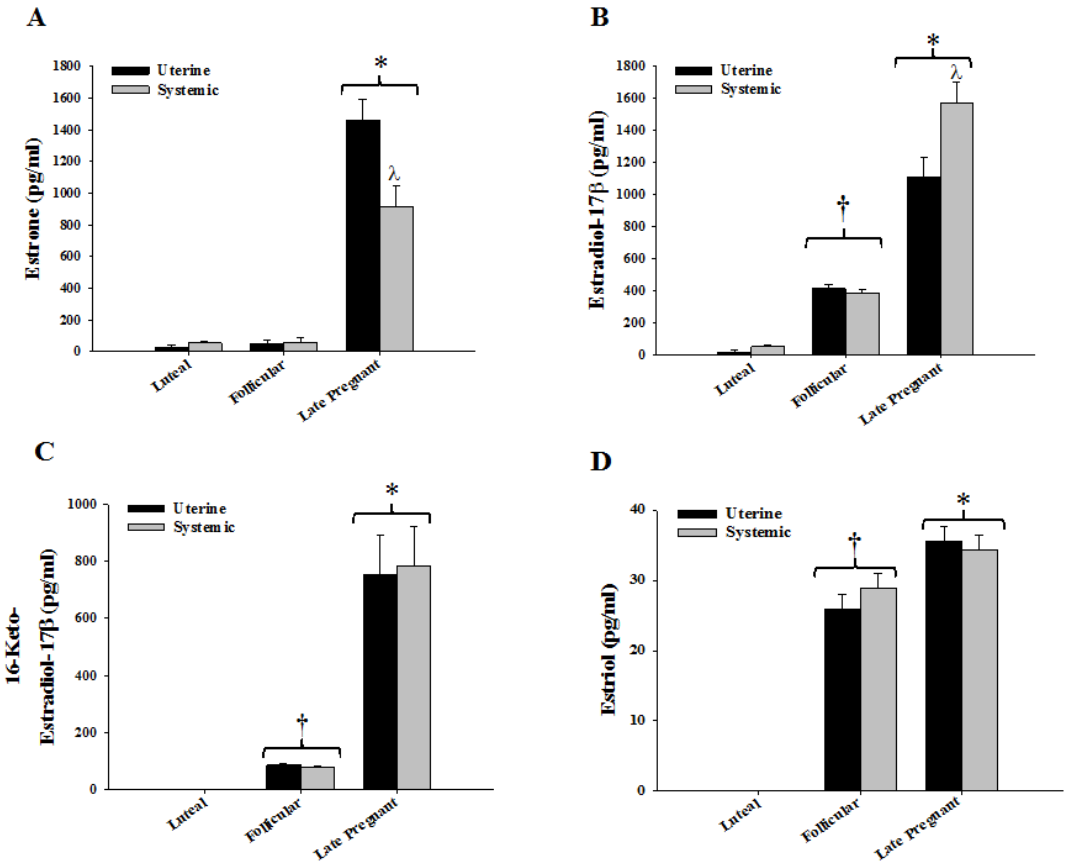

Figure 2.1: Products of primary synthesis: plasma levels of estrone, estradiol-17β, 16-keto-estradiol-17β, and estriol in nonpregnant luteal and follicular control ovine (n=4), versus late pregnant ovine (n=4). * Significantly different (P<0.001) in levels compared with respective nonpregnant luteal and follicular state ovine. † Significantly different (P<0.001) in levels compared with respective luteal state ovine. λ Significant different from (P<0.001) compared with local uterine levels.

Figure 2.2

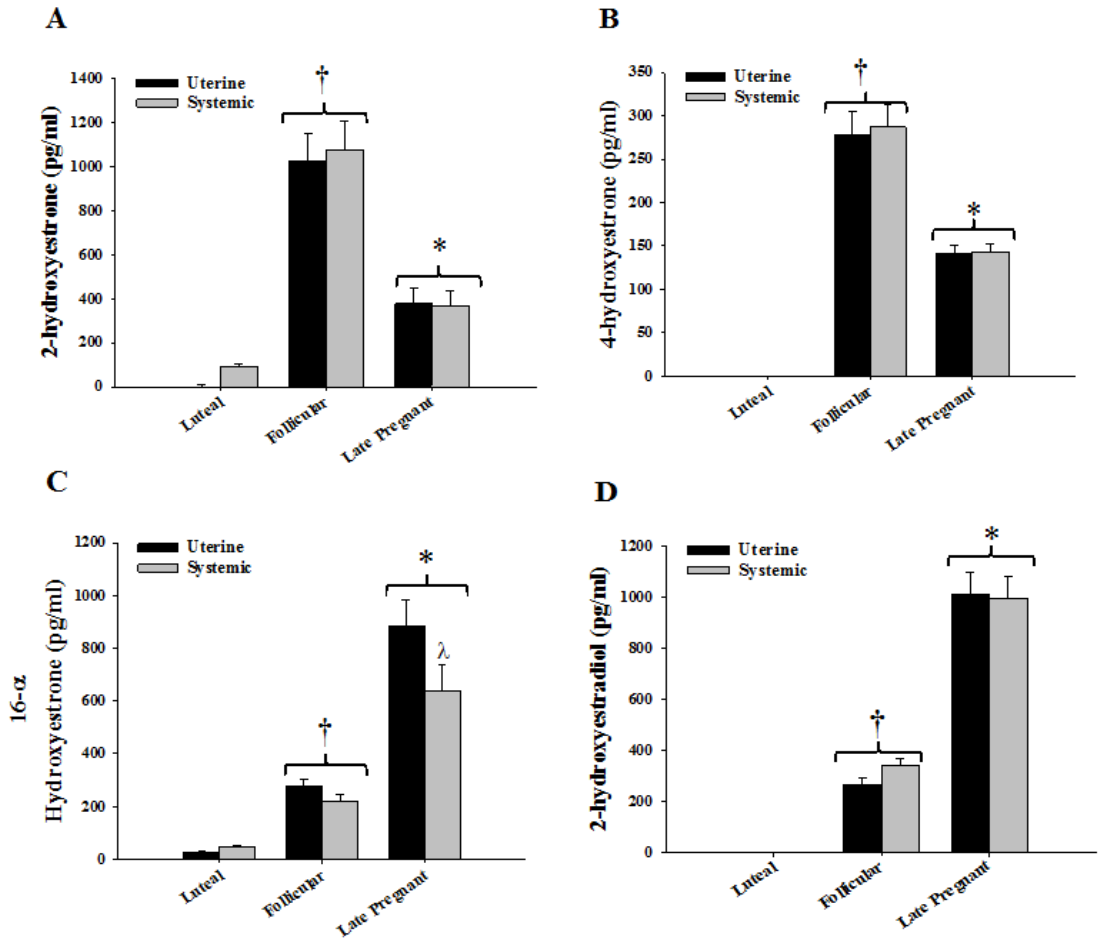

Figure 2.2: Products of hydroxylation: plasma levels of 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone and 2-hydroxyestradiol in nonpregnant luteal and follicular control ovine (n=4), versus late pregnant ovine (n=4). * Significantly different (P<0.001) in levels compared with respective nonpregnant luteal and follicular state ovine. † Significantly different (P<0.001) in levels compared with respective luteal state ovine. λ Significant different from (P<0.001) compared with local uterine levels.

Figure 2.3

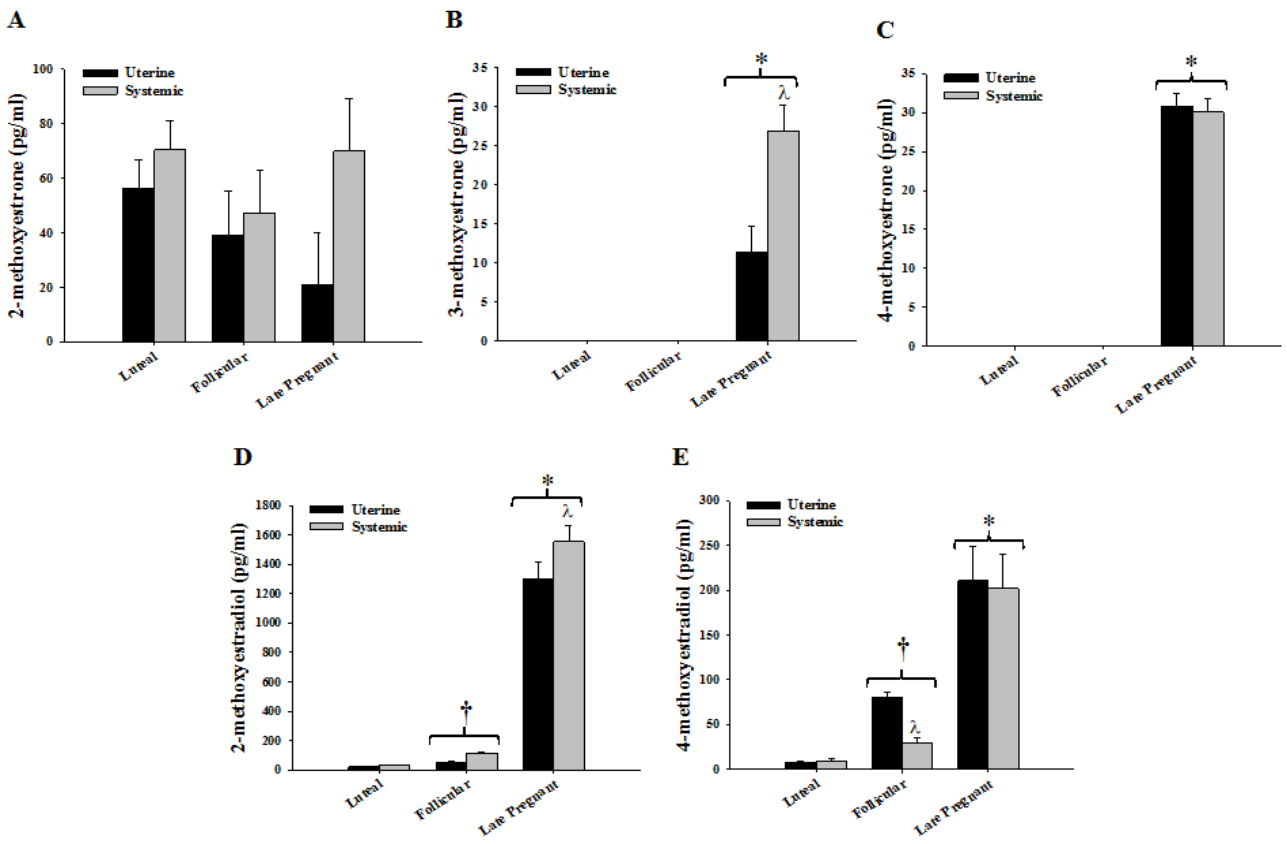

Figure 2.3: Products of *O*-methylation: plasma levels of 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol, and 4-methoxyestradiol in nonpregnant luteal and follicular control ovine (n=4), versus late pregnant ovine (n=4). * Significantly different (P<0.001) in levels compared with respective nonpregnant luteal and follicular state ovine. † Significantly different (P<0.001) in levels compared with respective luteal state ovine. λ Significant different from (P<0.001) compared with local uterine levels.

Figure 2.4

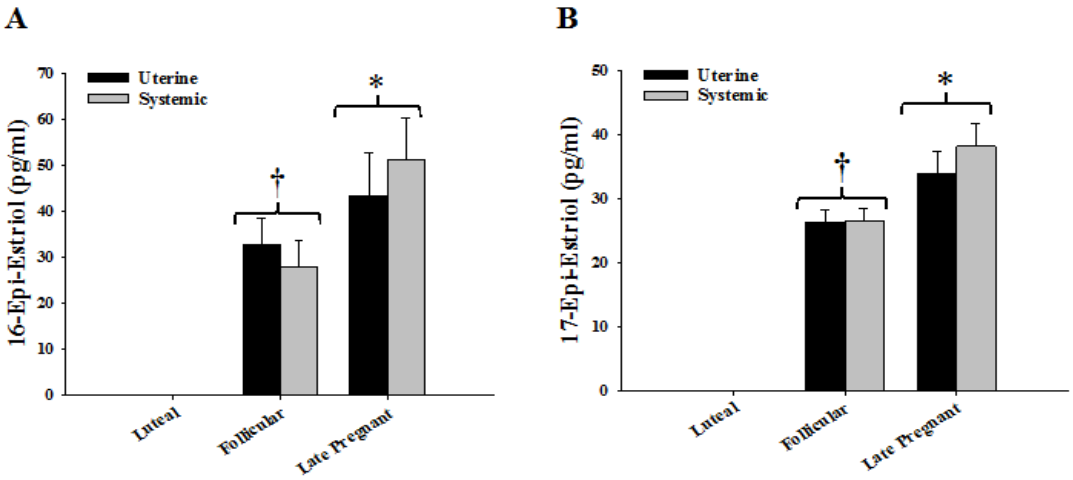

Figure 2.4: **Products of epimerization**: plasma levels of 16-epi-estriol and 17-epi-estriol in nonpregnant luteal and follicular control ovine (n=4), versus late pregnant ovine (n=4). * Significantly different (P<0.001) in levels compared with respective nonpregnant luteal and follicular state ovine. † Significantly different (P<0.001) in levels compared with respective luteal state ovine. λ Significant different from (P<0.001) compared with local uterine levels.

CHAPTER 3:

THE EFFECTS OF ESTRADIOL-17β AND ESTRADIOL METABOLITES ON UTERINE ENDOTHELIAL PROSTACYCLIN PRODUCTION: ROLE OF ER-α VERSUS ER-β

Estradiol-17β and Its CYP450s- and COMT–Derived Metabolites Selectively Stimulate Production of Prostacyclin in Uterine Artery Endothelial Cells: Role of ER-α versus ER-β

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ABSTRACT

Metabolism of estradiol-17β to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol, contributes importantly to the vascular effects of estradiol-17β in several vascular beds. However, little is known about the role of estradiol-17β metabolites via the different estrogen receptors (ER-α /ER-β) on de novo endothelial prostacyclin and thromboxane production. We hypothesized that estradiol-17β and its metabolites, via ER-α and/or ER-β can enhance the prostacyclin/thromboxane ratio through the classic phospholipase A_2, cyclooxygenase-1 and prostacyclin synthase pathway in ovine uterine artery endothelial cells (UAECs) in the pregnant (P-UAECs) versus the nonpregnant (NP-UAECs) state. Western analyses showed higher expression of phospholipase A_2, cyclooxygenase-1 and prostacyclin synthase in P-UAECs whereas thromboxane synthase was higher in NP-UAECs. In P-UAECs, estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol concentration and time-dependently increased prostacyclin compared to control. Prostacyclin increase in NP-UAECs was of a lower magnitude. Estradiol-17β and its metabolites stimulated higher prostacyclin /thromboxane ratio in P-UAECs compared to NP-UAECs. Estradiol-17β-induced increases in prostacyclin were abrogated by the antagonists SC-560 (COX-1), U-51605 (Prostacyclin synthase), ICI 182 780 (ICI; both ER-α/β) and MPP (ER-α), but not by PHTPP (ER-β). Prostacyclin increases induced by its metabolites was abolished by SC-560 and U-51605 but unaltered by ICI, MPP or PHTPP. Our findings demonstrate that estrogen via primarily ER-α and its metabolites via ER-independent mechanisms influence the de novo endothelial biosynthesis of prostacyclin, which may be important in the regulation of vascular tone. These findings also shed light on the complexities of estrogen signaling via its metabolism and the functional heterogeneity of the estrogen receptors.

INTRODUCTION

Prostacyclin (PGI_2) and thromboxane (TXA_2), two major vasoactive prostanoids that exert opposing effects on vascular tone, are end products of the sequential reactions catalyzed by cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-1/2 (COX-1/2) and either PGI_2 synthase (PGIS) or TXA_2 synthase (TXAS) enzymes respectively. [60] Classically, PGI_2, a powerful vasodilator is produced mainly by vascular endothelial cells, whereas TXA_2, a potent vasoconstrictor is principally produced by platelets.[60] In this regard, little is known about whether endothelial cells can produce TXA_2 and if this production plays a role in regulating the ratio of PGI_2 and TXA_2. PGI_2/TXA_2 ratio is considered of relevance in the regulation of physiologic and clinical vascular tone, with several studies showing that an imbalance in the generation of PGI_2 relative to TXA_2 is associated with hypertension, atherosclerosis and gestational vascular diseases such as preeclampsia.[72]

The dramatic rises in uterine blood flow during pregnancy are temporally associated with increases in *de novo* uterine vascular PGI_2 secretion and are accompanied by augmented expression of uterine artery endothelial $cPLA_2$, COX-1 and PGIS.[1, 65] These uterine blood flow rises are also partly mediated by increases in the plasma levels and actions of estrogen via the classical estrogen receptors (ERs).[1, 142, 220, 221] Infusion of estradiol-17β ($E_2\beta$) in sheep causes rises in uterine blood flow, increases the uterine arterial expression of $cPLA_2$, COX-1 and PGIS, which leads to the increase of the stable PGI_2 metabolite 6-keto-PGF1α.[66, 222] In human umbilical vein endothelial cells (HUVECs), $E_2\beta$ has also been shown to selectively stimulate PGI_2 production *in vitro* primarily via ER-α.[68] However, less is known about the influence of $E_2\beta$ on endothelial TXA_2 production and on the endothelial PGI_2/TXA_2 ratio.

The stimulatory effects of estrogen on uterine vascular endothelial PGI_2 production may be further modulated by its biologically active metabolites. $E_2\beta$ is sequentially metabolized by cytochrome P450s (CYP450s) to form the catecholestradiols, 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2), followed by metabolism of these catecholestradiols by catechol-*O*-methyltransferase (COMT) to form 2-methoxyestradiol (2-ME_2) and 4-methoxyestradiol (4-ME_2).[172] In cultured HUVECs, 2-ME_2 stimulates production of PGI_2.[223] Despite this knowledge, very little is known about the effects of $E_2\beta$ metabolites on endothelial PGI_2 levels. Furthermore, nothing is known about the roles of the classical ERs on $E_2\beta$ metabolite-induced endothelial PGI_2 levels. It is also unclear if $E_2\beta$ metabolites can alter TXA_2 production and the PGI_2/TXA_2 ratio in endothelial cells and if this is altered in pregnancy.

Thus, we hypothesized that $E_2\beta$ and its metabolites 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 can augment the PGI_2/TXA_2 ratio via ER-α and/or ER-β, through the classical $cPLA_2$-COX-1-PGIS pathway in uterine artery endothelial cells (UAECs) in the pregnant (P-UAECs) compared to the nonpregnant (NP-UAECs) state. Thus, we investigated: 1) the expression of $cPLA_2$, COX-1, PGIS and TXAS in P-UAECs versus NP-UAECs; 2) if treatments with $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4ME_2 can increase the PGI_2/TXA_2 ratio in pregnant uterine artery endothelial cells (P-UAECs) more than nonpregnant (NP)-UAECs; 3) the role of COX-1 and PGIS in P-UAEC PGI_2 levels; and 4) the roles of ER-α and/or ER-β on PGI_2 levels induced by $E_2\beta$ and its metabolites .

MATERIALS AND METHODS:

Cell Preparation and Culture

Animal use protocols and procedures were approved by the University of Wisconsin-Madison School of Medicine Research Animal Care Committee. Ovine NP-UAECs and P-UAECs were

isolated, validated and cultured from nonpregnant (luteal n=2 and follicular n=2) and late gestation (120 -130 days; term, 147 days; n=4) ewes as previously described.[28, 224, 225] At passage 4, ~70% confluence and serum starved, cells were lysed for Western blotting or transferred to 6-well plates for treatments as needed for respective experiments.

Protein Extraction and Western Immunoblotting

Protein extraction and Western Immunoblot analyses were performed as described previously.[28, 224, 225] $cPLA_2$, COX-1, PGIS and TXAS expressions were detected using mouse anti-$cPLA_2$, rabbit anti-COX-1, rabbit anti-PGIS or rabbit anti-TXAS (1:1000) and respective secondary antibodies (1:2000). β-actin was used as loading control. Positive control was only utilized for the expression of TXAS using human platelet lysates. Human platelet lysates were a generous donation from the Platelet and Neutrophil Immunology Lab, Blood Center of Wisconsin.

Experimental Treatments, Blockade of Enzymes and Receptors

All experiments were performed in quadruplicates and replicated in at least four different P-UAEC (n=4) and NP-UAEC (n=4) preparations. For time and concentration-response studies, P-UAECs in 6-well plates were serum starved (24 hours) in EBM, washed with serum free EBM and the medium was replaced with EBM vehicle (Control) or EBM containing 0.1, 1, 10 or 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, 4-ME_2 or Ca^{2+} Ionophore (A23187; positive control) for 0, 2, 4, 8, 12 or 24 hrs. For nonpregnant versus pregnant concentration-response studies, NP-UAECs and P-UAECs in 6-well plates were serum starved (24 hours) in EBM, washed with serum free EBM and medium was replaced with EBM vehicle (Control) or EBM containing 0.1, 1, 10 or 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, 4-ME_2 or A23187 for 12 hrs based on time course studies. COX-1 or PGIS blockade was performed by pretreating P-UAECs with SC-560 and U-51605 (1μmol/L, 1hr), respectively followed by $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, 4-

ME_2 or A23187 treatments at optimal dose (determined from concentration-response curves). ERs were blocked by pretreating P-UAECs for 1 hour with 1 μmol/L of the nonselective ER antagonist ICI 182,780, ER-α–selective antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyleth oxy)phenol]-1H-pyrazole dihydrochloride (MPP), or the ER-β–selective antagonist 4-[2-phenyl-5,7-bis (trifluoromethyl) pyrazolo[1,5-a]pyrim idin-3-yl]phenol (PHTPP) followed by treatments with EBM vehicle or EBM containing 0.1, 1, 10 or 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, 4-ME_2 or A23187 for optimal time determined from time courses.

PGI_2 and TXA_2 Assays

Following steroid treatments, media from individual 6-well plates were collected to measure production levels of PGI_2 or TXA_2 by using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Indices of both PGI_2 and TXA_2 levels were calculated from measuring their stable non-enzymatic hydrolysis products, 6-keto-PGF1α and thromboxane B_2 (TXB_2) respectively in duplicates. Furthermore, we calculated from other non-enzymatic hydrolysis products, 2, 3-dinor-6-keto-PGF1α. Productions were calculated per manufacturer's instructions after subtracting the value of the "blank" wells to remove background absorbance values. The levels of PGI_2 or TXA_2 in NP-UAECs and P-UAECs are expressed as the fold or ratio increases over untreated control corrected against a standard curve, non-specific binding, maximum binding and normalized to the amount of protein per well .

Statistical Analysis

Data are presented as a fold change of untreated control and expressed as Mean ± SEM. For the PGI_2/TXA_2 ratio, data are presented as the ratio of PGI_2/TXA_2 calculated from the absolute pg/ml concentrations from the same treatment wells in duplicates from all cell lines studied. Data were analyzed using a two-way ANOVA (SigmaPlot 11 Statistical Software). When

appropriate, an analysis of the simple effect was performed using one-way ANOVA followed by post hoc Student-Newman-Keuls test. Pairwise comparisons were performed using Student-Newman-Keuls test. Level of significance was established a *priori* at $P<0.05$.

RESULTS:

Expression of $cPLA_2$, COX-1, PGIS and TXAS in NP-UAECs versus P-UAECs

Western immunoblotting revealed the protein expression of $cPLA_2$, COX-1, PGIS and TXAS in both NP-UAECs and P-UAECs (Figure 1A). Densitometric analyses showed that the protein expressions of $cPLA_2$, COX-1 and PGIS were significantly higher in P-UAECs compared to NP-UAECs (Figure 1B). In contrast, the expression of TXAS was significantly reduced in P-UAECs compared to NP-UAECs (Figure 1B).

Basal and Calcium Ionophore Stimulated PGI_2 and TXA_2 Production in NP-UAECs and P-UAECs

Basal production of PGI_2 was higher by P-UAECs compared to NP-UAECs (Figure 2A). Unstimulated basal production of PGI_2 by P-UAECs at 8, 12 and 24 hours were 1.31 ± 0.23, 1.43 ± 0.23 and 1.53 ±0.23 folds respectively corresponding to 47, 58 and 68 pg/ml respectively at the same time points. A23187 stimulated significant production of PGI_2 in P-UAECs compared to NP-UAECs (Figure 2B). Maximum production by P-UAECs at 12 hours was 10.8 ± 0.39 fold of control compared to maximum production by NP-UAECs of 6.0 ± 0.37 fold of control.

Basal production of TXA_2 by P-UAECs was not different compared to production by NP-UAECs (Figure 2C). Unstimulated basal production of TXA_2 by P-UAECs at 8, 12 and 24 hours were 1.18 ± 0.19, 1.20 ± 0.19, 1.33 ± 0.36 compared to production by NP-UAECs at the same time points. However, A23187 stimulated significantly lower concentration-dependent production of TXA_2 in P-UAECs compared to NP-UAECs (Figure 2D). The maximum

production by P-UAECs at 12 hours was 1.49 ± 0.09 fold of control compared to maximum production by NP-UAECs at 1.93 ± 0.64 fold of control.

$E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 Stimulated a Time and Concentration-Dependent PGI_2 Increase in P-UAECs.

Time- and concentration-dependent PGI_2 increase was observed in P-UAECs after $E_2\beta$ treatment with highest responses observed at a concentration of 100 nmol/L at 12 hours treatment time (Figure 3A). Increase of PGI2 was noted after 4 hours in response to almost all concentration of $E_2\beta$ studied with the exception of 0.1 nmol/L. However, at 4, 8, 12, 24 hours, there were significant differences in PGI_2 levels in response to 10 and 100 nmol/L $E_2\beta$ which were higher compared to 0.1 or 1 nmol/L concentrations.

Similarly, exposure of P-UAECs to 2-OHE_2 and 4-OHE_2 treatments also stimulated a time and concentration-dependent PGI_2 increase by P-UAECs with maximum responses observed at a concentration of 100 nmol/L at 12 hours treatment time (Figure 3B and 3C). Nevertheless, PGI_2 increase was noted after 4 hours in response to all concentration of 2-OHE_2 and 4-OHE_2 studied and this increased in a time- and concentration-dependent manner compared to untreated control. No further increases in 2-OHE_2 and 4-OHE_2 -induced PGI_2 increase by P-UAECs was seen after 12 hours at all concentrations studied. There were significant differences in PGI_2 increase in response to 10 and 100 nmol/L of 2-OHE_2 and 4-OHE_2 which were higher compared to 0.1 or 1 nmol/L concentrations at 2, 4, 8, 12, 24 hours. However, 0.1 or 1 nmol/L concentrations stimulated significant increase of PGI_2 compared to untreated control at 2, 4, 8, 12, 24 hours.

The time and concentration course of PGI_2 levels in P-UAECs induced by 2-ME_2 and 4-ME_2 is shown in Figure 3D and 3E. Both methoxyestradiols increased PGI_2 in a time- and

concentration-dependent manner with maximal effects observed at a concentration of 100 nmol/L at 12 hours treatment time. However, increase of PGI_2 by P-UAECs was seen at all concentrations studied at 2 hours of treatment time or greater. There were significant differences in PGI_2 increase in response to 1, 10 and 100 nmol/L of $2\text{-}ME_2$ and $4\text{-}ME_2$ which were higher compared to 0.1nmol/L concentration at 4 hours of treatment time or greater.

$E_2\beta$, $2\text{-}OHE_2$, $4\text{-}OHE_2$, $2\text{-}ME_2$ and $4\text{-}ME_2$ Stimulated a Greater Concentration-Dependent PGI_2 Increase by P-UAECs more than NP-UAECs

A concentration-dependent increase in production of PGI_2 was observed by P-UAECs after $E_2\beta$ treatment with highest responses observed at a concentration of 100 nmol/L (Figure 4A). On the other hand, $E_2\beta$ also induced PGI_2 increases by NP-UAECs, however, the increase by NP-UAECs was significantly less compared to P-UAECs and no further concentration response was observed after the 10 nmol/L concentration (Figure 4A). Differences in PGI_2 increases between NP-UAECs and P-UAECs were observed at 1 nmol/L, 10 nmol/L, 100 nmol/L and 1 µmol/L concentrations.

Similar to $E_2\beta$, $2\text{-}OHE_2$ and $4\text{-}OHE_2$ treatments also stimulated greater concentration-dependent PGI_2 production increases by P-UAECs compared to NP-UAECs (Figure 4B and 4C). Maximum responses were observed at a concentration of 100 nmol/L with no further increases seen with higher concentrations. $2\text{-}OHE_2$ and $4\text{-}OHE_2$ treatments did stimulate elevations of the production of PGI_2 in NP-UAECs; however, these responses were significantly lower compared to the P-UAEC responses with no further increases seen after the 10 nmol/L concentration.

$2\text{-}ME_2$ and $4\text{-}ME_2$ treatments also stimulated greater concentration-dependent PGI_2 production increases by P-UAECs compared to NP-UAECs (Figure 4D and 4E. Maximum responses of P-UAECs to $2\text{-}ME_2$ was seen at 100 nmol/L with no further increases at greater

doses. On the other hand, 4-ME_2 induced maximum PGI_2 production increases by P-UAECs at a concentration of 10 nmol/L with no significant further increases with higher concentrations. Interestingly, 4-ME_2 was the only estrogen metabolite that stimulated a difference in PGI_2 production responses between NP-UAECs and P-UAECs at a concentration as low as 0.1 nmol/L. PGI_2 production was also noted by NP-UAECs in response to 2-ME_2 and 4-ME_2; however, these responses were of lower magnitude compared to P-UAEC responses with no further increases seen after the 10 nmol/L concentration.

The levels of TXA_2 production stimulated by $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 increased linearly but did not reach statistical significance compared to untreated control in either NP-UAECs or P-UAECs. Therefore, these TXA_2 productions are discussed within the context of the PGI_2/TXA_2 ratio.

The PGI_2/TXA_2 ratio: $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 Stimulated a Greater Concentration-Dependent PGI_2/TXA_2 ratio in P-UAECs more than NP-UAECs

As illustrated in the table 1, the basal PGI_2/TXA_2 ratio was higher in P-UAECs compared to NP-UAECs at 12 hrs treatment time. There was a concentration-dependent increase in the PGI_2/TXA_2 ratio when P-UAECs were exposed to $E_2\beta$ and its metabolites. .

$E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 Stimulated *de novo* PGI_2 Increase in P-UAECs via COX-1 and PGIS.

Antagonism with the COX-1 inhibitor SC-560 and the PGIS inhibitor U-51605 were tested at all concentrations (0.1, 1, 10 and 100 nmol/L) of $E_2\beta$ and its metabolites studied, however since all concentration yielded similar results, only the responses from the optimal concentration of 100 nmol/L at 12 hrs treatment time are shown here. Neither SC-560 nor U-51605 (1μmol/L, 1hr) pretreatments significantly altered basal control PGI_2 increase in P-UAECs (Figure 5A and 5B).

However, both antagonists completely abrogated the PGI_2 production in P-UAECs stimulated by 100 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2, 4-ME_2 and the Ca^{2+} Ionophore (A23187)); (data not shown) indicating that the PGI_2 production seen is *de novo* via the activities of COX-1 and PGIS (Figure 5A and 5B).

$E_2\beta$ but Not its Metabolites Stimulated PGI_2 Production in P-UAECs via the classic ERs

Antagonism with ICI 182,780 was tested at 0.1, 1, 10 and 100 nmol/L of $E_2\beta$ and its metabolites studied, however since all concentration yielded similar results, only the responses from the optimal concentration of 100 nmol/L are shown. Furthermore, since the NP-UAECs exhibited lower non-significant responses, antagonism studies were only carried out in P-UAECs. ICI 182,780 alone did not affect basal PGI_2 production in P-UAECs; however, it totally abrogated PGI_2 production in response to $E_2\beta$, indicating possibility of involvement of either ER-α and/or ER-β (Figure 6). In contrast, ICI 182,780 did not have an effect on P-UAEC PGI_2 production in response to 2-OHE_2, 4-OHE_2, 2-ME_2 or 4-ME_2 indicating ER-independent mechanisms. ICI 182,780 did not have an effect on the PGI_2 production of P-UAECs in response to nonreceptor stimulation with A23187 (data not shown) validating specific ER-mediated $E_2\beta$ responses (Figure 6).

$E_2\beta$ but Not its Metabolites Stimulated PGI_2 Production in P-UAECs Selectively via ER-α and Independent of ER-β

Similar to ICI 182,780 studies, antagonism with MPP and PHTPP were tested at all concentrations (0.1, 1, 10 and 100 nmol/L) of $E_2\beta$ and its metabolites studied; all concentrations examined yielded similar results, therefore only the data from the optimal concentration of 100 nmol/L are shown. In P-UAECs, ER-α blockade with 1 μmol/L of MPP completely abolished the PGI_2 increase stimulated by 100 nmol/L of $E_2\beta$ indicating a role for ER-α (Figure 7A). In

contrast, MPP did not abrogate the PGI_2 production stimulated by 100 nmol/L of 2-OHE_2, 4-OHE_2, 2-ME_2 or 4-ME_2 similar to the above ICI 182,780 (Figure 7A). In contrast, $E_2\beta$-induced PGI_2 production was not inhibited by 1 µmol/L of the ER-β selective antagonist PHTPP demonstrating a lack of requirement for ER-β in these responses (Figure 7B). Similar to MPP, PHTPP did not did not inhibit the PGI_2 production stimulated by 100 nmol/L of 2-OHE_2, 4-OHE_2, 2-ME_2 or 4-ME_2 (Figure 7B). Both MPP and PHTPP did not inhibit PGI_2 production of P-UAECs induced by the nonreceptor stimulation using Ca^{2+} Ionophore (A23187); (data not shown) further validating specific ER-αmediated $E_2\beta$ responses.

DISCUSSION:

The key findings observed from this study are: (1) Pregnancy induces higher protein expression of $cPLA_2$, COX-1 and PGIS in P-UAECs compared to NP-UAECs as well as a lower protein expression of TXAS in P-UAECs compared NP-UAECs; (2) $E_2\beta$ and its metabolites, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2, stimulate time and concentration-dependent increase in PGI_2 in P-UAECs ; (3) $E_2\beta$ and its metabolites stimulate a concentration-dependent increase in the PGI_2/TXA_2 ratio more in P-UAECs compared to NP-UAECs (4) $E_2\beta$ and its metabolites stimulate *de novo* PGI_2 production in P-UAECs via activities of COX-1 and PGIS; and (5) $E_2\beta$-induced PGI_2 production in P-UAECs is mediated primarily via ER-α and independent of ER-β, whereas 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 stimulate PGI_2 production in P-UAECs independent of either ER-α or ER-β.

We demonstrate herein that P-UAECs highly express the prostanoid system enzymes including $cPLA_2$, COX-1 and PGIS. These data are consistent with reports of increased expression of these enzymes in ovine uterine arteries *in vivo* and *ex* vivo[28, 63, 65, 226] and shows that the elevated expression during gestation are maintained to a great extent even through

passaging in culture. However, in the present study we demonstrate, for the first time, that TXAS is expressed in UAECs and that expression is lower in P-UAECs compared to NP-UAECs. Taken together, the data from our studies suggest that during pregnancy, the prostanoid enzyme system shifts its expression pattern in the favor of more PGI_2 production and less TXA_2 production in support of rises and/or maintenance of uterine blood flow. These results demonstrate that P-UAEC PGI_2 production is induced by gestational programming at the level of increased endothelial cell signaling, supporting earlier reports that pregnancy-specific programming in P-UAECs leads to increased basal and agonist-mediated responsiveness.[28] The retention of programming in P-UAECs, perhaps via epigenetic mechanims, may also be responsible for elevated responses to steroids in subsequent pregnancies *in vivo*. We also show herein that the calcium ionophore (A23187) induces significant increases of PGI_2 in P-UAECs and TXA_2 in NP-UAECs demonstrating that the prostanoid enzymes expressed in these endothelial cells are functional and capable of eliciting calcium-dependent and receptor-independent PGI_2 and TXA_2 production.

In this study, we demonstrate that $E_2\beta$ induces concentration-dependent increase of PGI_2 and PGI_2/TXA_2 ratio more in P-UAECs than in NP-UAECs. Although maximum responses are noted at a high concentration of 100 nmol/L, low physiological concentrations of 1 and 10 nmol/L stimulated significant concentration-dependent increase of PGI_2 and PGI_2/TXA_2 ratio more in P-UAECs than in NP-UAECs. In support of these findings are reports that $E_2\beta$ induces production of PGI_2 in other cultured endothelial cells such as HUVECs, bovine pulmonary artery and aortic endothelial cells.[68, 227-229] We have previously reported that the infusion of $E_2\beta$ in sheep significantly increases the *ex vivo* uterine arterial production of the stable PGI_2 metabolite 6-keto-PGF1α.[226] However, to the best of our knowledge, this is the first study to evaluate the *in*

vitro comparison of $E_2\beta$-induced endothelial PGI_2 production and elevated PGI_2/TXA_2 ratio in the pregnant versus the nonpregnant states. In this regard, these data are also consistent with our previous findings that P-UAECs exhibit pregnancy-specific PGI_2 production in response to ATP, basic fibroblast growth factor and epidermal growth factor.[28]

Our findings that 2-OHE_2 and 4-OHE_2 stimulate the increase of PGI_2 and PGI_2/TXA_2 ratio more in P-UAECs than in NP-UAECs supports our hypothesis that CYP450- derived metabolites of $E_2\beta$ may play a role in the regulation of vascular responsiveness during pregnancy. It has been previously demonstrated that the uterine arterial infusion of 2-OHE_2 and 4-OHE_2 in sheep and gilts causes vasodilation and increase in uterine blood flow.[195, 196] 2-OHE_2 and 4-OHE_2 also significantly augment endothelial-dependent vasodilation of preconstricted vascular beds in ZSF1 rats, an animal model for hypertension, Type 2 diabetes, hyperlipidemia, nephropathy, metabolic syndrome.[184, 230] Since 2-OHE_2 and 4-OHE_2 are rapidly converted to their methoxy derivatives in the presence of COMT, it is likely that the actions of the catecholestradiols on endothelial PGI_2/TXA_2 ratio maybe partly modulated by COMT expression and activity in these endothelial cells. Nevertheless, these observations suggest that the metabolism of $E_2\beta$ to the catecholestradiols, 2-OHE_2 and 4-OHE_2, may also play an essential role in the regulation of physiologic vascular responsiveness via production of endothelial-derived vasodilatory factors.

The observation that 2-ME_2 and 4-ME_2 enhance PGI_2 levels and PGI_2/TXA_2 ratio in P-UAECs more than in NP-UAECs supports evidence that methoxyestradiols may positively influence vascular responsiveness during pregnancy. Consistent with these findings is the report that 2-ME_2 induces PGI_2 production in HUVECs.[197] Low 2-ME_2 level has been implicated in preeclampsia, a disease characterized by low plasma and urinary PGI_2 and impaired uterine

blood flow.[231, 232] These observations suggest that 2-ME_2 may be a promising physiological as well as pharmacological agent capable of clinically improving vascular responsiveness. We demonstrate for the first time that, 4-ME_2 also stimulates PGI_2 increases *in vitro* greater in the pregnant compared to the nonpregnant state and indeed may play role in positive pregnancy-induced uterine vascular responsiveness. The potency of both 2-ME_2 and 4-ME_2 in inducing PGI_2 increases at low physiologic concentrations suggests that the methoxyestradiols maybe more potent under these conditions compared to $E_2\beta$ and the catecholestradiols and points to the notion that the vascular effects of locally produced and/or circulating estrogen metabolites may be more critical than previously thought compared to the vascular effects of the parent substrate.

Demonstrating a role for ER-α and/or ER-β, ICI which nonspecifically blocks ER-α and ER-β completely abrogated $E_2\beta$-induced PGI_2 production in P-UAECs. Previous studies have demonstrated that ICI also inhibits $E_2\beta$-induced production of PGI_2 in other cultured endothelial cells including HUVECs and ovine fetal pulmonary artery endothelial cells.[68] However, because of the potential relevance of ER subtype selectivity in vascular function, there is considerable interest in investigating whether the classical ERs exhibit functional heterogeneity in the regulation of $E_2\beta$-induced endothelial functions. Our data shows that $E_2\beta$-induced PGI_2 increases in P-UAECs is completely inhibited by the ER-α-specific MPP and unaffected by ER-β-specific PHTPP demonstrating that $E_2\beta$-induced PGI_2 production in P-UAECs is primarily mediated by ER-α. These data are in agreement with previous observations that PGI_2 production by HUVECs was seen when these cells were treated with an ER-α selective agonist 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT).[68] These data support the notions that whilst $E_2\beta$ binds and activates both ER-α and ER-β, it is the molecular and structural based differences in these

receptors allow for a wide range of functional heterogeneity which may partly explain the selective actions of $E_2\beta$.

In the present study, unlike $E_2\beta$, the effects of its metabolites on PGI_2 production in P-UAECs are not inhibited by the ER antagonists used in this study and thus may not be mediated via ER-α and/or ER-β. This is in agreement with several reports that the vascular physiology and pharmacology of $E_2\beta$ metabolites on many cell types including endothelial cells occur via ER-independent mechanisms.[184, 224, 225] Thus other receptor-mediated mechanisms not involving the classical ERs may mediate $E_2\beta$ metabolites-induced PGI_2 production in P-UAECs. Indeed we and others have reported that the genomic effects of $E_2\beta$ metabolites including proliferation of P-UAECs and suppression of pancreatic islet insulin release are mediated via the adrenergic receptors.[192, 225] Nevertheless, the exact mechanism of action of $E_2\beta$ metabolites on non-genomic PGI_2 increases in P-UAECs remains to be determined and may likely involve adrenergic receptors and/or other estrogen-associated receptors like G-protein-coupled receptor -30.

In conclusion, the findings from this current study demonstrates that the uterine endothelium exhibits pregnancy-specific increases in $cPLA_2$, COX-1 and PGIS and decreases in TXAS shifting in the endothelial PGI_2/TXA_2 ratio toward more PGI_2 production in association with rises and/or maintenance of uterine blood flow during pregnancy. Furthermore, we provide evidence that $E_2\beta$ primarily via ER-α and its metabolites via ER-independent mechanisms stimulate a higher *de novo* increase of the endothelial-derived vasodilator PGI_2 and PGI_2/TXA_2 ratio in the pregnant compared to the nonpregnant state. Although maximum production of PGI_2 and PGI_2/TXA_2 ratio was noted with the high concentration of 100 nmol/L, low physiologically relevant concentrations of 1 and 10 nmol/L of $E_2\beta$ and its metabolites also stimulated significant synthesis of these prostanoids more in P-UAECs compared to NP-UAECs. Collectively, the

selective responses of P-UAECs further illustrate pregnancy specific programming at the level of the uterine artery endothelium signaling resulting in enhanced $E_2\beta$ and its metabolites-mediated induction of PGI_2 synthesis in P-UAECs without significantly affecting TXA_2 production.

Perspectives:

The mechanisms by which estrogens regulate vascular tone and vasuclar responsiveness during pregnancy are not well understood. However, studies have shown that it likely involves ER-mediated stimulation of endothelial-derived vasodilatory factors including nitric oxide and/or PGI_2.[1,11] Herein, we demonstrate novel and compelling evidence that the vasoactive/vasoprotective effects of $E_2\beta$ during pregnancy may also involve its sequential conversion to catecholestradiols and methoxyestradiols which are capable of stimulating ER-independent endothelial PGI_2 synthesis. Additional studies are required to understand whether the ER-independent induction of endothelial PGI_2 synthesis by estrogen metabolites within uterine vasculature represents unappreciated signaling complexity of estrogens or just simply an evolutionary functional redundancy. Nevertheless, our findings necessitate the evaluation of catecholestradiols and methoxyestradiols in the regulation of vascular tone in physiology via endothelial-derived relaxing factors as well as dysregulation in the pathophysiology of vascular diseases such as hypertension, atherosclerosis and gestational vascular diseases such as preeclampsia.

Acknowledgements:

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Novelty and Significance:

What Is New?

- The uterine endothelium exhibits pregnancy-specific increases in phospholipase A_2, cyclooxygenase-1 and prostacyclin synthase and decreases in endothelial thromboxane synthase expression.
- Estradiol-17β primarily via estrogen receptor-α and its metabolites 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol via estrogen receptor-independent mechanisms stimulate higher *de novo* increase of the endothelial-derived prostacyclin/thromboxane ratio in the pregnant compared to the nonpregnant state.

What Is Relevant?

- The selective pregnancy-specific endothelial prostanoid enzyme expressions and increase in prostacyclin/thromboxane ratio demonstrates roles for catecholestradiols and methoxyestradiols in the regulation of vascular responsiveness in physiology via endothelial-derived relaxing factors as well as in the pathophysiology of vascular diseases characterized by blunted endothelium-dependent vasodilator responses such as hypertension, atherosclerosis and preeclampsia.

Summary

- These findings demonstrate novel and compelling evidence that the vasoactive/vasoprotective effects of estradiol-17β may involve its sequential conversion

to catecholestradiols and methoxyestradiols which are capable of stimulating estrogen receptor-independent endothelial prostacyclin synthesis

TABLES AND FIGURES

Table 3.1

Steroid	Cell Type	Steroid Concentration				
		Control	0.1 nmol/L	1 nmol/L	10 nmol/L	100 nmol/L
$E_2\beta$	NP-UAEC	1.49±0.054	1.49±0.057	1.76±0.05*	2.03± 0.13*	2.45 ± 0.01*
	P-UAEC	1.75±0.033†	2.27±0.024*†	4.39±0.02*†	5.45±0.17*†	10.06±0.20*†
2-OHE_2	NP-UAEC	1.46±0.057	1.76±0.050*	2.04±0.12*	2.52±0.076*	2.72±0.022*
	P-UAEC	1.94±0.018†	2.94±0.009*†	4.25±0.01*†	5.75±0.175*†	9.97±0.414*†
4-OHE_2	NP-UAEC	1.49±0.057	1.761±0.05*	2.03±0.10*	2.45±0.040*	2.61±0.016*
	P-UAEC	1.71±0.026†	2.78±0.023*†	4.67±0.01*†	5.76±0.027*†	9.78±0.092*†
2-ME_2	NP-UAEC	1.43±0.066	1.74±0.052*	1.98±0.11*	2.34±0.032*	2.498±0.04*
	P-UAEC	1.84±0.016†	2.83±0.007*†	4.66±0.02*†	6.54±0.045*†	12.59±0.25*†
4-ME_2	NP-UAEC	1.50±0.059	1.74±0.052*	1.98±0.11*	2.37±0.068*	2.529±0.03*
	P-UAEC	1.86±0.011†	2.96±0.010*†	4.67±0.06*†	6.72±0.029*†	11.72±0.15*†

*Increase (P<0.05) in UAEC PGI_2/TXA_2 ratio compared with untreated control. † Increase (P<0.05) in P-UAEC PGI_2/TXA_2 ratio compared to respective NP-UAEC group at specified concentration.

Table 3.1: **The Effects of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 on the PGI_2/TXA_2 Ratio in NP-UAECs versus P-UAECs**. A higher concentration-dependent increase in the PGI_2/TXA_2 ratio was observed in P-UAECs with $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 treatments compared to NP-UAECs. *Increase (P<0.05, n=4) in UAEC PGI_2/TXA_2 ratio compared with untreated control. † Increase (P<0.05, n=4) in P-UAEC PGI_2/TXA_2 ratio compared to respective NP-UAEC group at specified concentration.

Figure 3.1

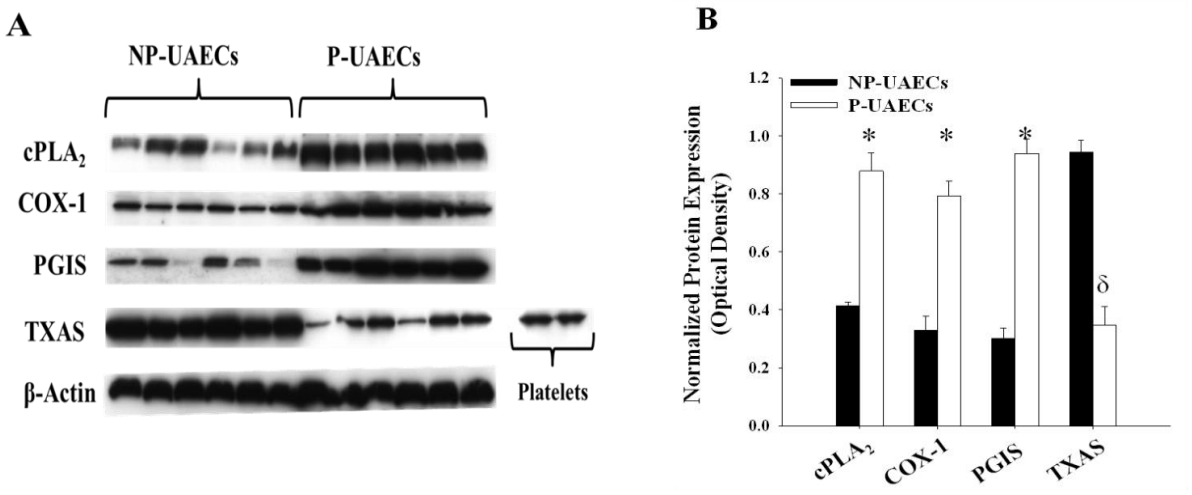

Figure 3.1: **Expression of the Prostanoid System Enzymes in NP-UAECs and P-UAECs.** (A)Western blots demonstrating expression of $cPLA_2$, COX-1, PGIS and TXAS in NP-UAECs and P-UAECs. (B) Densitometric analyses (normalized protein expression = enzyme protein expression optical density/β-actin optical density) showed pregnancy-specific increases of $cPLA_2$, COX-1, PGIS in P-UAECs compared to NP-UAECs whereas TXAS exhibited an decrease in P-UAECs versus NP-UAECs. The positive control lane shows protein expressions of TXAS in human platelet lysates. Blots are representative of duplicate blots and each band represents a different UAEC cell lines. *Increase (P<0.05; n=4) in P-UAEC compared to NP-UAEC. δ Decrease (P<0.05; n=4) in P-UAEC compared to NP-UAEC.

Figure 3.2

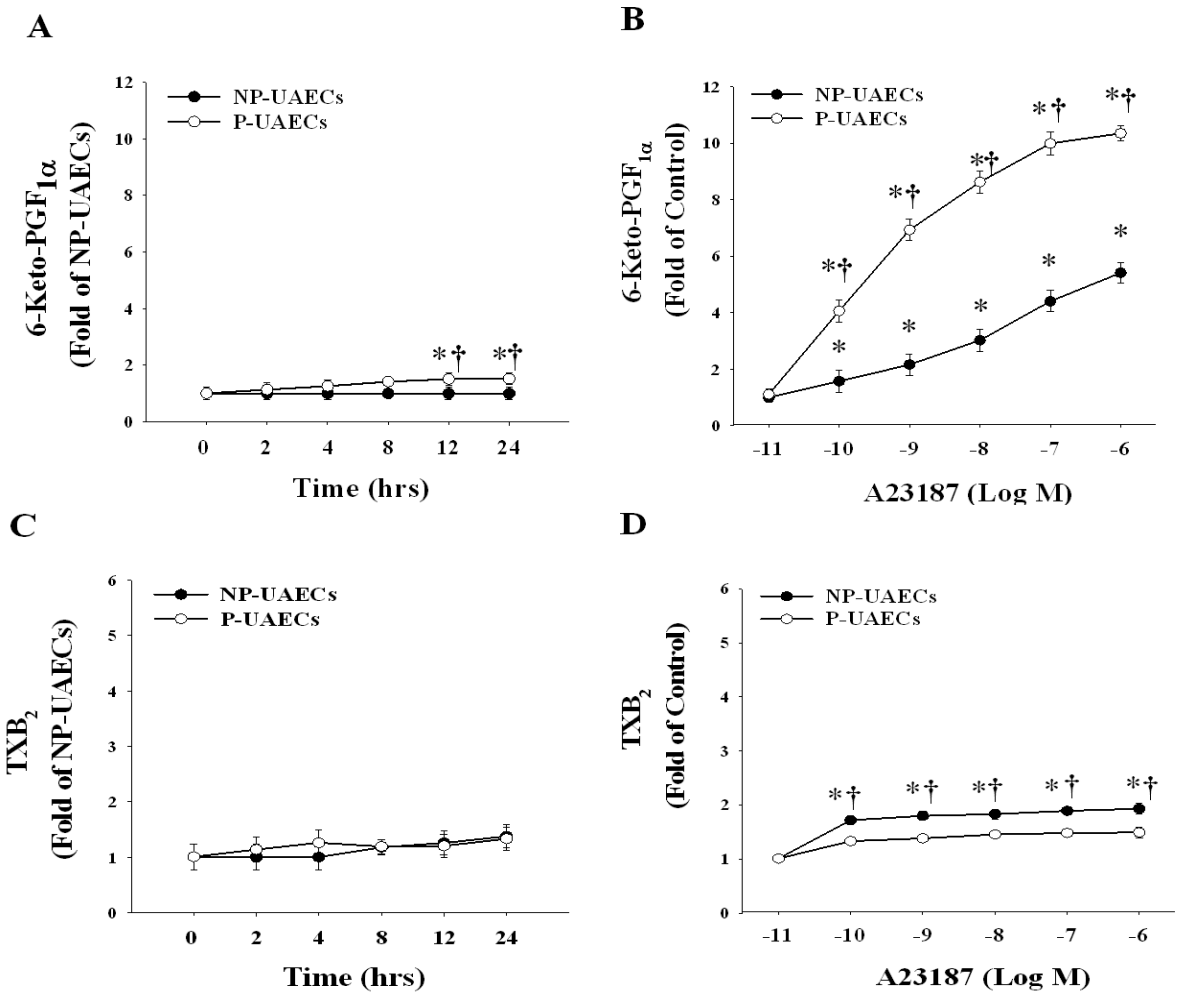

Figure 3.2: **Basal Unstimulated and Calcium Ionophore-Stimulated PGI_2 and TXA_2 Production in NP-UAECs and P-UAECs.** (A) Basal production of PGI_2 was higher by P-UAECs compared to NP-UAECs; (B) The calcium ionophore (A23187) stimulated more production of PGI_2 in P-UAECs compared to NP-UAECs; (C) Basal production of TXA_2 was not different in P-UAECs compared to NP-UAECs; (D) A23187 stimulated more production of TXA_2 in NP-UAECs compared to P-UAECs. *Increase in TXA_2 production (P<0.05; n=4) compared with untreated control and/or time 0. † Increase in TXA_2 production (P<0.05; n=4) compared to respective P-UAEC group at specified concentration.

Figure 3.3

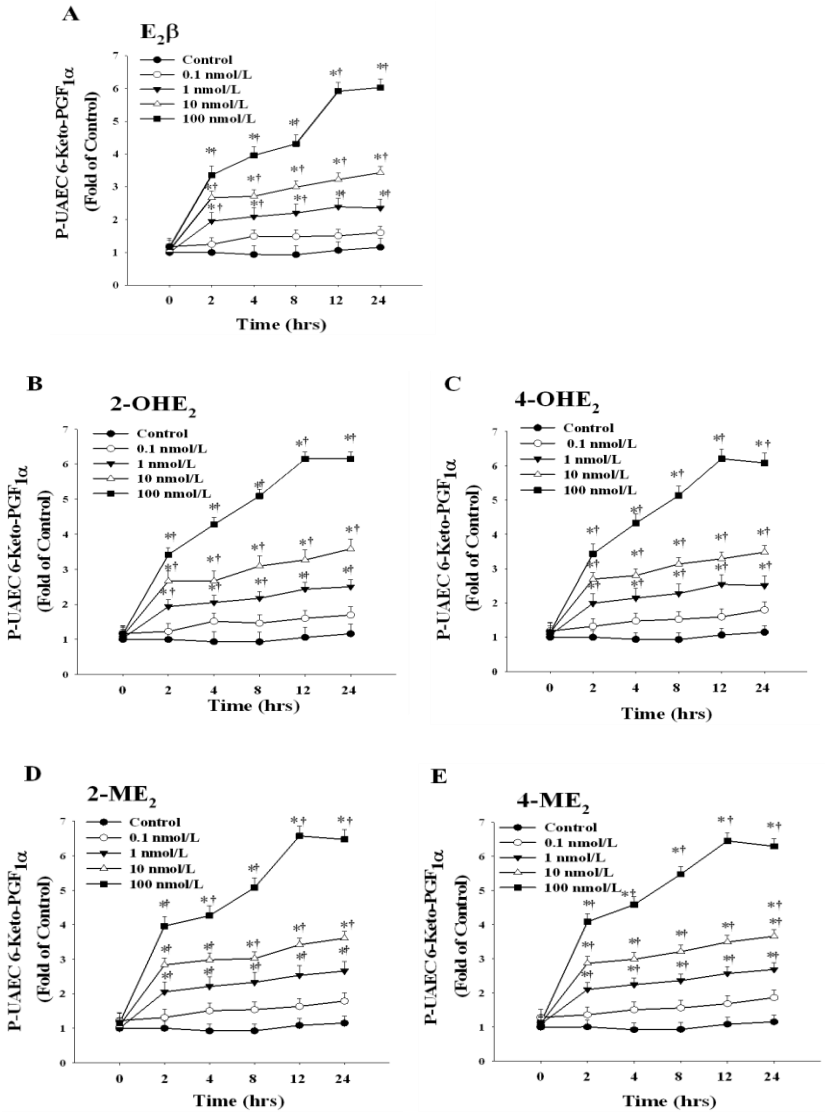

Figure 3.3: **Estradiol-17β and its Metabolites Induced Time and Concentration-Dependent Increases in Prostacyclin Production in P-UAECs.** Time and Concentration-dependent PGI_2 production by P-UAECs in response to (A) $E_2\beta$, (B) 2-OHE_2, (C) 4-OHE_2, (D) 2-ME_2, and (E) 4-ME_2. Time and concentration-dependent PGI_2 production responses were observed in P-UAECs in response to $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2. *Increase (P<0.05; n=4) in P-UAEC PGI_2 production compared with untreated control and time 0. † Increase in P-UAEC PGI_2 production compared to respective 0.1 and 1 nmol/L (P<0.05; n=4) group at specified time.

Figure 3.4

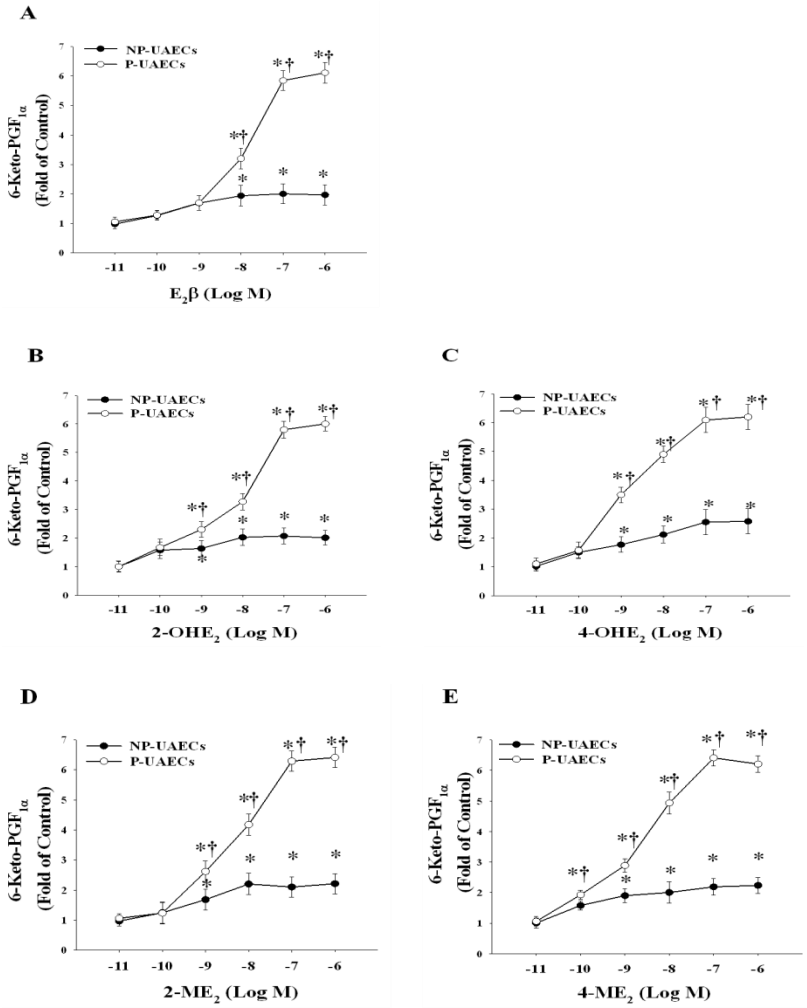

Figure 3.4: **Estradiol-17β and its Metabolites Induced Concentration-Dependent Increases in Prostacyclin Production in P-UAECs compared to NP-UAECs.** Concentration-dependent cell PGI_2 production in NP-UAECs and P-UAECs to (A) $E_2\beta$, (B) 2-OHE_2, (C) 4-OHE_2, (D) 2-ME_2, and (E) 4-ME_2. Concentration-dependent responses at 12 hrs were observed in P-UAECs in response to $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2; NP-UAECs exhibited lower PGI_2 production. *Increase (P<0.05; n=4) in UAEC PGI_2 production compared with untreated control. † Increase in P-UAEC PGI_2 production compared to respective NP-UAEC (P<0.05; n=4) group at specified concentration.

Figure 3.5

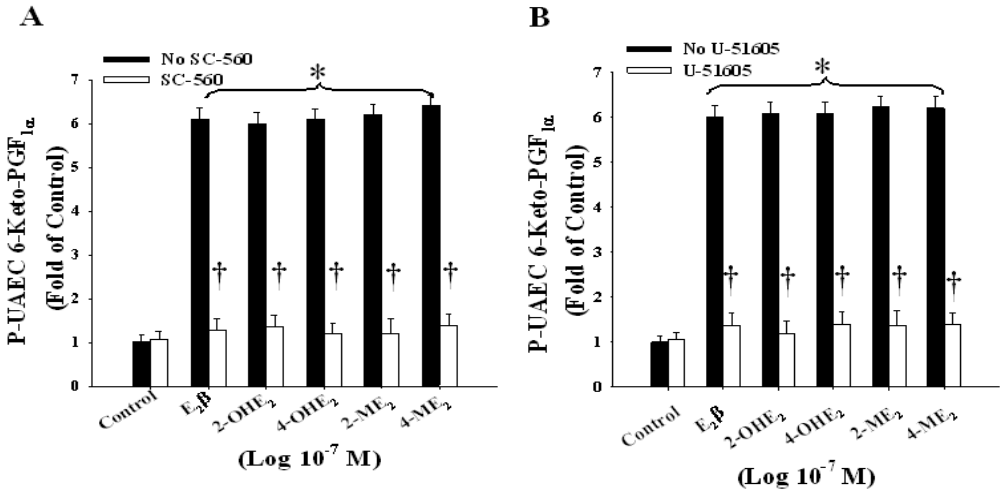

Figure 3.5: **Estradiol-17β and its Metabolites Stimulated *de novo* PGI_2 Production in P-UAECs via COX-1 and PGIS. (A)** The effects of the COX-1 antagonist SC-560 (1 μmol/L) on P-UAEC PGI_2 production to 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. SC-560 abrogated $tPGI_2$ production by P-UAECs in response to $E_2β$ and its metabolites respectively; (B) The effects of the PGIS antagonist U-51605 (1 μmol/L) on P-UAEC PGI_2 production to 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. U-51506 abrogated PGI_2 production by P-UAECs in response to $E_2β$ and its metabolites respectively. *Increase (P<0.05; n=4) in P-UAEC PGI_2 production compared with untreated control. † Indicates inhibition (P<0.05; n=4) of P-UAEC PGI_2 production with inhibitor.

Figure 3.6

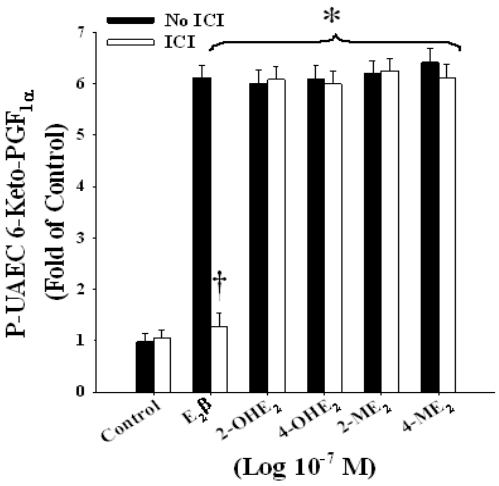

Figure 3.6: **Estradiol-17β but not its Metabolites Stimulated PGI_2 Production in P-UAECs via the classic ERs.** The effects of 1 µmol/L of ICI on P-UAEC PGI_2 production in response to 100 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. ICI abrogated PGI_2 production by P-UAECs in response to $E_2\beta$ but not in response to 2-OHE_2, 4-OHE_2, 2-ME_2 or 4-ME_2. *Increase (P<0.05; n=4) by P-UAEC PGI_2 production compared with untreated control. † Indicates inhibition (P<0.05; n=4) of P-UAEC PGI_2 production with ICI.

Figure 3.7

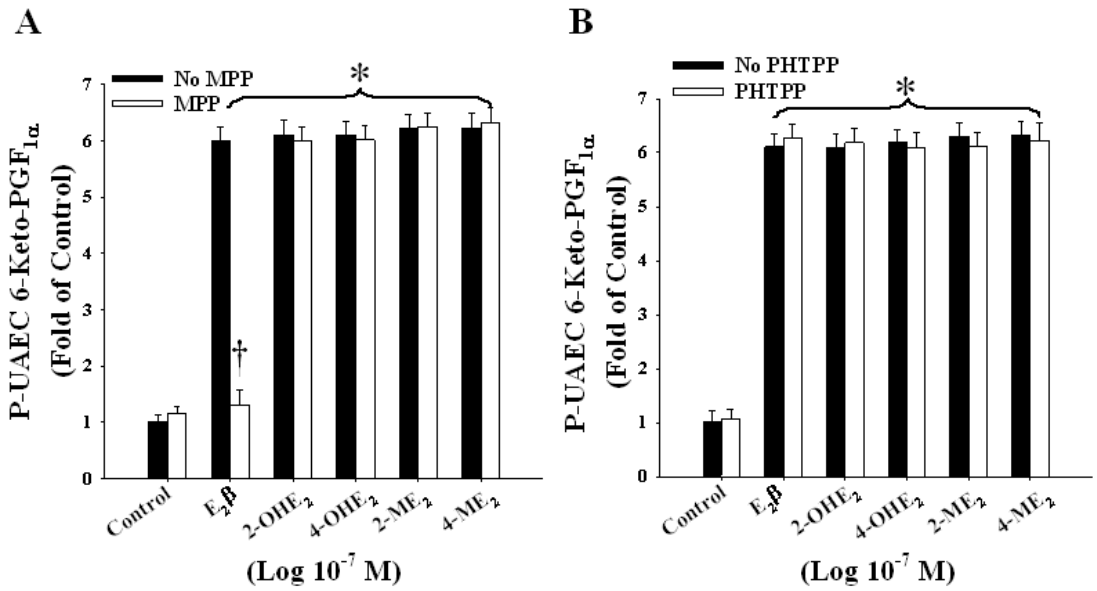

Figure 3.7: **Estradiol-17β but not its Metabolites Stimulated PGI_2 Production in P-UAECs Selectively via ER-α and Independent of ER-β. (A)** The effects of the ER-α antagonist MPP (1 μmol/L) on P-UAEC PGI_2 production to 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. MPP abrogated the PGI_2 production of P-UAECs to $E_2β$ but not in response to its metabolites. (B) The effects of the ER-β antagonist PHTPP (1 μmol/L) on P-UAEC PGI_2 production in response to 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. Neither a main effect of PHTPP nor an interaction was noted. *Increase (P<0.05; n=4) in P-UAEC PGI_2 production compared with untreated control. † Indicates inhibition (P<0.05; n=4) of PGI_2 production by P-UAEC with inhibitor

CHAPTER 4:

THE EFFECTS OF ESTRADIOL-17β AND ESTRADIOL METABOLITES ON UTERINE ENDOTHELIAL PROLIFERATION: ROLE OF ER-α VERSUS ER-β

Estradiol-17βand Its Cytochrome P450- and Catechol-O-Methyltransferase-Derived Metabolites Stimulate Proliferation in Uterine Artery Endothelial Cells: Role of Estrogen Receptor-*α* versus Estrogen Receptor-β

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ABSTRACT

Estradiol-17β and its metabolites which are synthesized by cytochrome P450s (CYP450s) and catechol-*O*-methyltransferase (COMT) to form 2 and 4-Hydroxyestradiol and 2 and 4-Methoxestradiol are elevated during pregnancy. We investigated whether CYP450s and COMT are expressed in uterine artery endothelial cells and if $E_2β$ and its metabolites modulate cell proliferation via classic estrogen receptors ER-α or ER-β and specifically play roles in physiologic uterine angiogenesis during pregnancy. Cultured ovine uterine artery endothelial cells from pregnant (P-UAECs) and nonpregnant (NP-UAECs) ewes were treated with increasing concentrations (0.1-100 nmol/L) of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2 and angiogenesis index evaluated using BrdU Proliferation Assay. Utilizing confocal microscopy and Western analyses to determine enzyme location and levels, we found that CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT were expressed in UAECs; however, expression levels were unaltered between NP- and P-UAECs. $E_2β$, 2-OHE_2, 4-OHE_2, and 4-ME_2 treatment concentration-dependently stimulated proliferation in P-UAECs but not NP-UAECs whereas 2-ME_2 did not stimulate proliferation of either cell type. The cell proliferative responses of P-UAEC to $E_2β$ were solely mediated by ER-β whereas responses to $E_2β$ metabolites were neither ER-α nor ER-β mediated. We demonstrate an important vascular pharmacologic role for $E_2β$, its CYP450- and COMT-derived metabolites and ER-β in uterine angiogenesis regulation during pregnancy that may be dysfunctional in preeclampsia and other cardiovascular disorders.

INTRODUCTION

Pregnancy is associated with dramatic rises in uterine blood flow (UBF) resulting from substantial vascular adaptations including vasodilatation and angiogenesis.[1] These adaptations are critical in pregnancy since their dysfunctions are implicated directly in pathologic pregnancies such as preeclampsia which complicate 6-8% of all pregnancies in the United States and account for 50,000 maternal deaths per year worldwide.[204]

Regulation of vascular adaptations during pregnancy is mediated in part by estrogens, which are significantly elevated during gestation.[1] Infusion of estradiol-17β ($E_2β$) in pregnant sheep markedly reduces uterine and systemic vascular resistance causing a rise in uterine and systemic blood flows.[66] The uterine arterial administration of the nonselective estrogen receptor (ER) antagonist ICI 182,780 in pregnant sheep lowers UBF, demonstrating endogenous estrogen via ERs helps maintain uterine perfusion.[221] $E_2β$ promotes proliferation of human umbilical vein endothelial cells (HUVECs), an index of angiogenesis.[233] Further, $E_2β$ stimulates proliferation of human myometrial microvascular endothelial cells.[234]

The effects of estrogen on uterine vascular adaptations may be further modulated by its biologically active metabolites. $E_2β$ metabolism catalyzed by cytochrome P450s (CYP450s) and Catechol-*O*-methyltransferase (COMT) produces the catecholestradiols 2-Hydroxyestradiol (2-OHE_2) and 4-Hydroxyestradiol (4-OHE_2), and the methoxyestradiols 2-Methoxestradiol (2-ME_2) and 4-Methoxyestradiol (4-ME_2).[172] Strong evidence supports the involvement of $E_2β$-derived metabolites in pregnancy and in the regulation of angiogenesis. For example, 2-ME_2 and COMT-deficient mice exhibit preeclampsia-like symptoms including impaired angiogenesis and hypertension.[231] Furthermore, treatment with low concentration of 2-OHE_2, 4-OHE_2, 2-ME_2 or 4-ME_2 induces proliferation of cultured HUVECs whereas high concentration of 2-OHE_2 or 2-

ME_2 inhibits proliferation. [198] Thus, CYP450s and COMT might be expressed in the uterine vasculature and $E_2\beta$ and its metabolites may participate in the regulation of uterine artery angiogenesis during pregnancy.

In this study, we tested the hypothesis that $E_2\beta$, its CYP450s and COMT-derived metabolites participate in the regulation of "physiologic" uterine angiogenesis during pregnancy. Ovine uterine artery endothelial cells (UAECs) from late pregnant (P-UAECs) and nonpregnant (NP-UAECs) ewes consistently express ER-α and ER-β and exhibit pregnancy-specific responses to the angiogenic ligands demonstrating that they are a good model to evaluate direct receptor mediated actions of $E_2\beta$ and its metabolites on the endothelium. [52, 235] The objectives for this study were to investigate: 1) the expression and intracellular distribution of CYP450s and COMT in P-UAECs compared to NP-UAECs; 2) whether $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 stimulate proliferation of P-UAECs greater than in NP-UAECs, utilized here as an *in vitro* index of angiogenesis; and 3) examine if $E_2\beta$ and its metabolites induce proliferative responses via ER-α and/or ER-β.

MATERIALS AND METHODS

Cell Preparation and Culture:

All cell preparations and culture were approved by the University of Wisconsin-Madison School of Medicine and Public Health Research Animal Care Committee and performed as previously shown. UAECs were isolated and validated from late gestation (P); (120-130 days; term= 147 days; n=6) and nonpregnant (NP; luteal n=5 and follicular n=2) ewes. At passage 5 and ~ 70% confluence, cell were transferred to slides, 96 well plates, or lysed for protein extraction as needed for respective experiments.

Protein Extraction and Western Immunoblotting:

Western immunoblotting was performed as previously described. CYP1A1, CYP1A2, CYP1B1, CYP3A4, COMT and ER-β expressions were detected using mouse anti-CYP1A1, rabbit anti-CYP1A2, rabbit anti-CYP1B1, rabbit anti-CYP3A4, rabbit anti-COMT or rabbit anti-ER-β antibodies. GAPDH was utilized as a loading control.

Immunofluorescence Confocal Microscopy:

Immunofluorescence confocal microscopy was performed as previously described.[14] Cells were 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% Triton-X for 3 min, blocked for 30 min with goat serum, incubated with primary antibodies for CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT expressions were detected using the same antibodies as those used for immunoblotting for 20 min. Subsequently, cells were incubated with secondary antibodies Alexa Fluor 488 goat anti-mouse or Alexa Fluor 488 goat anti-rabbit IgGs for 30 min. Scanning was done with a radiance 2100 MP Rainbow confocal/multiphoton laser scan microscope system (Bio-Rad, Hercules, CA).

Experimental Treatments and Blockade and Activation of ER-α and ER-β:

All UAEC cell proliferation experiments were performed in quadruplicates and replicated in at least six NP-UAEC and P-UAEC preparations and n values for each experiment are shown in corresponding figure legends. For concentration response studies, medium was replaced with EBM alone or EBM containing 0.1, 1, 10 or 100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 for 24 hrs. ERs were blocked by pretreating UAECs for 1 hr with 1μmol/L of the pure ER antagonist ICI 182, 780 (ICI), or ER-α selective receptor antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyleth oxy)phenol]-1H-pyrazole dihydrochloride (MPP), or ER-β

selective antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrim idin-3-yl]phenol (PHTPP). Additional concentration response studies were performed using 0, 0.1, 1, 10 or 100 nmol/L of the pure ER-α selective agonist 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) or the ER-β selective agonist 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN). We also studied the effects of 0.1 nmol/L PPT + 0.1 nmol/L DPN and 1 μmol/L PHTPP + 0.1 nmol/L DPN to further evaluate receptor activation, additive effects and specificity of ER-β selective agonist receptor activation, respectively.

BrdU Cell Proliferation Assays:

BrdU label was added for 16 hrs during the 24 hrs of steroid treatment and an *in vitro* index of proliferation was evaluated. Plates were read using Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Results were expressed as fold increases over untreated control after subtracting the value of the blank.

Statistical Analysis:

Data (means ± SEM) were analyzed using a 2-way ANOVA with "pregnancy effect" and "concentration effect" as two "between" factors. When appropriate, analyses of simple effects were performed using one-way ANOVA followed by post-hoc Student-Newman Keuls test. Pairwise comparisons were performed when appropriate using Bonferroni or Student-Newman-Keuls test. Biphasic concentration response (deviation from the standard monotonic sigmoid shape) description was determined by nonlinear regression using the logarithm of the concentration of the agonists against various responses. A level of significance was established *a priori* at $P<0.05$.

RESULTS

CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT are expressed in UAECs:

Western analyses indicated the presence of CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT in NP-UAECs and P-UAECs (Figure 1A). However, no differences were found in their levels of expression between NP-UAECs and P-UAECs (Figure 1B). Confocal microscopy also revealed no difference in intracellular distribution patterns of the enzymes between NP-UAECs and P-UAECs. Therefore, unless noted, P-UAECs images are shown. Expressions of CYP1A1, CYP1A2 and CYP3A4 were localized in cytoplasmic and nuclear compartments of P-UAECs (Figure 2A, B and D). CYP1B1 expression was localized in the nuclear region whereas COMT was localized in the cytoplasmic compartment (Figure 2C, 2E).

P-UAEC proliferation in response to $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2:

A biphasic concentration response was observed in P-UAECs in response to $E_2\beta$ with a maximum proliferation in P-UAECs being observed at the physiologic concentration of 0.1 nmol/L (Figure 3A). In contrast, NP-UAECs did not exhibit a proliferative response to any concentration of $E_2\beta$. Similarly, P-UAECs but not NP-UAECs exhibited a biphasic proliferative response to 2-OHE_2 and 4-OHE_2 (Figure 3B, 3C). The magnitude of P-UAECs proliferation at 0.1 nmol/L of $E_2\beta$, 2-OHE_2 and 4-OHE_2 were 2.07 ± 0.16, 1.79 ± 0.02 and 1.78 ± 0.02 fold of control, respectively.

At any concentration, 2-ME_2 did not stimulate proliferation in either P-UAECs or NP-UAECs (Figure 3D). 4-ME_2 at all concentrations induced proliferation in P-UAECs but not in NP-UAECs (Figure 3E). Proliferation of P-UAECs at the physiologic concentration of 0.1 nmol/L of 4-ME_2 was 1.50 ± 0.16 fold of control (Figure 3E). However, response to 4-ME_2 was not biphasic and the maximum proliferation of 1.74 ± 0.04 fold was observed at a high

pharmacological concentration of 100 nmol/L. An additional validation of cell proliferation utilizing ViaLight Plus Kit (Lonza Inc., Rockland, ME) was performed to confirm increases in total cell numbers after treatment with $E_2\beta$ or its metabolites.

Proliferation of P-UAECs via classic ERs:

Antagonism with ICI was tested at two physiologic concentrations of 0.1 and 1.0 nmol/L $E_2\beta$ and its metabolites; both agonist concentrations yielded similar results. Therefore, only 0.1 nmol/L agonist responses are shown in Figure 4. Treatment with ICI alone had no effect on P-UAEC proliferation. However, ICI pretreatment totally abrogated cell proliferation responses to $E_2\beta$ indicating the requirement of ER-α and/or ER-β (Figure 4). We then tested the effects of ICI on P-UAEC proliferation in response to 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2. None of the estrogen metabolites required either ER-α or ER-β to stimulate proliferation in P-UAECs (Figure 4). These results demonstrated that at 0.1 nmol/L, $E_2\beta$ was more potent than 2-OHE_2, 4-OHE_2 and 4-ME_2 which were equipotent in stimulating P-UAECs proliferation.

Proliferation of P-UAECs occurs via ER-β and is independent of ER-α:

In P-UAECs, blockade of ER-α with 1 μmol/L MPP did not abolish the proliferative effects of $E_2\beta$, 2-OHE_2, 4-OHE_2, or 4-ME_2 ($P= 0.396$); (Figure 5). In contrast, $E_2\beta$-induced proliferation was completely inhibited by 1 μmol/L of the ER-β selective antagonist PHTPP ($P<0.05$; Figure 6). However, PHTPP did not alter the P-UAECs cell proliferative responses to any of the estrogen metabolites.

We further evaluated if the ER-β-mediated cell proliferative responses in P-UAECs are due to an increase in ER-β expression levels between NP-UAECs, P-UAECs and P-UAECs treated with 0.1 nmol/L $E_2\beta$ or its metabolites. As shown in Figure S1 (please see http://hyper.ahajournals.org), ER-β expression was not different among these groups ($P=0.943$).

ER-β activation not ER-α stimulates proliferation in P-UAECs:

Treatment of P-UAECs with PPT did not induce proliferation at any concentration (Figure 7A). In contrast, DPN stimulated cell proliferation by 1.50 ± 0.05 fold of control at all concentrations (Figure 7B). Because these cell proliferative responses were less than that of $E_2\beta$ alone and did not exhibit a concentration-dependent response, we treated with the combination of ER-α selective agonist PPT (0.1 nmol/L) and ER-β selective agonist DPN (0.1 nmol/L) (Figure 7C). No further increase in P-UAEC proliferative response was observed. Moreover, pretreatment with 1 μmol/L PHTPP completely inhibited DPN-induced responses in P-UAECs ($P<0.05$); (Figure 7C).

DISCUSSION

The novel findings observed from this study are: 1) CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT are all expressed in UAECs; 2) $E_2\beta$, 2-OHE_2, 4-OHE_2, and 4-ME_2 stimulate proliferation of P-UAECs, but not that of NP-UAECs; and 3) $E_2\beta$-induced cell proliferative responses are primarily mediated via ER-β whereas $E_2\beta$-derived metabolites proliferative responses are independent of ER-α and ER-β.

We demonstrate for the first time that UAECs constitutively express enzymes that may metabolize $E_2\beta$ to its active hydroxy- and subsequently methoxy- metabolites including CYP1A1, CYP1A2, CYP3A4, CYP1B1 and COMT. Consistent with our findings, are reports showing that CYP450s and COMT are expressed in endothelial cells from other vasculatures including umbilical vein, aorta and coronary artery.[184] To our knowledge, this is the first characterization of the localized intracellular expression of CYP450s in vascular endothelial cells. Our data also support findings that COMT is primarily an intracellular cytosolic enzyme.[213] Although very little is known about the intracellular localization of these CYP450s in

endothelial cells, it is possible that intracellular compartmentalization of these enzymes may be directly associated with enzymatic function.

Physiologic plasma concentration of $E_2\beta$ in women ranges from 0.1-2.2 nmol/L and increases dramatically during pregnancy. Here, we demonstrate that at a concentration of 0.1 nmol/L, $E_2\beta$ stimulates proliferation of P-UAECs, but not that of NP-UAECs. The P-UAECs proliferative response is also similar to the responses of HUVECs and retinal microvascular endothelial cells to estrogenic stimulation.[233] It has also been demonstrated that $E_2\beta$ promotes *in vivo* endometrial endothelial cell proliferation in mice.[21,22] These data are also consistent with our previous findings[23] showing that $E_2\beta$ increases [H^3]-thymidine incorporation and tube formation in UAECs; maximum responses of UAECs to $E_2\beta$ stimulation were seen at a concentration of 1 nmol/L[23] and not 0.1 nmol/L as observed in the current study and NP-UAECs were not evaluated. Collectively, these results demonstrate that P-UAEC responses may be induced by gestational programming at the level of vascular endothelial cell signaling, and support reports that pregnancy-induced programming in P-UAECs leads to increased responsiveness to agonists and these effects are retained in cultured primary cell lines.[15] In contrast, the complete lack of mitogenic response of NP-UAECs is specific to $E_2\beta$ since NP-UAECs show proliferation in response to VEGF, bFGF, EGF, and high (≥5%) serum.[15, 24, 25] However, the mechanistic significance of pregnancy-induced estrogenic programming on physiologic angiogenesis in P-UAECs remains to be elucidated.

Plasma levels of catecholestrogens in pregnant women are 10-fold more than that of nonpregnant.[26] Our finding that low levels of 2-OHE_2 and 4-OHE_2 stimulate proliferation of the P-UAECs, but not that of NP-UAECs also supports the idea that CYP450s- and COMT-derived metabolites of $E_2\beta$ may also play roles in the regulation of uterine angiogenesis during

pregnancy. It has been reported that low concentrations of 2-OHE_2 and 4-OHE_2 stimulate proliferation of HUVECs and direct infusion of 2-OHE_2 into the uterine artery of nonpregnant sheep causes vasodilatation whereas 4-OHE_2 interacts directly with calcium channels to locally increase blood flow in gilts.[13,27,28] Collectively, these findings demonstrate that catecholestradiols 2-OHE_2 and 4-OHE_2 may play roles in pregnancy-induced vascular adaptations.

The *O*-methylation of catecholestradiols to methoxyestradiols is proposed to produce less potent and antiproliferative metabolites of $E_2\beta$.[10] Interestingly, we demonstrated that 4-ME_2 stimulated proliferation of P-UAECs but not NP-UAECs which is consistent with observations that low concentrations of 4-ME_2 stimulate proliferation in HUVECs. In contrast, 2-ME_2 is not a direct mitogen on uterine vascular endothelial cells; supporting numerous earlier reports on its antiproliferative effects .[29,30,31,32] The exact reason for the divergent pattern in proliferative rates between 2-ME_2 and 4-ME_2 is not known. However, 2-ME_2 has been shown to disrupt tubulin polymeration and induce cell cycle arrest in the mitotic phase in endothelial and smooth muscle cells.[33,34] Thus, the differences in the association of 2-ME_2 and 4-ME_2 with the regulators of mitosis, may account for the difference in their proliferative responses.

Administration of the nonselective ER antagonist ICI completely abrogated $E_2\beta$-induced P-UAEC proliferation demonstrating a role for ER-α and ER-β. This supports our previous observations that ICI blocks $E_2\beta$-induced P-UAEC [H^3]-thymidine incorporation and tube formation[23] and $E_2\beta$-induced VEGF-mediated proliferation.[9] However, antagonism of ER-β with PHTPP completely abrogated $E_2\beta$ proliferative effects demonstrating an ER-β mediated effect. Treatment with the ER-α agonist PPT stimulates proliferation of human myometrial microvascular endothelial cells.[35] However, activation of ER-α with PPT did not stimulate proliferation of P-UAECs, whereas ER-β stimulation with DPN induced proliferation. It is

possible that the differences in cell proliferation in response to PPT, DPN or $E_2\beta$ may be due to their distinct differences in affinity for the ERs in association with other co-activators and nature of ER-ligand complexes.[36,37] Nevertheless, inhibition of both $E_2\beta$- and DPN-induced P-UAEC proliferation by PHTPP validates our conclusion that the effects of $E_2\beta$ on P-UAECs are primarily ER-β -mediated and completely independent of ER-α . Importantly, NP-UAECs, P-UAECs and P-UAECs treated with $E_2\beta$ express similar levels of ER-β under these conditions (Figure S1, please see http://hyper.ahajournals.org.) suggesting that the ER-β-mediated $E_2\beta$ effects may not be strictly dependent on ER-β expression levels but perhaps on other gestational-programming factors at the level of P-UAECs signaling.

Unlike $E_2\beta$, the effects of its metabolites on P-UAECs proliferation are not mediated via ER-α or ER-β even though $E_2\beta$ metabolites possess little affinity for the classical ERs.[10,38] These results also indirectly suggests that CYP450s and COMT expressed in the UAECs may not possess high enough enzymatic activity *in vitro* under these conditions to metabolize $E_2\beta$ to hydroxy and methoxy metabolites. $E_2\beta$ metabolites may induce proliferative effects via other estrogen associated receptors like GPR30 found in vascular and uterine endothelial cells.[39,40] Nonetheless, the exact mechanism of action of estrogen metabolites on uterine vascular endothelial cells remains to be determined.

Perspectives

It is well established that $E_2\beta$ and its metabolites possess vascular protective effects on the cardiovascular system.[41,42,43] However, little is known about a possible link between estrogen metabolism and the regulation of uterine angiogenesis during pregnancy. Therefore, understanding the biochemistry of $E_2\beta$ metabolism and the vascular pharmacology of $E_2\beta$ and its metabolites on the uterine endothelium may provide clues for our understanding of normal

pregnancy-associated vascular adaptations and the dysfunction of endothelia in the pathophysiology of preeclampsia and other cardiovascular disorders.[3,4]

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TABLES AND FIGURES

Figure 4.1

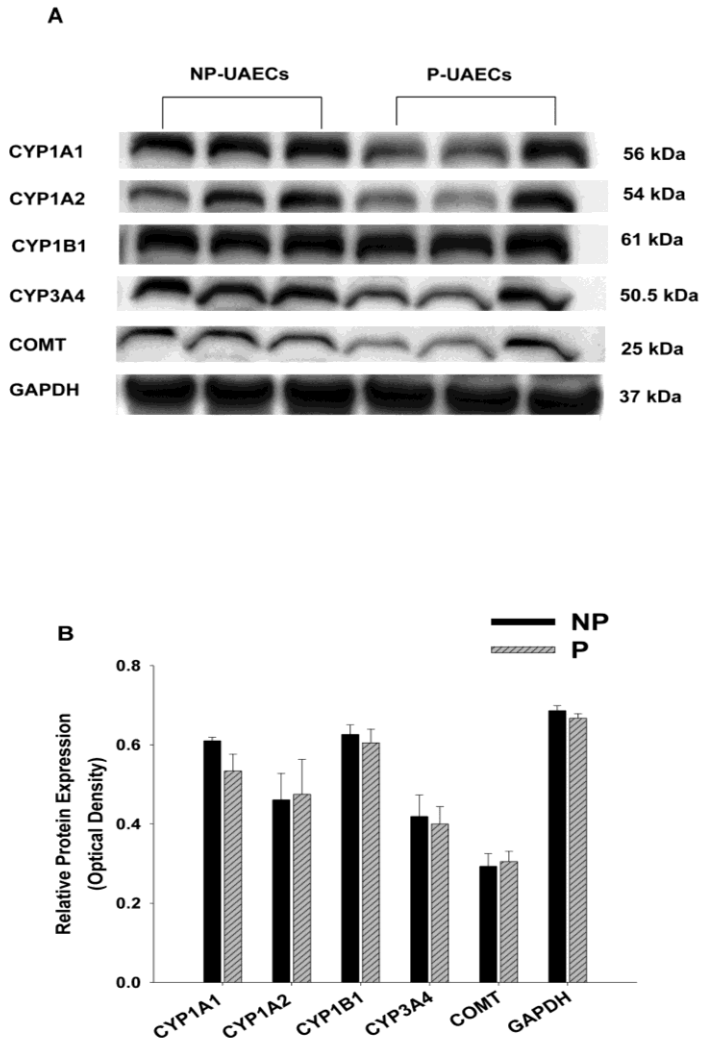

Figure 4.1: (A) Immunoblot showing expression of CYP1A1, CYP1A2, CYP1B1, CYP3A4, COMT and GAPDH (loading control) in NP-UAECs and P-UAECs. (B) Densitometric analyses (normalized protein expression = enzyme expression optical density /GAPDH optical density) showed no difference between NP-UAECs (n=6) and P-UAECs (n=6); (P=0.949, One-Way ANOVA).

Figure 4.2

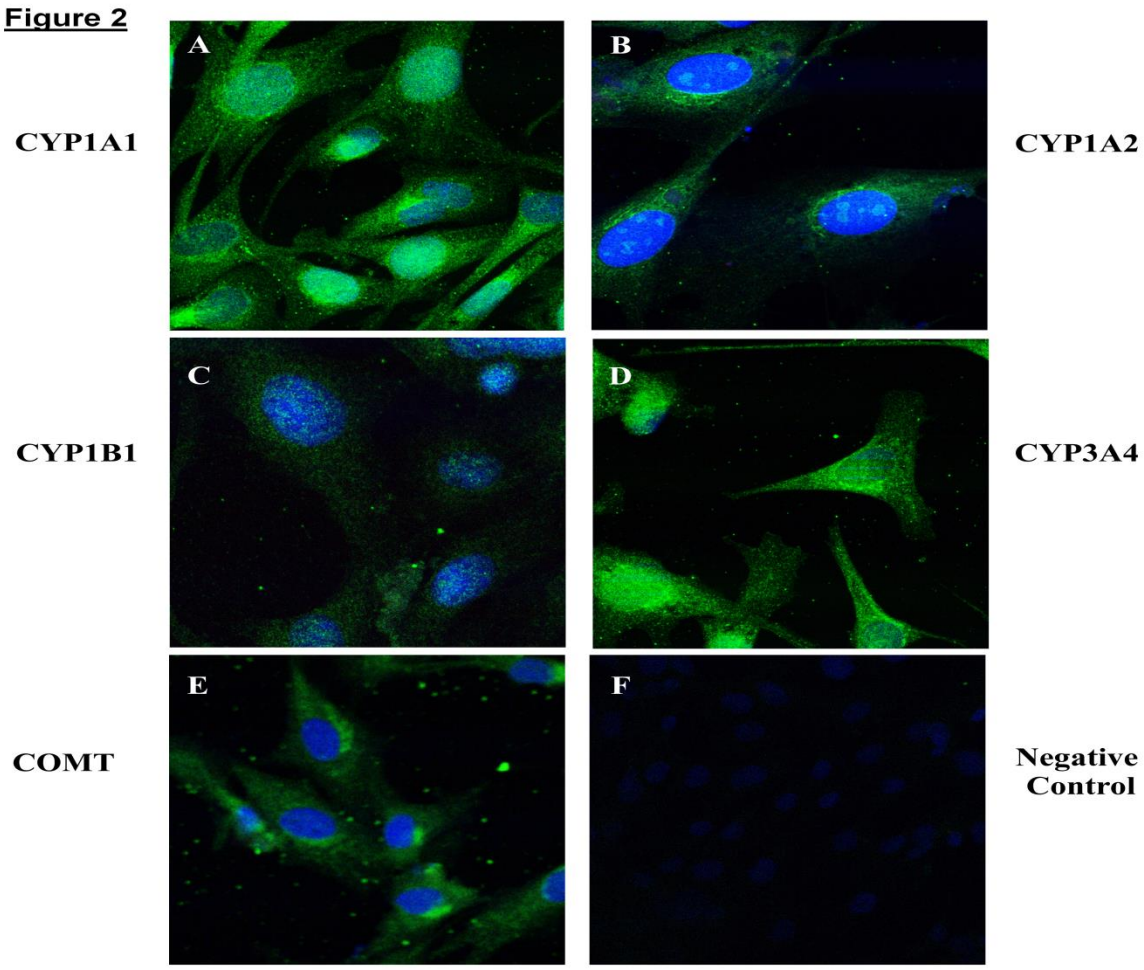

Figure 4.2: Immunofluorescence microscopy demonstrating the spatial intracellular localization of (A) CYP1A1, (B) CYP1A2, (C) CYP1B1, (D) CYP3A4 and (E) COMT in P-UAECs. Positive staining is green fluorescence with nuclei depicted in blue (DAPI). Negative controls showed no fluorescence labeling (F). Pictures are representative of three experiments.

Figure 4.3

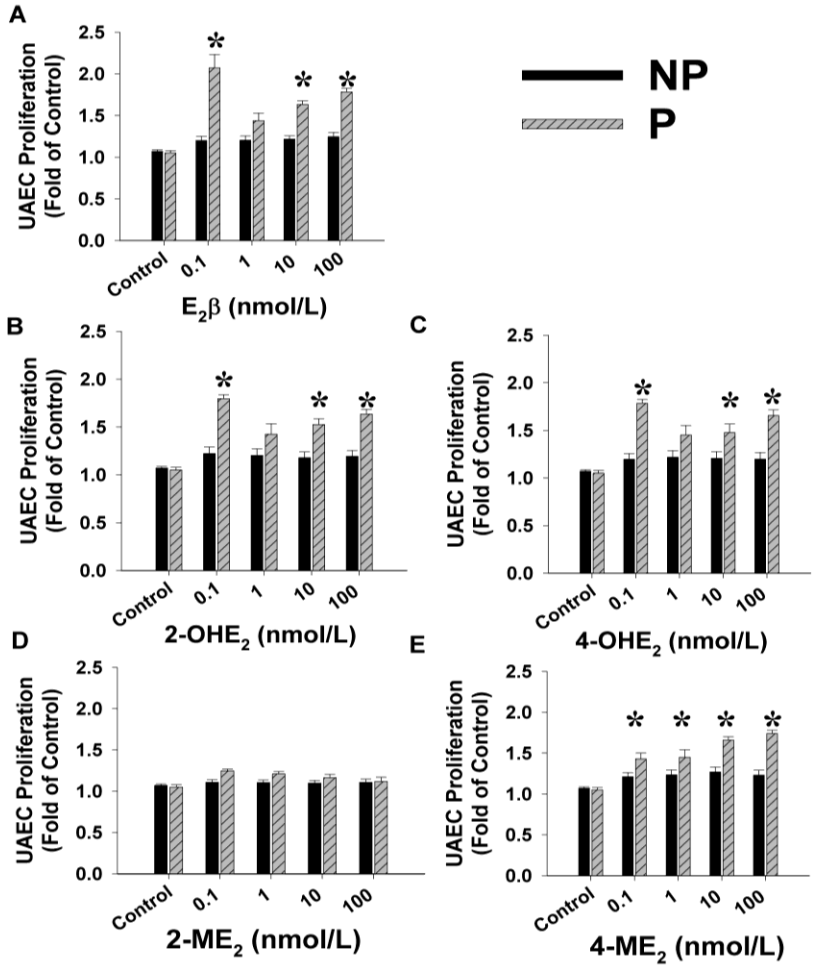

Figure 4.3: Concentration-dependent cell proliferation responses of NP-UAECs and P-UAECs to (A) $E_2\beta$, (B) 2-OHE_2, (C) 4-OHE_2, (D) 2-ME_2 and (E) 4-ME_2. A biphasic proliferative response was observed in P-UAECs in response to $E_2\beta$, 2-OHE_2, and 4-OHE_2 but not 4-ME_2 compared to control with maximum responses at a physiologic concentration of 0.1 nmol/L (2-way ANOVA; Pregnancy x Concentration interaction effect; $E_2\beta$, $F_{4,40}=8.16$, $P<0.0001$; 2-OHE_2, $F_{4,40}=4.07$, $P=0.0073$;, 4-OHE_2, $F_{4,40}=3.69$, $P=0.0119$; and 4-ME_2, $F_{4,40}=5.05$, $P=0.002$). NP-UAECs did not respond to $E_2\beta$ or its metabolites. No proliferation effect was observed with $2ME_2$. *Indicates an increase ($P<0.05$; n=6) in P-UAEC proliferation compared with both the respective NP-UAEC (n=7) group and untreated control.

Figure 4.4

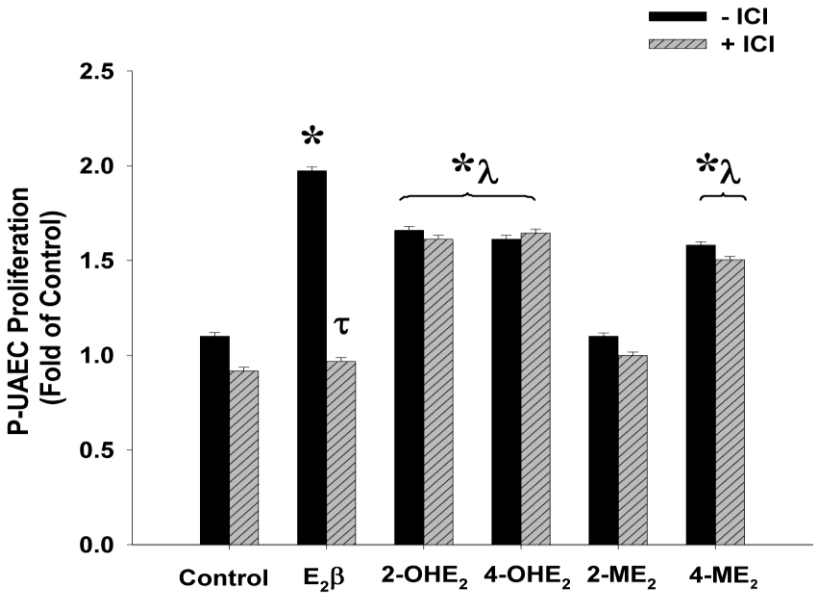

Figure 4.4: The effects of 1 μmol/L ICI pretreatment on P-UAECs proliferation responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2. ICI abrogated the response of P-UAECs to $E_2\beta$ but not in response to 2-OHE_2, 4-OHE_2 and 4-ME_2 respectively (2-way ANOVA; antagonist x group interaction effect; $F_{5,60}$=25.272, P<0.001.*Indicates an increase (P<0.05, n=6) in P-UAEC proliferation compared to untreated control. τ indicates a complete inhibition (P<0.05) of P-UAEC proliferation in the presence of ICI; λ indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ alone responses.

Figure 4.5

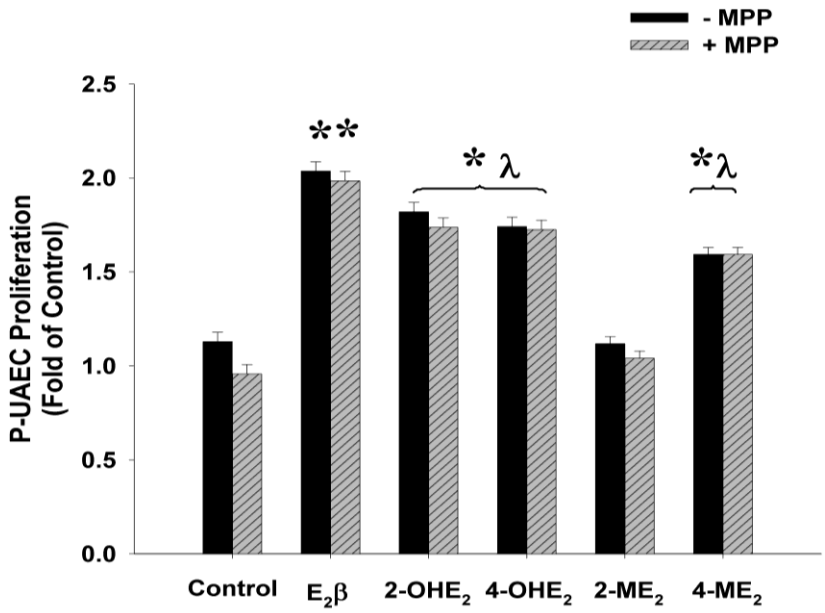

Figure 4.5: The effects of the ER-α antagonist MPP (1μmol/L) pretreatment P-UAECs proliferation responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2. MPP had no effect on the proliferation responses of P-UAECs to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 (2-way ANOVA, group effect, $F_{5,60}$=14.315, P<0.001). Neither a main effect of the antagonist nor an interaction was noted. *Indicates an increase (P<0.05; n=6) in P-UAEC proliferation compared to untreated control; λ indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ alone responses.

Figure 4.6

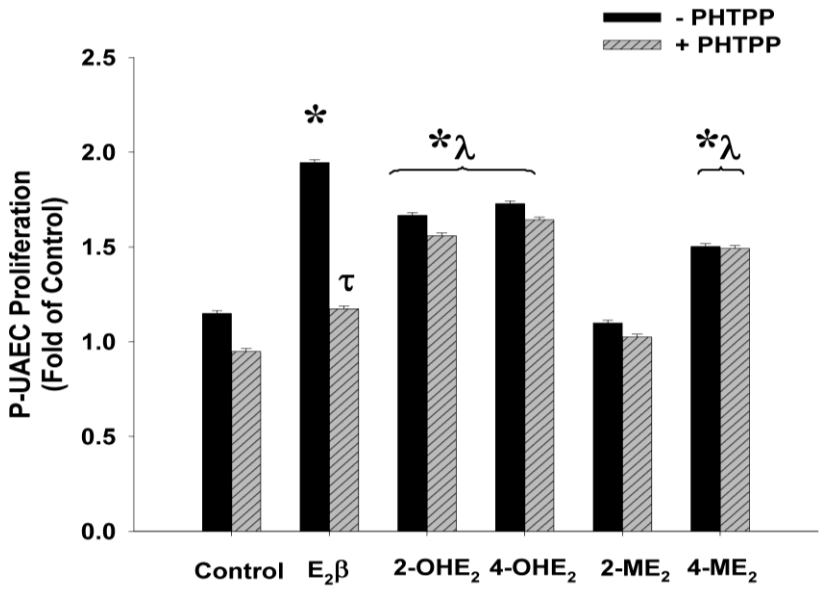

Figure 4.6: The effects of the ER-β antagonist PHTPP (1μmol/L) pretreatment on P-UAECs proliferative responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 (2-way ANOVA; antagonist x group interaction effect; $F_{5,60}$=17.517, P<0.001. *Indicates an increase (P<0.05; n=6) in P-UAEC proliferation compared to untreated control. τ indicates a complete inhibition (P<0.05) of P-UAEC proliferation in the presence of PHTPP. λ indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ alone responses.

Figure 4.7

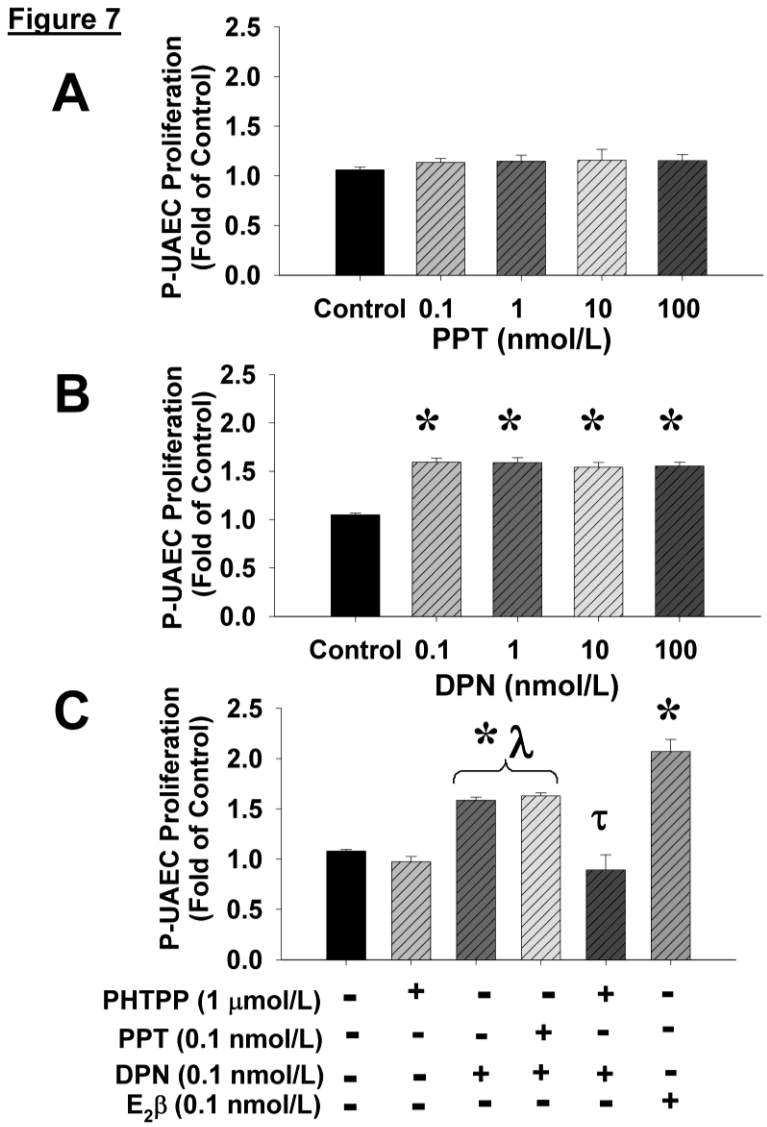

Figure 4.7: Concentration-dependent effects of (A) ER-α agonist PPT or (B) ER-β agonist DPN and (C) their combination on cell proliferation responses of P-UAECs. Blockade of ER-β with PHTPP (1μmol/L) pretreatment prior to treatment with ER-β agonist DNP is shown in (C). * Indicates an increase ($P<0.05$; n=7) in P-UAEC proliferation compared to untreated controls. λ indicates a difference ($P< 0.05$) in P-UAEC proliferation in response to DPN or the combination of DNP and PPT compared to $E_2\beta$ responses. τ indicates complete inhibition ($P<0.05$) of P-UAEC proliferation in the presence of PHTPP.

CHAPTER 5:

THE EFFECTS OF CATECHOLAMINES AND ADRENERGIC RECEPTORS ON ESTRADIOL-17β AND ESTRADIOL METABOLITES-INDUCED UTERINE ENDOTHELIAL PROLIFERATION

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

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ABSTRACT

Sequential conversion of estradiol-17β to its biologically active catecholestradiols 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) contributes importantly to its angiogenic effects on uterine artery endothelial cells derived from pregnant (P-UAECs), but not nonpregnant (NP-UAECs) ewes via estrogen receptor-independent mechanism. Because catecholestradiols and catecholamines exhibit structural similarities and have high affinity for α- and β-adrenergic receptors (ARs), we investigated if the endothelial α- or β-ARs mediate catecholestradiols-induced proliferation of P-UAECs and whether catecholamines alter these responses. Western analyses revealed expression of specific AR subtypes in NP-UAECs and P-UAECs including α_2-, β_2- and β_3-ARs; not α_1- and β_1-ARs. Levels of β_2-ARs and β_3-ARs were unaltered by pregnancy; whereas α_2-ARs were decreased. Norepinephrine and epinephrine increased P-UAEC, but not NP-UAEC proliferation and these effects were suppressed by propranolol (β-AR blocker) but not phentolamine (α-AR blocker). Catecholamines combinations with 2-OHE_2 or 4-OHE_2 enhanced P-UAEC mitogenesis. Catecholestradiol-induced P-UAECs proliferation was also inhibited by propranolol but not phentolamine. β_2-AR and β_3-AR antagonists (ICI 118,551and SR 59230A respectively) abrogated the mitogenic effects of both 2-OHE_2 and 4-OHE_2. Stimulation of β_2-ARs and β_3-ARs using Formoterol and BRL37344 dose-dependently stimulated P-UAEC proliferation which was abrogated by ICI 118,551and SR 59230A, respectively. Proliferation effects of both catecholamines and catecholestradiols were only observed in P-UAEC (not NP-UAEC) and were mediated via β_2-ARs and β_3-ARs. We demonstrate for the first time convergence of the endothelial AR and estrogenic systems in the regulating endothelial proliferation, thus providing a distinct evolutionary advantage for modulating uterine perfusion during stressful pregnancies.

INTRODUCTION

Estradiol-17β ($E_2β$) has physiologic/pathophysiologic effects on the cardiovascular system via diverse mechanisms including local conversion to catecholestradiols 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2 by cytochrome P450s (CYP450s).[172, 184] Catecholestradiols improve endothelial function and alleviate hypertension in ZSF1 rats; animal model for hypertension and metabolic syndrome.[230] We reported that $E_2β$, 2-OHE_2 and 4-OHE_2 increase proliferation in ovine uterine artery endothelial cells derived from pregnant (P-UAECs) but not the nonpregnant ewes (NP-UAECs).[224] Unlike $E_2β$, 2-OHE_2 and 4-OHE_2 induced P-UAEC proliferation were not blocked by ICI-182,780 demonstrating that catecholestradiols induce P-UAEC proliferation was estrogen receptor (ER)-independent.[204]

Catecholestradiols exhibit close structural similarities (Figure 1) and functional interactions with the norepinephrine and epinephrine.[186, 236] Owing to the shared phenolic A ring "catechol" moiety, catecholestradiols interact directly with catecholamine responses, a property not shared by $E_2β$.[236] Catecholestradiols compete with high affinity for binding to neuroendocrine enzymes and α-adrenergic receptors (ARs) and β-ARs in hypothalamus, anterior pituitary, corpus striatum and liver.[194, 237, 238] 3D structural and functional analyses demonstrate that catecholamines "catechol" moiety is functionally important in AR activation.[239, 240] Thus, it is conceivable that the ER-independent mitogenic effects of 2-OHE_2 and 4-OHE_2 on the uterine endothelium may be mediated by α-ARs or β-ARs and they may directly interact with the catecholamines which endogenously activate ARs. Nevertheless, there are no reports on the potential role of an endothelial AR system in catecholestradiol-induced proliferation.

We hypothesized that catecholestradiols stimulate P-UAECs proliferation via α-ARs and/or β-ARs and that catecholamines which directly activate these ARs will alter these

responses. We investigated: 1)NP-UAEC versus P-UAEC subtype specific expression of α_1-, α_2-, β_1-, β_2- and β_3-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 catecholestradiols and catecholamines stimulate P-UAEC proliferation via α-ARs or β-ARs; 5) if ARs exhibit subtype specificity in catecholestradiol-induced P-UAEC proliferation; and 6) subtype specific pharmacological activation of ARs on P-UAEC proliferation. We report for the first time specific β_2-AR and β_3-AR subtype-mediated mechanisms for the novel convergence of the endothelial AR system with estrogen metabolites in regulating P-UAEC not NP-UAEC proliferation. We discovered in P-UAECs specific a co-dependence via conserved catechol phenolic moieties (derivatives of both $E_2\beta$ and tyrosine) of a unique β-AR coupled system. This may provide for a distinct gestational evolutionary advantage by modulating uterine angiogenesis to help/maintain fetal developmental well-being during periods of repeated bouts of stress releases of catecholamines as seen during "fight or flight" responses.

MATERIALS AND METHODS

For complete details, please see http://hyper.ahajournals.org.

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 blotting or transferred to 96-well plates for experimental treatments.

Western Analyses

Western Analyses were performed using rabbit anti-α_1-AR, anti-α_2-AR, anti-β_1-AR, anti-β_2-AR,

or anti-β_3-AR antibodies (1:500) and secondary antibodies (1:2000). β-actin/GAPDH used as loading controls.

5-Bromodeoxyuridine Cell Proliferation Assays

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

Experimental Treatments

Proliferation experiments were performed in quadruplicates and replicated in ≥ four preparations. NP-UAECs and P-UAECs in 96-well plates were serum starved (24 hours) and washed in endothelial basal media (EBM) and medium was replaced with EBM containing 0, 0.1, 1, 10 or 100 nmol/L norepinephrine or epinephrine (24 hours). Additional P-UAEC studies investigating interactions were performed by combination treatments (0.1 nmol/L) of 2-OHE_2 or 4-OHE_2 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) propranolol followed by norepinephrine, epinephrine or 2-OHE_2 or 4-OHE_2 (0.1 nmol/L; 24 hours). Based on Western analyses of specific AR subtypes, we conducted AR subtype specific blockade using (10 μmol/L; 1 hour) yohimbine (α_2-AR), ICI 118,551(β_2-AR) and SR 59230A (β_3-AR) followed by catecholestradiol treatments (0.1 nmol/L; 24 hours). We validated AR-subtype specific inhibition for P-UAEC mitogenic responses (0, 0.1, 1, 10, 100 nmol/L) using specific β_2-AR agonist Formoterol and β_3-AR agonist BRL 37344. ICI 118,551 and SR 59230A (1 μmol/L) effects respectively on Formoterol and BRL 37344 (100 nmol/L) for specificity validation of β_2-AR and β_3-AR agonists in P-UAECs. For antagonists/agonist specificities, see http://hyper.ahajournals.org.

Statistical Analysis

Data as Means ± SEM are presented as a fold of untreated control. Overall group differences

were determined by one-way or two-way 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 Adrenergic Receptor Subtypes

Western immunoblotting showed α_2-AR, β_2-AR and β_3-AR not α_1-AR or β_1-AR subtypes in P-UAECs (Figure 2A). Positive controls show protein expressions of α_1-ARs, α_2-ARs, β_1-ARs, β_2-ARs and β_3-ARs in vascular smooth muscle cells (VSM), left ventricular cardiomyocytes (CMC) and adipose tissue (AT), thus validating the absence of α_1-ARs or β_1-ARs. Western analyses and densitometric analyses (data not shown) showed the equal expression of β_2-ARs and β_3-ARs between NP-UAECs and P-UAECs; however, α_2-ARs were higher in NP-UAECs versus P-UAECs.

Catecholamines Increase P-UAEC but not NP-UAEC Proliferation and Increase Catecholestradiol-Induced of P-UAECs Proliferation

Neither norepinephrine nor epinephrine stimulated in NP-UAECs 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 physiologic concentrations (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 was 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 were similar to catecholestradiols (1.86± 0.02-, vs. 1.81± 0.02-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 either 2-OHE_2 (2.22 ± 0.07- and 2.24±0.07-fold, respectively) or 4-OHE_2 (2.15±0.07- and 2.23±0.07-fold, respectively).

β-ARs but not α-ARs Mediate Catecholamine and Catecholestradiol-Induced P-UARC Proliferation

Phentolamine and propranolol antagonism (10μmol/L) of P-UAEC proliferation using both 0.1 (Figure 4A) and 100 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 α-ARs using phentolamine. In contrast, nonselective blockade of β-ARs using propranolol completely abrogated catecholamine mediated P-UAEC proliferation ($P < 0.05$).

Confirming our previous report,[4] the magnitude of proliferation of P-UAECs in responses to 2-OHE_2 (1.89±0.02-fold), and 4-OHE_2 (1.88±0.02-fold) were similar (Figure 4B). Increases in P-UAEC proliferation seen with 0.1 nmol/L 2-OHE_2 and 4-OHE_2 were unaltered ($P >0.05$) by nonselective 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 $E_2\beta$-induced P-UAEC proliferation, we evaluated these α-AR and β-AR antagonists on $E_2\beta$-induced proliferation of P-UAECs. The $E_2\beta$-induced rise (0.1 nmol/L) in P-UAEC proliferations was not altered ($P > 0.05$) by either phentolamine or propranolol.

β_2-ARs and to a Lesser Extent β_3-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 α_2-AR subtype with Yohimbine did not inhibit 2-OHE_2 and 4-OHE_2-induced P-UAEC proliferation. In contrast, the selective antagonists of β_2-AR (ICI 118,551) or β_3-AR (SR 59230A) respectively either blocked ($P < 0.01$) or only partially attenuated ($P<0.05$) the proliferation induced by 0.1 nmol/L 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 β_2-ARs. In contrast combination of SR59230A and yohimbine only partially decreased catecholestradiol-induced P-UAEC proliferation demonstrating only partial β_3-AR subtype involvement. These combination inhibitor studies neither support dimerization nor significant cross talk between these AR subtypes.

Stimulation of β_2- and β_3-ARs Promote P-UAEC Proliferation

To further evaluate β_2-ARs vs. β_3-ARs, we tested the actions of specific β-AR agonists. Both of the subtype specific β_2-AR (Formoterol) and β_3-AR agonist (BRL 37344) 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- 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 β_2-AR (ICI 118,551) but not β_3-AR (SR 59230A) antagonist. β_3-AR antagonist completely abrogated responses by BRL 37344, not by Formoterol.

DISCUSSION

Recently we reported that unlike their parent substrate hormone $E_2\beta$, 2-OHE_2 and 4-OHE_2 do not stimulate P-UAEC proliferation via classical ERs.[4] 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 are responsible for catecholestradiol- and catecholamine-mediated proliferation, a critical process for angiogenic-mediated uterine perfusion during gestation. These data provide a novel previously not described model by which estrogen metabolites function as potential circulating β-AR mimetic agonists. Therefore, modifying phenolic A ring of estrogens to a "catechol" moieties generate an endogenous β-mimetic agent with angiogenic and possibly other cardiovascular capabilities. Our key findings are that: 1) in NP-UAECs and P-UAECs there are distinct AR subtypes expressed including α_2-ARs, β_2-ARs and β_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 P-UAEC proliferation; 3) neither catecholestradiols nor catecholamines induce P-UAEC proliferation α-ARs, but rather solely via β-ARs; 4) 2-OHE_2 and 4-OHE_2 modulate P-UAEC proliferation primarily via β_2-ARs and to a lesser-extent via β_3-ARs; and 5) pharmacologic agonists for either β_2-ARs or β_3-ARs specifically

stimulate P-UAEC proliferation suggesting similar coupling mechanisms and/or signaling convergence.

We report, for the first time, *in vitro* expressions of several specific AR subtypes α_2-ARs, β_2-ARs, and β_3-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.[241-243] When compared to NP-UAECs, β_2-AR and β_3-AR expressions were unaltered by pregnancy status, whereas α_2-ARs were reduced. It is unclear whether co-expression 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 β_2-ARs are abundantly localized in the P-UAEC caveolae domain, a "hub" for compartmentalizing signal transduction for regulation of multiple functions.[244] Therefore, demonstration of specific AR expression relative to the subcellular localization of α_2, β_2 and β_3-ARs in NP-UAECs versus P-UAECs needs to be determined. This may fulfill distinct physiologic and pathophysiologic functional significance for expression relative to localization of multiple AR subtypes in endothelium. .

Since ARs are present on the endothelium, they are undoubtedly exposed to circulating endogenous norepinephrine and epinephrine released from the adrenal medulla. Normal physiologic circulating catecholamine concentrations are 1-2 nmol/L and increase dramatically in pathologic cardiovascular conditions and during "fight or flight" stress responses.[185, 243] Hence, we demonstrated that even at a low physiologic concentration (0.1 nmol/L) of both norepinephrine and epinephrine significantly increases P-UAEC, not NP-UAEC, proliferation suggesting that catecholamines indeed may play roles in regulating physiologic angiogenesis during gestation. Consistent with these finding, catecholamines augment *in vivo* angiogenesis in

dopamine β-hydroxylase knockout mice deficient in plasma catecholamines.[245] Confirming our recent report, a low physiologic concentration (0.1 nmol/L) of 2-OHE_2 and 4-OHE_2 stimulate P-UAEC proliferation.[225] 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 physiologic and/or pathologic angiogenesis during gestation. These data therefore demonstrate that catecholamines play a complementary and even an additive role to 2-OHE_2 and 4-OHE_2 as positive β-AR-mediated modulators of physiologic angiogenesis. These data also implied that catecholamines and catecholestradiols should exhibit similar AR-subtype-specific signaling pathways to induce P-UAEC proliferation. Catecholestradiols have been previously shown to competitively bind to AR subtypes in rat cerebral cortex, striatum, and anterior pituitary as well as to guinea-pig hypothalamic membranes.[194, 236] Therefore, our data show that "catechol" moieties of catecholestradiols and catecholamines are very important for the binding and activation of β-ARs signaling.

The lack of alteration of P-UAEC proliferation when the nonspecific α-AR antagonist phentolamine and α_2-AR specific blocker yohimbine was used show that α_2-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 of α_2-ARs regulating endothelial cell proliferation. However, α_2-ARs have been closely associated with nitric oxide signaling in endothelial cells [246], suggesting functional relevance of α_2-AR expression in endothelial-mediated vasodilatation.

Consistent with our novel findings that propranolol abrogated 2-OHE_2 and 4-OHE_2– induce P-UAEC proliferation, are reports showing that stimulation of β-ARs by various

pharmacokinetic compounds stimulate proliferation of endothelial cells.[247, 248] Classically, β-ARs are prototypical G-protein coupled receptors triggering diverse signaling cascades through α, β and γ G-protein subunits, adenylate cyclase, intracellular cAMP and protein kinase A and C.[249] 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 adenylate cyclase, cAMP and protein kinase A and C.[250-252] 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 β_2-AR antagonist ICI 118,551 abolished P-UAEC proliferation stimulated by 2-OHE_2 and 4-OHE_2 suggests β_2-AR coupling whereas the partial inhibition by β_3-AR blocker SR 59230A also implies potential involvement of β_3-ARs. In contrast, both the specific β_2-AR (Formoterol) and β_3-AR (BRL 37344) agonists equally induced P-UAEC proliferation which were 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 since a similar concentration of SR 59230A induced significant abrogation of β_3-ARs in response to BRL 37344. Activation of either β_2-AR and/or β_3-AR have been shown to play a role in endothelial cell proliferation from human umbilical vein, retina and bovine aorta.[247, 248] However, ours is the first report to demonstrate that β_2-AR and β_3-AR mediate the catecholestradiol-induced proliferation of endothelial cells. P-UAECs express similar levels of β_2-ARs and β_3-ARs compared to 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 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 30-50 fold-rises in uterine blood flow, such that by term the uterine vascular bed receives nearly 20% of the also greatly expanded cardiac output and blood volume.[1, 84] Furthermore, maternal uterine perfusion is maintained 1-2 fold in excess of the needs of the parallel, but separate, fetoplacental circulation.[253] We previously suggested that during an acute gestational "flight or fight" response when catecholamines are greatly elevated -far in excess of the efficacious levels utilized herein- cardiac output redistributes away from the uterine vascular bed (α-AR-mediated) to the muscles and other tissues (β-AR-mediate) for survival of the mother and her fetus, thus providing a distinct short term survival advantage for placental mammals.[87, 254]

Perspectives:

The current study sheds new light on the existence of a previously unrecognized two 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 physiologic 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 necessitates the further investigation of estrogen metabolites such as catecholestradiols in the vascular systems which do not signal via the classical estrogen receptors.

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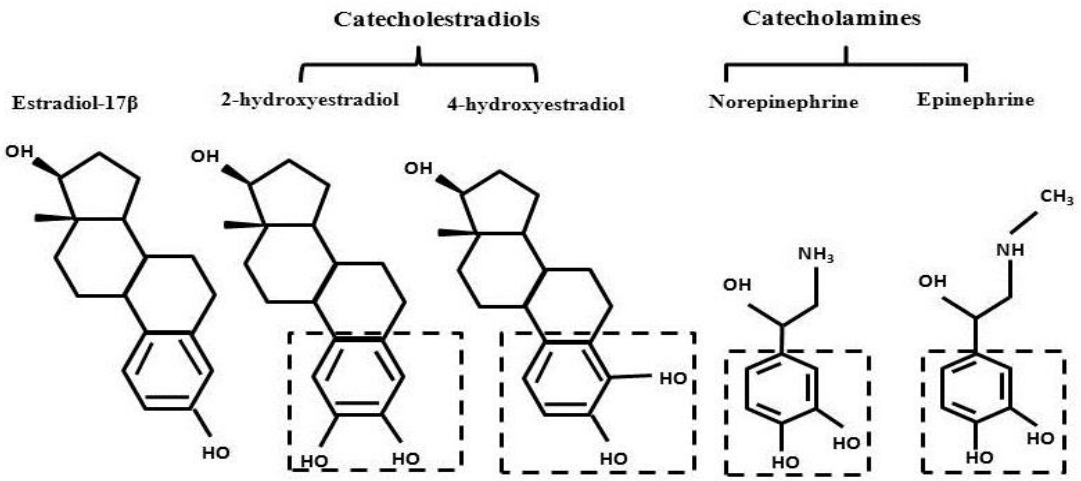
TABLES AND FIGURES

Figure 5.1



Receptors	Estradiol-17β	Catecholestradiols	Catecholamines
ER-α/ER-β	YES	NO	NO
α-AR/β-AR	NO	??	YES

Figure 5.1: Adding one OH group modifies the estrogenic phenolic A ring forming "catechol" moieties potentially making it an "AR-mimetic" agent for inducing ER-independent angiogenesis. The boxes outline shared phenolic A ring "catechol" moiety between 2-hydroxyestradiol, 4-hydroxyestradiol, norepinephrine and epinephrine. ?? Indicates hypotheses tested.

Figure 5.2

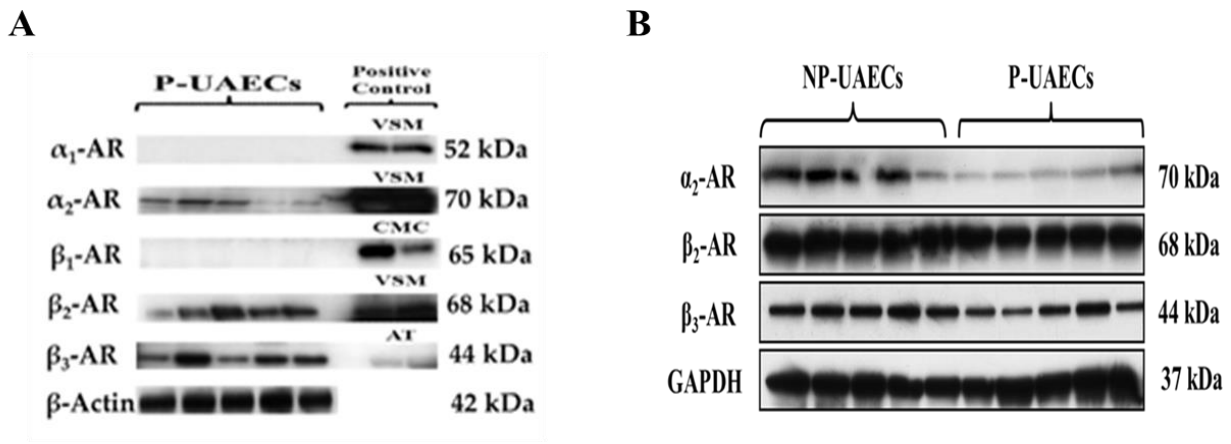


Figure 5.2: **AR subtypes in UAECs. (A)** Western blots demonstrating expression of α_2-ARs, β_2-ARs and β_3-ARs; but not α_1-ARs or β_1-ARs subtypes in P-UAECs. Positive control lanes are vascular smooth muscle cells (VSM), left ventricle cardiomyocytes (CMC) and adipose tissue (AT). **(B)** Expression α_2-ARs, β_2-ARs and β_3-ARs in **NP-UAECs versus P-UAECs**. Blots are representative of two independent experiments from individual UAEC cell lines.

Figure 5.3

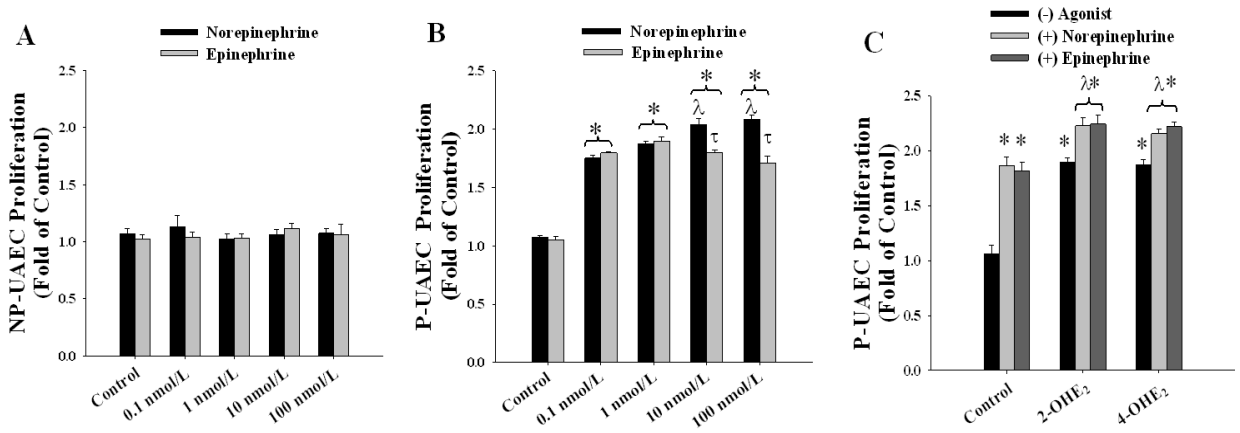


Figure 5.3: **Catecholamines stimulate of P-UAECs but not NP-UAECs Proliferation and Augment Catecholestradiol-induced P-UAEC Proliferation. (A)** Concentration response of NP-UAECs to 0, 0.1, 1, 10 and 100 nmol/L norepinephrine and epinephrine (two-way ANOVA; concentration x group; $F_{8,45} = 0.306$, $P = 0.960$; $n = 6$). **(B)** Concentration response of P-UAECs to 0, 0.1, 1, 10 and 100 nmol/L norepinephrine and epinephrine (two-way ANOVA; concentration x group; $F_{8,45} = 10.52$, $P < 0.001$; $n = 6$). **(C)** Combinations of 0.1 nmol/L norepinephrine or epinephrine with either 2-OHE_2 or 4-OHE_2 augmented P-UAEC proliferation responses (two-way ANOVA; group x agonist; $F_{4,27} = 3.73$, $P = 0.015$; $n = 4$) *Increase vs. control. λ Increase vs. 0.1 and 1 nmol/L. τ norepinephrine > epinephrine. ψ Increase vs. catecholestradiols or catecholamines alone

Figure 5.4

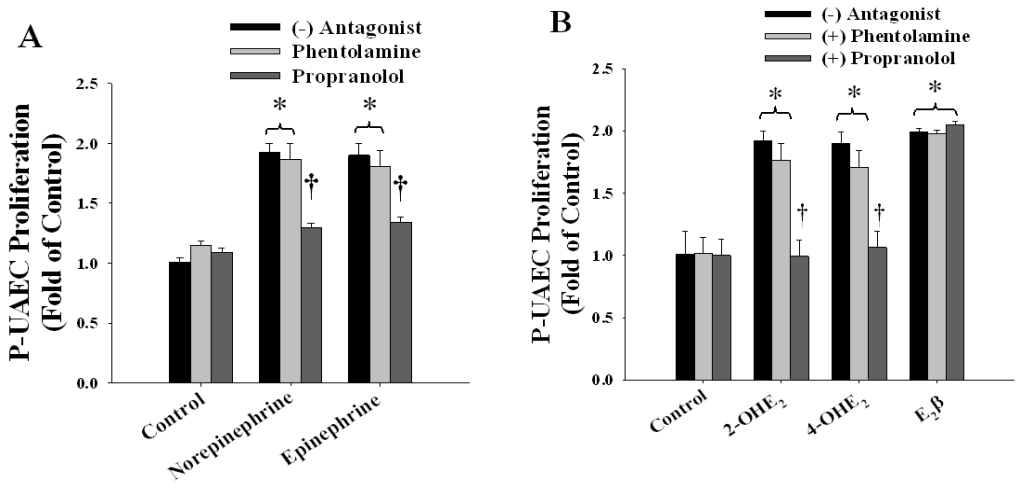


Figure 5.4: **β-ARs, but not α-ARs, mediate Catecholamine and Catecholestradiol-induced P-UAEC Proliferation. (A)** Effects of phentolamine or propranolol on P-UAECs proliferation to 0.1 nmol/L norepinephrine or epinephrine. Phentolamine had no effect; whereas propranolol completely abrogated catecholamine-induced P-UAEC proliferation (two-way ANOVA; antagonist x group; $F_{8,45} = 9.12$, $P < 0.001$; n = 4). **(B)** Effects of phentolamine or propranolol on P-UAECs proliferative responses to 2-OHE_2, 4-OHE_2 or $E_2\beta$ (0.1 nmol/L). Phentolamine had no effect; whereas propranolol completely abrogated 2-OHE_2- and 4-OHE_2- , but not $E_2\beta$-induced P-UAECs proliferative responses (two-way ANOVA; antagonist x group; $F_{6,36} = 7.88$, $P < 0.001$; n = 4). *Increase vs. untreated. † Complete inhibition.

Figure 5.5

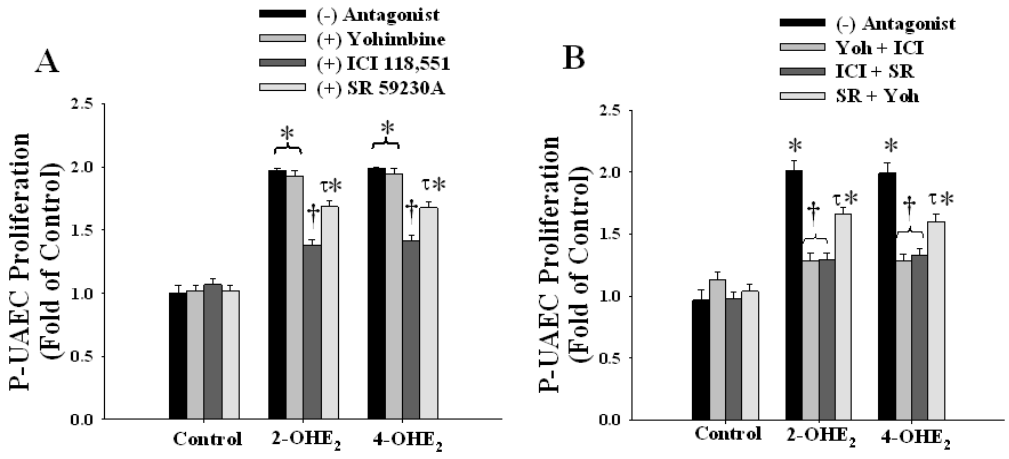


Figure 5.5: **β_2-ARs and to a Lesser Extent β_3-ARs Mediate Catecholestradiol-induced P-UAEC Proliferation. (A)** Effects of yohimbine, ICI 118,551 or SR59230A on P-UAEC proliferation to 2-OHE_2 or 4-OHE_2 (0.1 nmol/L). Yohimbine had no effect; whereas ICI 118,551 attenuated and SR59230A partially inhibited catecholestradiol-mediated P-UAEC proliferation (two-way ANOVA; antagonist x group; $F_{8,33}$= 7.871, $P < 0.001$; n = 4). **(B)** Effects of yohimbine, ICI 118,551, and SR59230A combinations on P-UAEC proliferative responses to catecholestradiols. ICI 118,551 in all combinations completely blocked P-UAEC proliferation responses to catecholestradiols (two-way ANOVA; antagonist combination x group; $F_{8,33}$ = 9.551, $P < 0.001$; n = 4). *Increase vs. untreated. † Complete inhibition. τ Partial inhibition.

Figure 5.6

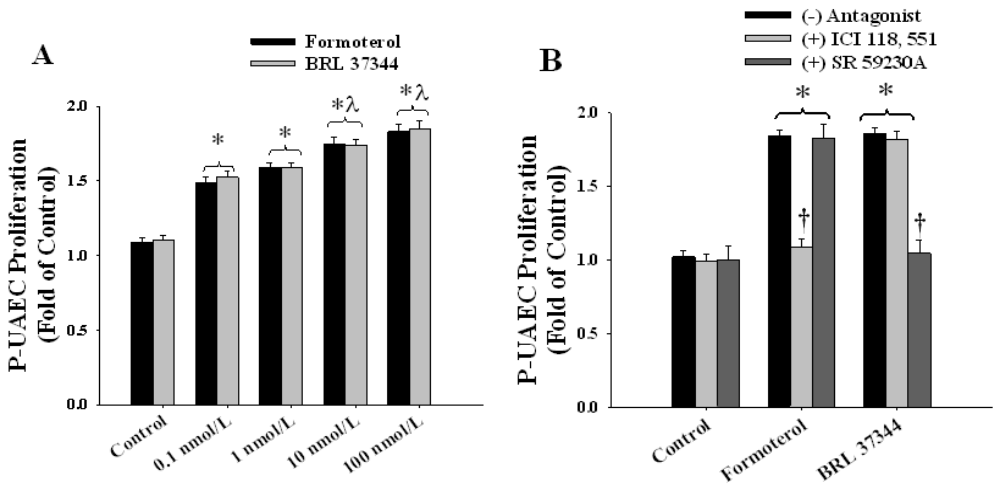


Figure 5.6: **Stimulation of β_2- and β_3-ARs promotes P-UAEC proliferation.** (**A**) Concentration response of P-UAECs to 0, 0.1, 1, 10 and 100 nmol/L of β_2- and β_3-AR agonists Formoterol and BRL 37344 (two-way ANOVA; concentration x group; $F_{4,30} = 3.01$, $P < 0.001$; n = 6). (**B**) Effects of ICI 118, 551 or SR59230A on P-UAEC proliferative responses to Formoterol and BRL 37344 (two-way ANOVA; antagonist x group; $F_{8,45} = 20.53$, $P < 0.001$; n = 6). *Increase vs. untreated. λ Increase vs. 0.1 and 1 nmol/L□ † Complete inhibition.

Chapter 6:

THE ROLE OF SIGNALING PATHWAYS IN ESTRADIOL-17β AND ESTRADIOL METABOLITES-INDUCED UTERINE ENDOTHELIAL CELL PROLIFERATION

Activation of Mitogen-Activated Protein Kinases Facilitate Estrogen Metabolites Actions on Uterine Artery Endothelial Cell Proliferation

[Manuscript in preparation, *Hypertension*]

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ABSTRACT

Experimental evidence demonstrates that estrogen via the classical estrogen receptors activates phosphoinositide 3-kinase (PI3K) or mitogen-activated protein kinases (MAPK) to stimulate proliferation in vascular endothelial cells. We have shown, however, that estrogen metabolites stimulate proliferation in uterine artery endothelial cells (P-UAECs) independent of the classical estrogen receptors and via adrenergic or other unidentified receptors; thus it is unknown whether signaling mechanisms are involved in facilitating these diverse and differential receptor-mediated actions. We therefore, tested whether similar signaling pathways modulate the effects of estrogen versus its metabolites on P-UAECs proliferation. Cultured P-UAECs were pretreated with the inhibitors LY294002 (PI3K), or PD98059 (p42/44 MAPK), or SB203580 (p38 MAPK), or SP600125 (JNK MAPK) followed by exposure to vehicle or 0.1 nmol/L of estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol. Using the selective inhibitors, we demonstrate that despite the role of different receptors, estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol induce P-UAEC proliferation similarly via p42/44, p38 and JNK MAPK pathways and independent of the PI3K pathway. Exposure of P-UAECs to estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol induced distinct temporal and transient phosphorylation of p42/44 MAPK but a temporal and non-transient phosphorylation of p38 or JNK MAPKs. Our data demonstrates that MAPK signaling pathways contribute importantly to actions of estrogen and its metabolites on P-UAEC proliferation, which suggests that similar molecular mechanisms may regulate the complex and diverse receptor-mediated actions of estrogen and estrogen metabolites on P-UAEC proliferation. Further studies are needed to provide additional details on the interaction among these signaling pathways to control endothelial cell proliferation.

INTRODUCTION

Classically, estrogen (estradiol-17β) is thought to act via its nuclear receptors, ER-α and/or ER-β, to induce events such as endothelial cell proliferation.[233] In this regard, we have previously reported that estradiol-17β-induced proliferation of uterine artery endothelial cells (P-UAECs) occurs primarily via ER-β.[224] Recently, however, increasing evidence demonstrates that estradiol-17β can also modulate endothelial events such as proliferation via activation of intracellular signaling kinases such as phosphatidylinositol 3-kinase (PI3K), p42/44, p38 and/or c-Jun NH(2)-terminal (JNK) mitogen-activated protein kinase (MAPK) pathways.[115, 255, 256] For example, in aortic, retinal, coronary artery and umbilical vein endothelial cells, activation of PI3K, p42/44, p38 or JNK MAPKs by estradiol-17β have been attributed to regulation of proliferation.[257-260] Indeed, estrogen-induced signaling kinase activation and pattern depends on the endothelial cell type studied, the conditions used and the time-course of estrogen exposure. Nevertheless, the precise signaling pathways facilitating estradiol-17β-mediated P-UAEC proliferation are not well understood and have not been studied.

Evidence is increasing that many of the physiological and pharmacological effects of estrogen are mediated via the actions of its biologically active metabolites. Estrogen metabolism is catalyzed by the sequential actions of cytochrome P450s (CYP450s) and catechol-*O*-methyltransferase (COMT) to produce the catecholestradiols 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) and the methoxyestradiols 2-methoxestradiol (2-ME_2) and 4-methoxyestradiol.[172, 184] Estrogen metabolites also enhance proliferation of endothelial cells derived from several vascular beds.[198, 224] Indeed, we have reported in a previous study that compared to the parent substrate, 2-hydroxyestradiol and 4-hydroxyestradiol induced proliferation of P-UAECs via β_2- and β_3-adrenergic receptors and independent of the classical

estrogen receptors.[225] In addition, we also reported that unlike 2-methoxyestradiol which did not induce mitogenesis, 4-methoxyestradiol-stimulated proliferation of P-UAECs was unaltered by ICI 182,780, propranolol or phentolamine, suggesting that the proliferative effects of this estrogen metabolite occurs independent of ER-α, ER-β or α- or β-adrenergic receptors.[224, 225] Therefore, it is important to delineate the exact signaling pathways mediating these complex and differential receptor-mediated actions of estrogen metabolites to shed light on the complexity and diversity of estrogen signaling via its metabolites.

In the present study, we evaluated whether estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol stimulate proliferation of P-UAECs via activation of PI3K, p42/44, p38 or JNK MAPK signaling cascades and to determine whether common intermediates in these signaling pathway are involved in the complex and differential receptor-mediated actions of these steroids. Furthermore, we examined the temporal phosphorylations of these signaling kinases following exposure to estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol to evaluate the precise signaling activation and patterns of these kinases.

MATERIALS and METHODS

Cell Preparation and Culture

All cell preparations and culture were approved by the University of Wisconsin-Madison School of Medicine and Public Health Research Animal Care Committee and performed as previously described.[224] P-UAECs were isolated and validated from late gestation (120-130 days; term= 147 days; n=6) ewes. At passage 4 and ~ 70% confluence, cell were transferred to slides, 96 well plates, or lysed for protein extraction as needed for respective experiments.

Experimental Treatments and Blockade of PI3K, p42/44, p38 and JNK MAPKs

All P-UAEC cell proliferation experiments were performed in quadruplicates and replicated in at least four P-UAEC preparations. p42/44, p38, JNK MAPKs and PI3K were blocked by pretreating P-UAECs for 1 hr with 2.5 or 5 μmol/L of the pure antagonists LY294002 (PI3K), PD98059 (p42/44 MAPK), SB203580 (p38 MAPK) or SP600125 (JNK MAPK) and followed by optimal 0.1 nmol/L concentrations of estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol

Protein Extraction and Western Immunoblotting

Western immunoblotting was performed as previously described.[224, 225] Following treatment with 0.1 nmol/L concentrations of estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol phosphorylated and total p42/44, p38 and JNK MAPK expressions were detected using rabbit anti- p42/44, rabbit anti- p38, rabbit anti- JNK. GAPDH and/or β-actin were utilized as a loading control.

BrdU Cell Proliferation Assays

BrdU label was added for 16 hrs during the 24 hrs of steroid treatment and an *in vitro* index of proliferation was evaluated. Plates were read using Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Results were expressed as fold increases over untreated control after subtracting the value of the blank.

Statistical Analysis

Data (means ± SEM) were analyzed using a 2-way ANOVA with "concentration of blocker" and "blocker effect" as two "between" factors. When appropriate, analyses of simple effects were performed using one-way ANOVA followed by post-hoc Student-Newman Keuls test. Pairwise comparisons were performed when appropriate using Bonferroni or Student-Newman-Keuls test. A level of significance was established *a priori* at $P<0.05$.

RESULTS

Role of p42/44, p38 and JNK MAPK pathways in proliferation induced by estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol

In P-UAECs, blockade of PI3K with LY 294002 did not abolish the proliferative effects of estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol. Treatment with PD98059 alone had no effect on P-UAEC proliferation. However, PD98059 pretreatment totally abrogated cell proliferation responses to estradiol-17β indicating the requirement of p42/44 MAPK in estradiol-17β -induced proliferation. We then tested the effects of PD98059 on P-UAEC proliferation in response to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol or 4-methoxyestradiol. PD98059 pretreatment inhibited all proliferation responses to 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol. 2-methoxyestradiol did not stimulate proliferation in P-UAECs. These results demonstrated that estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol induce proliferation via activation of p42/44 MAPK.

Treatment with SB203580 alone had no effect on P-UAEC proliferation. However, SB203580 pretreatment totally abrogated cell proliferation responses to estradiol-17β indicating

the requirement of p38 MAPK in estradiol-17β-induced proliferation. We then tested the effects of SB203580 on P-UAEC proliferation in response to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol or 4-methoxyestradiol. SB203580 pretreatment inhibited all proliferation responses to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol. Similar to the LY294002 and PD98059 experiments, 2-methoxyestradiol did not stimulate proliferation in P-UAECs.

Treatment with SP600125 alone had no effect on P-UAEC proliferation. However, SP600125 pretreatment totally abrogated cell proliferation responses to estradiol-17β indicating the requirement of JNK MAPK in estradiol-17β-induced proliferation. We then tested the effects of SP600125 on P-UAEC proliferation in response to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol. SP600125 pretreatment inhibited all proliferation responses to 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol. These results demonstrated that estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol induce proliferation via activation of JNK MAPK.

Temporal activation of phosphorylated and total p42/44, p38 and JNK MAPK induced by E_2β, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2

Phosphorylations of target kinases were only evaluated for ones that completely inhibited proliferation when blocked. Time-course activation of phosphorylation of p42/44 MAPK was seen in response to 0.1 nmol/L estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol, albeit with some nuances. Specifically, temporal activation of p42/44 MAPK was biphasic and transient and seen at 15 minutes and 12 hours in response to estradiol-17β, 2-hydroxyestradiol and 4-hydroxyestradiol. On the other hand, the temporal phosphorylation of p42/44 MAPK in response to 4-methoxyestradiop was monophasic and seen at only the 15

minute time point. Total levels of p42/44 MAPK was not changed in response to 0.1 nmol/L of estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol.

Temporal response of p38 MAPK phosphorylation was time dependent, non-transient and increased with time in response to 0.1 nmol/L estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol. Total levels of p38 MAPK was not changed in response to estrogen or its metabolites. Time-course dependent responses of phosphorylated JNK MAPK was changed and time dependent in response to 0.1 nmol/L estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol; whilst total levels of total JNK was unchanged in response to time-course treatments of 0.1 nmol/L estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol or 4-methoxyestradiol.

DISCUSSION

In the present study, we demonstrated that p42/44, p38 and JNK MAPKs, as key mediators, are required for estradiol-17β action on P-UAEC proliferation. Our results are consistent with other studies that have demonstrated that estrogen can induce endothelial proliferation *in vitro* by stimulation of p42/44, p38 and/or JNK MAPK pathways.[114, 257, 261, 262] We also showed herein that activation of PI3K does not play a role estradiol-17β-induced proliferation of P-UAECs. Activation of PI3K has been shown by other investigators to be involved in estradiol-17β-mediated proliferation of other endothelial cells.[260, 263] It is important to note that only the phosphorylation of p42/44 MAPK was temporal and transient; the phosphorylation of p38 and JNK MAPKs were only temporal. The differences regarding the involvements and specificities of signaling kinases in response to estradiol-17β exposure are unknown. Nevertheless, the fact that estrogen can activate MAPK signaling kinases to facilitate endothelial proliferation indicates that these signaling pathways are another component of the myriad of estrogenic actions on

endothelial cells that should be further assessed.

We also show herein that activation of p42/44, p38 and JNK MAPKs is necessary for 2-hydroxyestradiol and 4-hydroxyestradiol action on P-UAECs proliferation. We demonstrate that the effects of these catecholestradiols on P-UAEC proliferation occurs independent of PI3K. To the best of our knowledge, this is the first report to show that catecholestradiols stimulate proliferation specifically via MAPKs. The pattern of p42/44 MAPK phosphorylation was similar to that of estradiol-17β with two transient distinct peaks at 15 minutes and at 12 hours. Since catecholestradiols stimulate proliferation of P-UAECs via β_2- and to a lesser-extent β_3-adrenegric receptors, these data suggests that despite the absence of common receptors, estradiol-17β and its catecholestradiol metabolites stimulate proliferation via similar signaling pathways.[225] These observations also point to partial redundancy in the mechanisms that may mediate endothelial proliferation in the uterine vasculature.

Our findings also demonstrate that 4-methoxyestradiol also stimulates proliferation of P-UAECs via activation of p42/44, p38 and JNK MAPKs and independent of PI3K. However, the distinct phosphorylation of p42/44 MAPK, demonstrating only a single peak at 15 minutes, shows that this estrogen metabolite may stimulates proliferation differently from estradiol and the catecholestradiols. In our previous study, 4-methoxyestradiol-induced proliferation of P-UAECs was not inhibited by ICI 182,780, propranolol or phentolamine, suggesting that the proliferative effects of 4-methoxyestradiol are estrogen and adrenergic receptor-independent or involve an unidentified receptor.[224, 225] Therefore, our observation collectively suggests the complexity and diversity of estrogen signaling via different receptors and different metabolites yet via activation and convergence of similar signaling pathways.

In conclusion, our data show an association of p42/44, p38 and JNK MAPK pathways

with P-UAEC proliferation following treatment with estrogen or its metabolites. These findings raise the possibility that these MAPKs are involved in the activation and or regulation of angiogenesis during pregnancy. However, whether there is cross-talk amongst these pathways or simply functional redundancy remains unclear. Since several kinases are inextricably linked to the endothelial dysfunction in gestational vascular diseases such as preeclmapsia, these observations may be important in bringing together components of the estrogen metabolism, impaired angiogenesis and the signaling pathways regulating endothelial cell proliferation and function.

PERSPECTIVES

The results from these studies demonstrate that the actions of estrogen and its metabolites on uterine artery endothelial cells are facilitated via the activation of p42/44, p38 and JNK MAPKs, although it is unclear whether convergence with receptors are required to mediate these actions. Additional studies are required to provide further evidence of the specific role and/or convergence of each of the signaling kinases in the events observed. These results indicate that alterations in signal transduction pathways by estrogen metabolites represent another mechanism of action in a complex chain of events mediated by the parent substrate on endothelial cells. These findings deepen our understanding of estrogen signaling via its metabolites, and may have important implications for the intricacies of estrogen replacement therapy and vascular diseases where endothelial proliferation and estrogen play major roles.

NOVELTY and SIGNIFICANCE

What is New?

We report that MAPK signaling pathways contribute importantly to actions of estrogen and its metabolites on endothelial proliferation, which suggests that similar molecular mechanisms may

regulate the complex and diverse receptor-mediated actions of estrogen and estrogen metabolites.

What is Relevant?

Sequential conversion of estradiol-17β to its biologically active metabolites, contributes importantly to its angiogenic effects on uterine artery endothelial cells via different receptors. Therefore, it is unknown what signaling pathways mediate these diverse and differential receptor-mediated actions.

Summary

These results indicate that alterations in MAPK signal transduction pathways by estrogen metabolites represent another mechanism of action in a complex chain of events mediated by the parent substrate on endothelial cells.

TABLES AND FIGURES

Figure 6.1

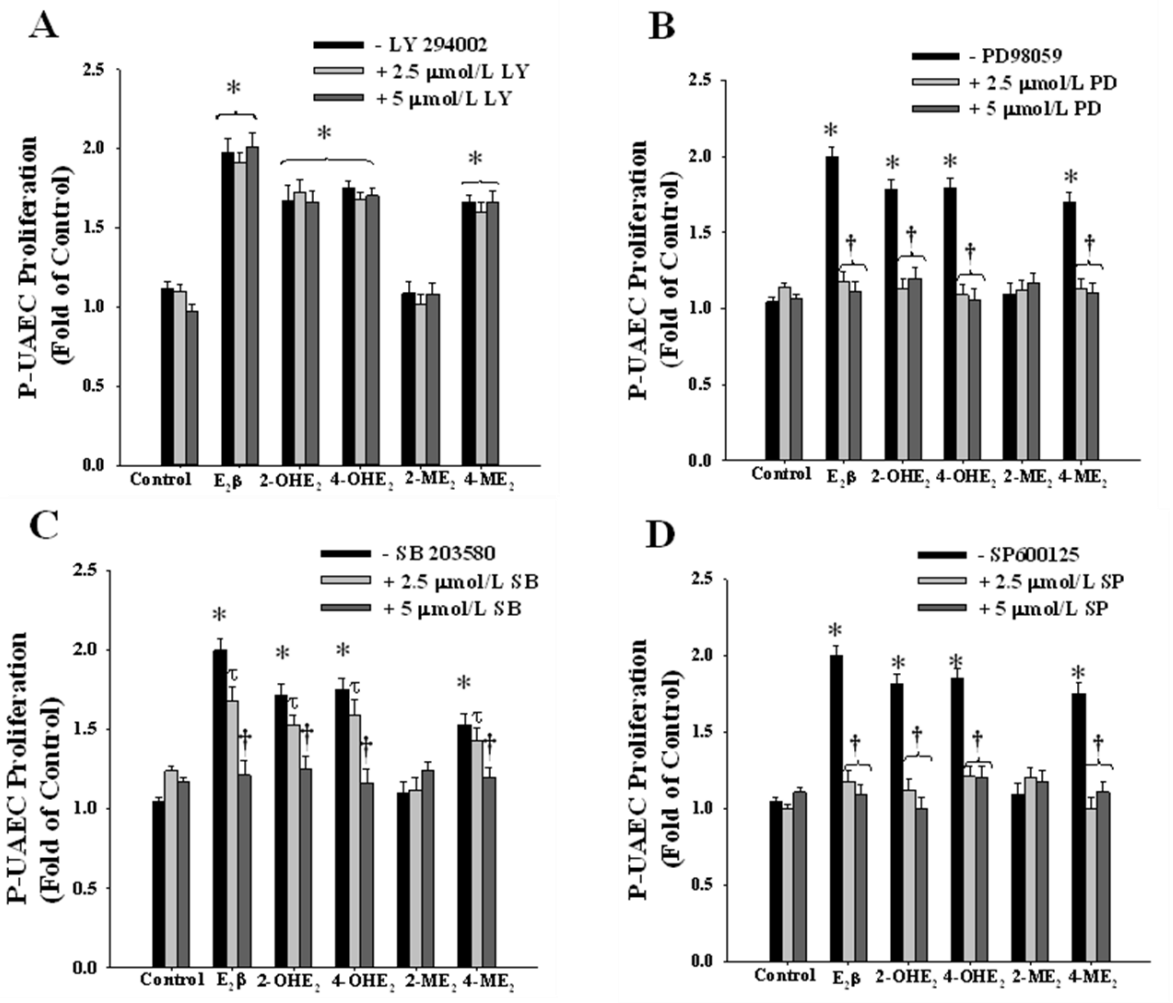

Figure 6.1: Effects of (A) PD98059 or (B) LY 294002 or (C) SB 203580 or (D) SP600125 on P-UAEC proliferation to estradiol-17β or 2-hydroxyestradiol or 4-hydroxyestradiol, 2-methoxyestradiol or 4-methoxyestradiol (0.1 nmol/L). LY 294002 had no effect; whereas 5µmol/L PD98059, SB 203580 and SP600125 inhibited P-UAEC proliferation in response to $E_2\beta$ or 2-OHE_2 or 4-OHE_2, 2-ME_2 or 4-ME_2.2-ME_2 had not proliferative effect on P-UAECs. *Increase in proliferation compared to untreated control. † Complete inhibition. τ Partial inhibition/decrease.

Figure 6.2

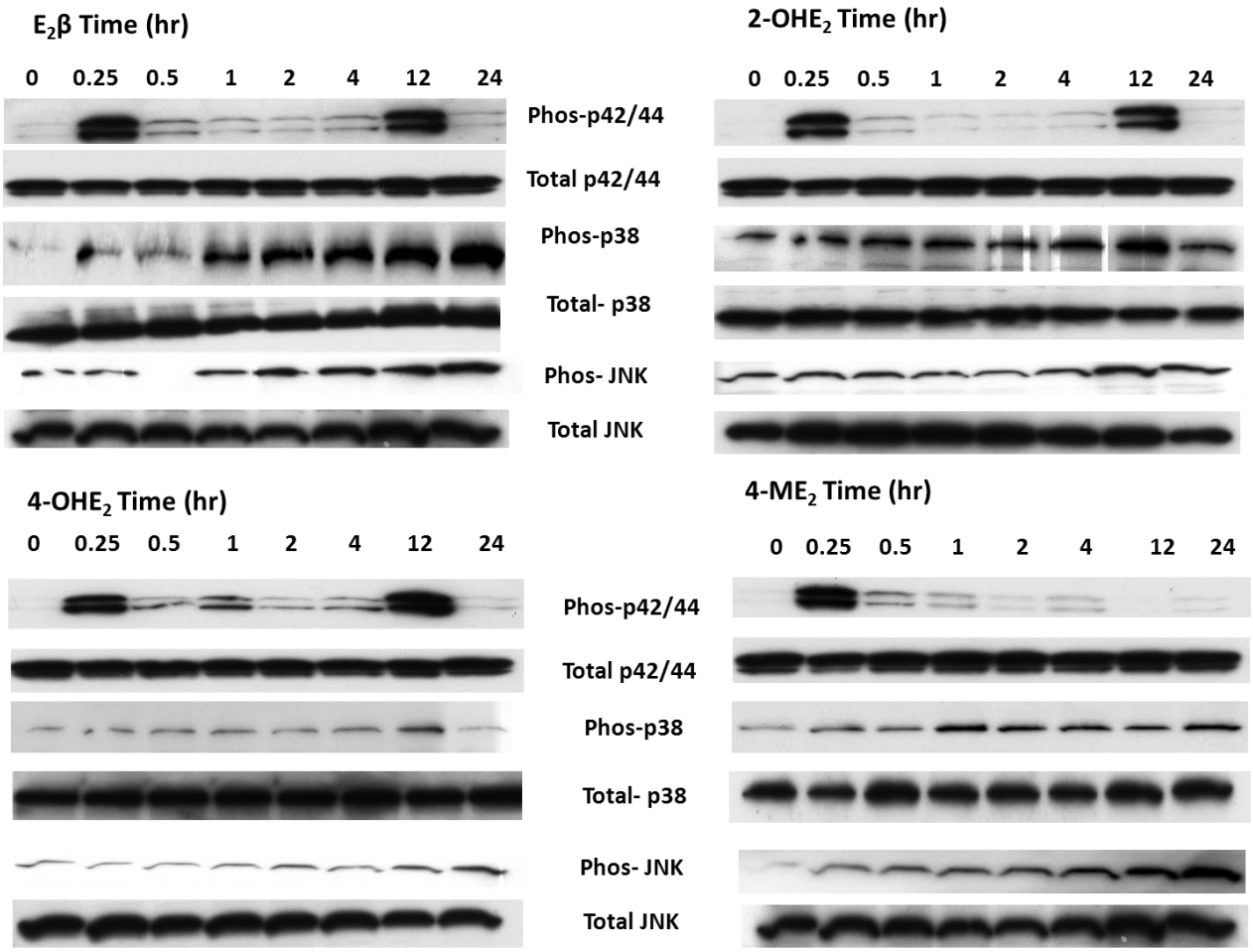

Figure 6.2: Temporal induction of phosphorylation in P-UAECs by estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol (0.1 nmol/L). Phosphorylation of p42/44 MAPK was biphasic with two significant peaks at 15 min and 12 hr for estradiol-17β, 2-hydroxyestradiol and 4-hydroxyestradiol and monophasic for 4-methoxyestradiol. Phosphorylation of p38 MAPK increased with time in response to estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol. Phosphorylation of JNK MAPK was time dependent in response to estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol. Total levels of p42/44, p38 and JNK MAPK were not temporally changed

CHAPTER 7:

THE SYSTEMIC PLASMA PROFILE OF ESTROGENS AND ESTROGEN METABOLITES IN PREECLAMPSIA

Aberrant Synthesis, Metabolism and Plasma Accumulation of Circulating Estrogens and Estrogen Metabolites in Preeclampsia: Implications for Vascular Dysfunction

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ABSTRACT

Estrogens and estrogen metabolites have important functions in cardiovascular and other physiology, yet the patterns of estrogen synthesis, metabolism and the individual plasma profile of estrogens and estrogen metabolites during human pregnancy as well as in preeclampsia remain undetermined. We performed liquid chromatography mass spectrometry on plasma samples from normotensive pregnant women (normP; n = 8), women with mild (mPE; n = 8) and severe (sPE; n = 8) preeclampsia at labor. Compared to normP, estrone was lower in sPE, whereas plasma level of estradiol-17β was significantly lower in women with mPE and sPE. Estriol was lower in sPE but not in mPE. Although, 2-hydroxyestrone was lower in mPE and sPE, 4-hydroxyestrone was high in sPE. 16-α-hydroxyestrone was higher in mPE but not in sPE. 2-hydroxyestradiol in women with mPE and sPE were lower compared to normP. Compared to 2-methoxyestrone in normP, levels were lower in sPE. 3-methoxyestrone and 4-methoxyestrone were unchanged. 2-methoxyestradiol was lower in mPE and sPE; however, 4-methoxyestradiol was low only in sPE. Compared to normP, 16-keto-estradiol levels were significantly higher in sPE whereas 16-epi-estriol and 17-epi-estriol were lower in women with sPE.

Aberrant synthesis and metabolism of estrogens and estrogen metabolites in preeclampsia provides functional insight into the role of these steroids in pregnancy and highlights previously unknown and underappreciated critical links between preeclampsia and the cardiovascular and other physiology of these estrogens and estrogen metabolites.

Key Words: preeclampsia, estrogens, estrogen metabolites, steroid synthesis and metabolism

INTRODCUTION

Preeclampsia is a hypertensive disorder of pregnancy that affects 5% to 8% of pregnancies, thus remaining a significant cause of maternal and fetal morbidity and mortality as well as greater susceptibility and earlier onset of future cardiovascular disease in both mother and baby.[207, 264, 265] The etiology of preeclampsia remains elusive; however, contributing factors manifest in the form of impairment of several normal maternal cardiovascular adaptations seen during pregnancy.[204, 266]

We and others have demonstrated that regulation of maternal cardiovascular adaptation during pregnancy is mediated in part by the primary and classical estrogens, estrone, estradiol-17β and estriol, which are synthesized by the uteroplacental unit using circulating steroid precursors from both the maternal and fetal adrenal glands.[53, 220] Evidence also supports a role for these primary estrogens in preeclampsia[208-210]; however, whether primary estrogens can be useful biomarkers for maternal and fetal well-being in adverse pregnancies including preeclampsia has been a subject of controversy. Several studies support evidence that serum and urinary primary estrogens maybe useful for screening for adverse pregnancy outcomes[208], whereas others contend that measurement of these estrogens is of little value.[211] Nevertheless, the levels and plasma profile of circulating primary estrogens in preeclampsia are, at best, unclear and this is further complicated by the lack of information on specific functional estrogen metabolites thus hindering our comprehensive understanding of their role(s) in its pathophysiology.

Primary estrogens are converted in the uteroplacental unit by cytochrome P450s (CYP450s) into multiple hydroxylated metabolites defined by the position of hydroxylation such

as 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone, 2-hydroxyestradiol and 4-hydroxyestradiol (Figure 1).[172] Hydroxylated primary estrogens especially the catecholestrogens (hydroxylated estrogens at the carbon 2 and 4 positions) undergo enzymatic *O*-methylation by catechol-*O*-methyltransferase (COMT) to form methoxyestrogens such as 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol (Figure 1).[172] Other metabolites of primary estrogens formed from enzymatic pathways include 16-keto-estradiol, 16-epi-estriol and 17-epi-estriol (Figure 1). There is increasing strong support for the concept that regulation of maternal cardiovascular adaptation during pregnancy is partly mediated by estrogen metabolites.[212, 213] Estrogen metabolites may also play a role in preeclampsia because pregnant mice deficient in COMT, an enzyme that catalyzes the methylation of catecholestrogens to methoxyestrogens, exhibit a preeclampsia-like phenotype.[182] However, the metabolism of estrogens is very complex and necessitates that a plethora of other functional metabolites of estrogens be properly accounted for to achieve a more comprehensive knowledge of their potential collective contributions to the pathogenesis of preeclampsia.

Therefore, the objective of this study was to compare plasma levels of total primary estrogens and estrogen metabolites in normotensive pregnant women (normP) compared to women with mild (mPE) and severe (sPE) preeclampsia at term. Because of the cardiovascular and other physiologic functions of estrogens and estrogen metabolites including during gestation, we hypothesized that preeclampsia, a disease marred by widespread cardiovascular dysfunction, is characterized by aberrant synthesis, metabolism and accumulation of estrogens and estrogen metabolites. In this report, we highlight several specific connections that exist between estrogens and estrogen metabolites with regards to cardiovascular adaptations seen during

pregnancy and highlight possible comprehensive and integrated roles for these estrogens and estrogen metabolites in the pathophysiology preeclampsia (Figure 6). Moreover, we also present a working hypothesis that 16-keto-estradiol maybe a possible interconversion metabolite between estradiol-17β and estriol and may play a role in the circulating levels of both primary estrogens (Figure 1).

MATERIALS AND METHODS

Subjects

All subjects signed a written informed consent, and all study-related procedures and protocols were approved by the Institutional Review Boards at the University of Wisconsin and Meriter Hospital, Madison, Wisconsin. The women were recruited at the time of admission to Labor and Delivery and thus all subjects were at term.

Inclusion Criteria

The normP control group included pregnant women with no preexisting medical diseases or antenatal complications. Preeclampsia criterion for inclusion was based as described in Creasy and Resnisk's Maternal-Fetal Medicine: Principles and Practice, 6th Edition Chapter 35. Mean gestational ages at enrollment for normP, mPE and sPE women were 38.9±0.1, 36.6±1.0 and 35.5±1.2 weeks respectively. 24 hour total urine protein was measured in only women that showed ≥1+ on urine dipstick and/or elevated blood pressure on two separate assessments more than 4 hours apart.

Exclusion Criteria

Subjects were excluded if they exhibited any of the following: Lupus, Antiphospholipid Antibody Syndrome, Chorioamnionitis, Placental Abruption or Meconium stained fluid

Sample Collection

Blood samples were collected from normP (n = 8), mPE (n = 8) and sPE (n = 8) subjects via venipuncture of the brachial vein, prepared, centrifuged and plasma aliquots were stored at -80 °C until quantitative measurement.

Liquid chromatography-tandem mass spectrometry

Plasma levels of estrogens and estrogen metabolites were measured by a sensitive, specific and precise high performance liquid chromatography-tandem mass spectrometry method utilizing selected reaction monitoring for measuring the absolute quantities of total (conjugated and unconjugated) estrogens and estrogen metabolites as previously described[267] and performed at Primera Analytical Solutions Corp., Princeton, NJ. The unit for the calculated level of estrogens and estrogen metabolites was pg/ml.

Statistical Analysis

Data are presented as Means ± SEM. The difference in mean values between experimental groups was determined by one-way ANOVA (SigmaPlot 12 Statistical Software) followed by post-hoc Student-Newman- Keuls multiple pairwise comparisons. Level of significance was established a *priori* at $P<0.05$.

RESULTS

Characteristics of the Subjects

Compared to normP, sPE but not mPE women had significantly higher body weight and body-mass index (Table 1). Per inclusion criteria, both women with mPE and sPE demonstrated higher than normal systolic and diastolic blood pressures. Moreover, women with sPE had significantly higher systolic and diastolic blood pressure compared to mPE women (Table 1). Per inclusion criteria, compared with normP women who had negative dip stick protein levels, both women with mPE and sPE had higher protein in their urine. In addition, women with sPE had

significantly higher proteinuria compared to mPE women. Gestational ages at time of admission were not significantly different amongst the women.

Plasma Profile of Primary/Classical Estrogens and Estrogen Metabolites in Preeclampsia

NormP and mPE women had similar levels of plasma estrone (4503 ±740 and 5341 ±1255 pg/ml respectively) (Figure 2A); however, women with sPE had significantly lower level of plasma estrone (1163 ±162 pg/ml) (Figure 2A). Estradiol-17β levels were lower in women with mPE (5326±213 pg/ml) compared to levels in normP (9441±626 pg/ml) (Figure 2B). Moreover, plasma estradiol-17β in women with sPE (1975±162 pg/ml) was lower compared to both normP and mPE (Figure 2B). Levels of 16-keto-estradiol in normP and mPE women (861±22 and 652±229 pg/ml respectively) were not different, whereas levels in sPE women (1894±100 pg/ml) were significantly higher (Figure 2C). Plasma levels of estriol were similar between normP women and women with mPE (2599±162 and 2589±203 pg/ml respectively); however, women with sPE had significantly lower estriol (1163±34 pg/ml) (Figure 2D).

Levels of plasma 2-hydroxyestrone in normP and mPE were 315±43 pg/ml and 215±22 pg/ml respectively (Figure 3A); however, levels in sPE were significantly lower (138±21 pg/ml) (Figure 3A). Compared to normP (129±28 pg/ml), levels of plasma 4-hydroxyestrone were increased in mPE and sPE (180±10 and 202±10 pg/ml respectively) (Figure 3B). Plasma level of 16-α-hydroxyestrone in mPE women (6781±286 pg/ml) was significantly higher compared to normP women (3346±1354 pg/ml) and sPE women (4227±856 pg/ml) (Figure 3C). We observed that plasma level of 2-hydroxyestradiol in women with mPE was significantly lower compared to levels in normP women (Figure 3D). In addition, the levels of plasma 2-hydroxyestradiol in women with sPE (170±25 pg/ml) was significantly lower compared to both normP (502±26 pg/ml) and mPE women (231±7 pg/ml) (Figure 3D).

Plasma levels of 2-methoxyestrone were similar between normP women and mPE (769±45 and 723±109 pg/ml respectively); however, women with sPE had significantly lower 2-methoxyestrone (530±26 pg/ml) (Figure 4A). Plasma levels of 3-methoxyestrone (168±16, 187±56 and 132±6 pg/ml respectively) and 4-methoxyestrone (26±9.0, 36±5.9 and 20±2.7 pg/ml respectively) were not significantly different amongst normP, mPE and sPE (Figure 4B and 4C). Plasma levels of 2-methoxyestradiol in women with mPE (1813±133 pg/ml) were significantly lower compared to levels in normP (2186±156 pg/ml) (Figure 4D). In addition, the levels of plasma 2-methoxyestradiol in women with sPE (982±55 pg/ml) were significantly lower compared to both normP and mPE (Figure 4D). NormP and mPE did not have different levels of 4-methoxyestradiol (903±168 and 1008±30 pg/ml respectively) (Figure 4E). However, women with sPE had lower 4-methoxyestradiol (393±18 pg/ml) compared to normP and mPE (Figure 4E).

The plasma levels of 16-epi-estriol (1104±39 pg/ml) and 17-epi-estriol (189±48 pg/ml) were different in sPE compared to normP (2268±457 and 627±58 pg/ml respectively) and mPE women (2958±124 and 521±16 pg/ml respectively) (Figure 5A and 5B).

DISCUSSION

Our findings demonstrate that mild and severe preeclampsia are characterized by aberrant synthesis, metabolism and levels of estrogens and estrogen metabolites. Although plasma levels of these estrogens and estrogen metabolites suggest aberrant synthesis and metabolism, they are particularly specific and distinct- therefore, potentially useful in our understanding of the pathophysiology of preeclampsia and exploration of better clinical management and outcomes.

In normal pregnancy, plasma levels of estrone, estradiol-17β and estriol are increased and parallel some of the increases in uteroplacental blood flow as well as development of an

extensive uteroplacental vascular bed.[53, 220] During this time, the placenta is the main source of these estrogens using circulating steroid precursors from the maternal uterine compartment, adrenal glands as well as fetal adrenal glands.[220, 268] Placental biosynthesis of primary estrogens during pregnancy is complex and results from interaction and interdependence of separate maternal, placental and fetal systems that individually do not possess the necessary enzymatic capabilities to make these critical estrogens.[269] Because the placenta does not express 17α-hydroxylase, the obligatory synthesis of C19-steroid precursors is not possible for estrogen synthesis.[269] Therefore, the primary classical estrogens are synthesized from dehydroepiandrosterone and dehydroepiandrosterone-sulfate precursors synthesized from the maternal and fetal adrenal glands.[270] It is important to note that at or near term, half of placental estrone and estradiol-17β is derived from dehydroepiandrosterone and dehydroepiandrosterone-sulfate precursors from maternal adrenal glands whilst the other half is derived from the fetal adrenal zone.[269, 270] In contrast, over ninety percent of placental estriol is derived from dehydroepiandrosterone and dehydroepiandrosterone-sulfate precursors from the fetal adrenal zone whilst only ten percent is derived from maternal sources at or near term.[269] Our findings are consistent with studies, albeit controversial, by other investigators that estrone, estradiol-17β and estriol may be low in preeclampsia.[208, 271] Estrone and estradiol-17β have been demonstrated to be potent vasodilators in the uterine and systemic vascular beds by reducing vascular resistance and elevating perfusion.[141, 142, 272] Since preeclampsia is characterized partly by impaired uterine and systemic vascular vasodilatory responsiveness and decreased uterine perfusion, our findings suggest the possibility that aberrant activities of uteroplacental aromatase, 17β-hydroxysteroid dehydrogenase, placental sulfatase and/or fetal 16α- and 17α-hydroxylase leading to low estrone, estradiol-17β and estriol maybe a primary mechanisms responsible for

some of the clinical manifestations of this disease. Low levels of estrone, estradiol-17β and estriol observed in this study may also be due to reduced circulating steroid precursors from the maternal uterine compartment, adrenal glands as well as fetal adrenal glands. However, previous studies have shown that preeclampsia is not associated with low levels of C-19 steroid precursors including androstenedione, and dehydroepiandrosterone.[208, 273]

We report herein, for the first time, that the plasma level of 16-keto-estradiol is significantly higher in women with severe preeclampsia. The concomitant higher levels on 16-keto-estradiol-17β with low levels of estradiol-17β and estriol as well as structural and kinetic evaluations led us to the novel hypothesis that this metabolite maybe an interconversion metabolite between estradiol-17β and estriol (see Figure 1 schematic).[274, 275] Thus, collectively, our observations suggest that elevated levels of 16-keto-estradiol-17β may be used as a predictive biomarker in preeclampsia indicating levels of both estradiol-17β and estriol.

The present findings also show, for the first time, that plasma levels of 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone and 2-hydroxyestradiol levels are altered in preeclampsia. The low level of both 2-hydroxyestrone and 2-hydroxyestradiol in women with preeclampsia suggests low and/or aberrant activity of CYP1A1, CYP1A2 and CYP3A4 which primarily hydroxylate estrogens in the C-2 position to form catecholestrogens.[172]. We report abberrant levels of 4-hydroxyestrone and 16-α-hydroxyestrone in preeclampsia supporting our hypothesis of dysregulation of estrone hydroxylation pathways. There is also the possibility that the low levels of hydroxylated estrogens may be partly due to the low levels of estrone and estradiol-17β as subtrates for the uteroplacental CYP450 enzymes. Collectively, these data suggest that formations of hydroxylated estrogens, which have been demonstrated to possess several uteroplacental vascular effects including vasodilatory activities,[276] induction of

endothelial cell proliferation,[198, 212] generation of prostacyclin[197] and synergistic effects with nitric oxide,[213] are highly dysregulated in preeclampsia which would contribute to the reduced uterine perfusion seen in pregnancies complicated by preeclampsia.

Enzymatic *O*-methylation of catecholestrogens by COMT forms several methoxyestrogens.[172] Preeclampsia is also associated with low activity of COMT in human placentas[4] and pregnant COMT-deficient mice exhibit a preeclampsia-like phenotype.[182] In support of this hypothesis, we show herein that apart from 2-methoxyestradiol, other physiologically relevant COMT-derived metabolites including 2-methoxyestrone and 4-methoxyestradiol are also decreased in preeclampsia. It is possible that apart from low activity of COMT, low levels of methoxyestrogens in preeclampsia may also be due to low levels of catecholestrogen subtrates as well as the primary estrogens as discussed above. Nevertheless, since methoxyestrogens induce various positive vascular effects via vasoactive and intracellular molecules that have been implicated in preeclampsia such as nitric oxide, prostacyclin, endothelin-1, cyclic nucleotides, HIF-1α and adhesion molecules, our findings suggest that the effects of COMT in preeclampsia could be more critical than previously thought and necessitates further investigation. Many compounds with "catechol" structures are substrates for COMT including the vasoactive catecholamines epinephrine and norepinephrine. We have also previously proposed that this property of COMT also points to the potential relevance of the convergence of the sympathetic catecholamine system and estrogen metabolism systems in the dysregulation of vascular responsiveness in preeclampsia.[225]

We noted, for the first time, that 16-epi-estriol and 17-epi-estriol are both significantly decreased in severe preeclampsia but only 17-epi-estriol was low in mild preeclampsia suggesting that in addition to low estriol levels, epimerization metabolism of estriol may also be

aberrant in preeclampsia. 16-epi-estriol has been demonstrated to possess strong anti-inflammatory effects without profound immunosuppressive or glycogenic activities.[277, 278] 17-epi-estriol possesses negative effects on inflammatory and adhesion by suppressing TNFα-induced and nitric oxide-mediated VCAM-1 expression.[279] Thus, since preeclampsia is associated with abnormal increased expression of adhesion molecules and increased levels of pro-inflammatory cytokines that induce alterations in vascular vasodilatory responsiveness,[280] our findings suggest the first possibility that low 16-epi-estriol and 17-epi-estriol may potentially contribute to perturbations in inflammatory and impaired adhesion responses in preeclampsia.

In summary, our findings provide the first complete evidence that preeclampsia is associated with aberrant synthesis, metabolism and distinct levels of 15 estrogens and estrogen metabolites therefore suggesting important functional roles for a plethora of estrogens and estrogen metabolites in the pathogenesis of pregnancies complicated with preeclampsia (Figure 6). These findings also necessitate the need for prospective longitudinal studies covering early, mid and late trimesters to further assess and validate whether these estrogens and estrogen metabolites can be useful biomarkers in our understanding of the pathophysiology of preeclampsia, early prediction and identification.

Perspectives

Evidence is increasing that the multiple and diverse pathways by which primary estrogens are converted to multiple metabolites may contribute to numerous cardiovascular functions attributed to these estrogens in several vascular beds. The connection between estrogen metabolites and preeclampsia was first noted when the activity COMT was shown to be lower in the placentas of patients with severe preeclampsia and that pregnant mice deficient in COMT exhibit a preeclampsia-like phenotype. However, the diverse pathways that extensively convert

estrogens to multiple metabolites are extremely complex. This necessitates the importance to investigate a plethora of other functional estrogen metabolites to achieve a more comprehensive knowledge of their contributions, if any, in preeclampsia and other hypertensive diseases. We show herein that aberrant synthesis and metabolism of estrogens and estrogen metabolites in preeclampsia which suggests a connection and provides insight into several previously unknown and underappreciated critical links between the clinical pathophysiology of the disease with the cardiovascular and other functional physiology of these estrogens and estrogen metabolites (Figure 6).

Acknowledgements

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Novelty and Significance

What is New?

Preeclampsia is characterized by distinct aberrant synthesis and metabolism of estrogens and estrogen metabolites including estrone, estradiol-17β, 16-keto-estradiol, estriol, 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol, 4-methoxyestradiol, 16-epi-estriol and 17-epi-estriol.

What is Relevant?

Preeclampsia is a hypertensive disorder of pregnancy with an elusive etiology and affects 5% to 8% of pregnancies, thus remaining a significant cause of maternal and fetal morbidity and mortality as well as greater susceptibility and earlier onset of future cardiovascular disease in both mother and baby

Summary

Our findings demonstrate that preeclampsia is characterized by specific and distinct aberrant synthesis, metabolism and plasma accumulation of 15 individual estrogens and estrogen metabolites. The functional implications of these findings are potentially useful in our understanding of the pathophysiology of preeclampsia and exploration of better clinical management and outcomes.

TABLES AND FIGURES

Table 7.1

Clinical data and characteristics of the normP, mPE and sPE women

Clinical data and characteristics of the normP, mPE and sPE women			
Characteristic	**normP (n=8)**	**mPE (n=8)**	**sPE (n=8)**
Age (yr.)	33.6 ±1.8	33.0±1.4	25.5 ±2.0*†
Height (in)	66.8±1.0	66.0±1.4	66.0±0.7
Weight (lbs.)	213.0±17.4	200.1±11.7*	242.8±18.5*†
Body mass index	33.3±2.2	32.3±1.8	39.1±2.7*†
Systolic Blood Pressure (mmHg)	111.62±2.7	149.3± 3.9*	167.2± 7.0*†
Diastolic Blood Pressure (mmHg)	65.2±2.9	95.8±2.9*	105.0±4.9*†
24 Hour Total Urinary Protein (mg)		960.3± 398.2	4572.2±2499.9†
Gestational Age at Admit (wks.)	38.9±0.1	36.6±1.0	35.5±1.2
Gestational Age at Delivery (wks.)	38.9±0.1	37.8±0.4	35.4±1.2
Values are means ±SEM			
*Different (P<0.05) compared with normP group. † Different (P<0.05) compared to mPE group.			

Figure 7.1

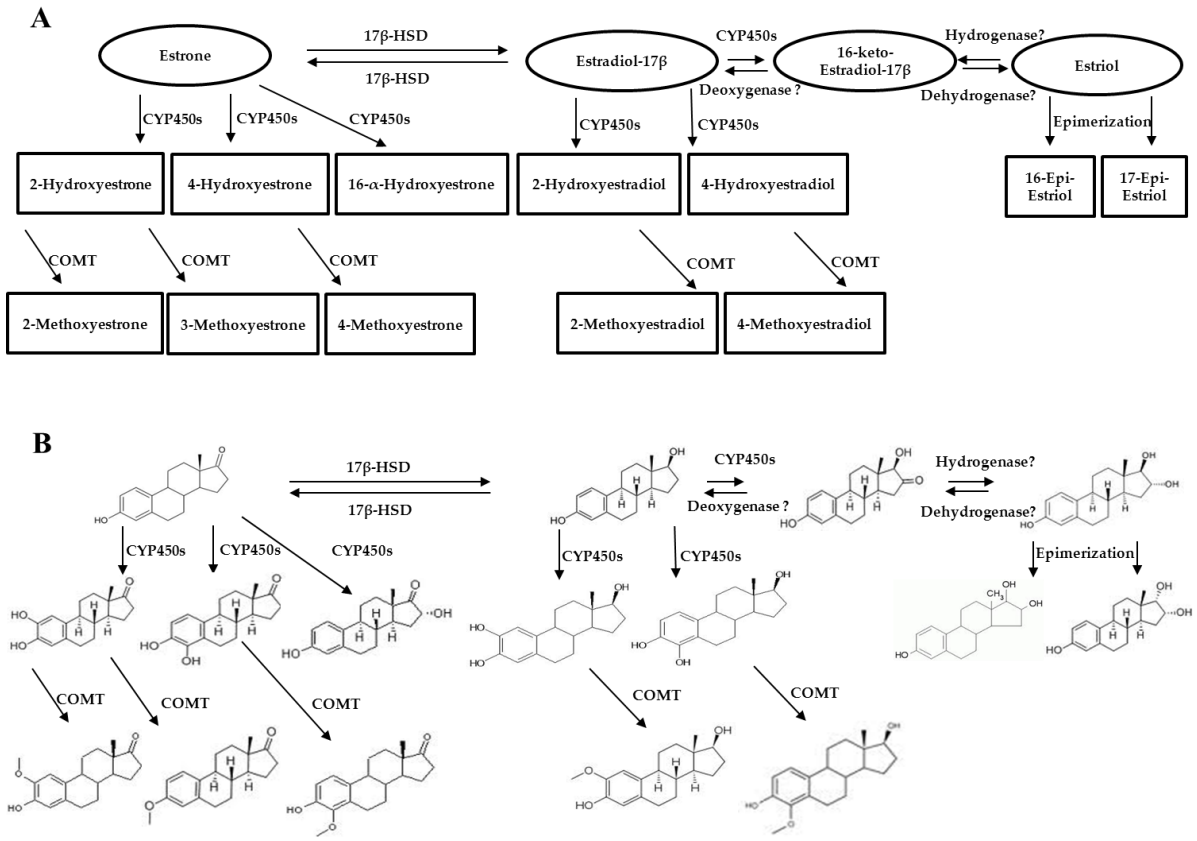


Figure 7.1: Schematic (A) and structural (B) representation of the complexities of the synthesis and multiple mechanisms of metabolism of estrogens and estrogen metabolites. Figure also depicts our novel working hypothesis that 16-keto-estradiol maybe a possible interconversion metabolite between estradiol-17β and estriol through either the sequential actions of CYP450s and a hydrogenase or the sequential actions of a dehydrogenase and a deoxygenase. 17β-HSD (17β-hydroxysteroid dehydrogenase), CYP450s (cytochrome P450) and COMT (catechol-*O*-methyltransferase)

Figure 7.2

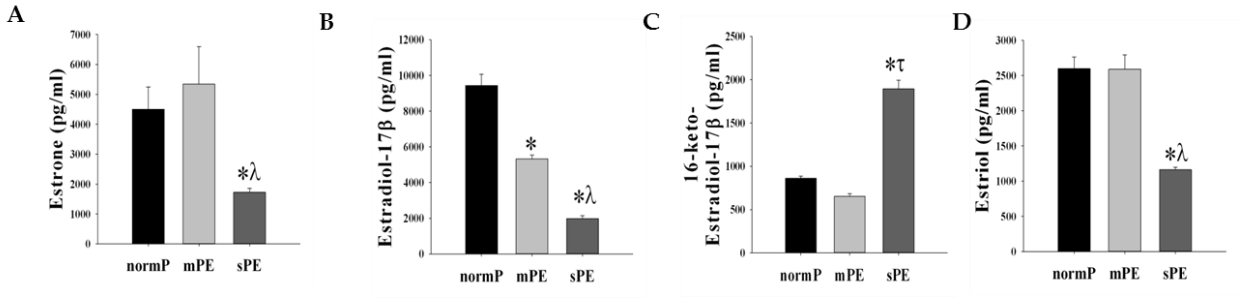


Figure 7.2: Plasma levels of estrone, estradiol-17β, 16-keto-estradiol-17β and estriol in normotensive pregnant control women (normP; n=8), women with mild preeclampsia (mPE; n=8), and women with severe preeclampsia (sPE; n=8). *Significantly different ($P< 0.001$) in levels compared to normP. λ Significantly different ($P< 0.001$) in levels compared to mPE. τ Significant increase ($P< 0.001$) in levels compared to normP.

Figure 7.3

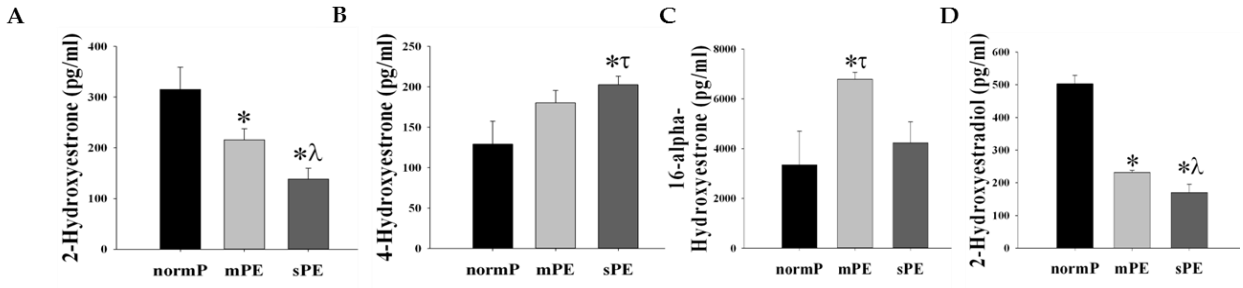


Figure 7.3: Plasma levels of 2-hydroyestrone, 4-hydroyestrone, 16-α-hydroxyestrone and 2-hydroxyestradiol-17β, in normotensive pregnant control women (normP; n=8), women with mild preeclampsia (mPE; n=8), and women with severe preeclampsia (sPE; n=8). *Significantly different ($P< 0.001$) in levels compared to normP. λ Significantly different ($P< 0.001$) in levels compared to mPE. τ Significant increase ($P< 0.001$) in levels compared to normP.

Figure 7.4

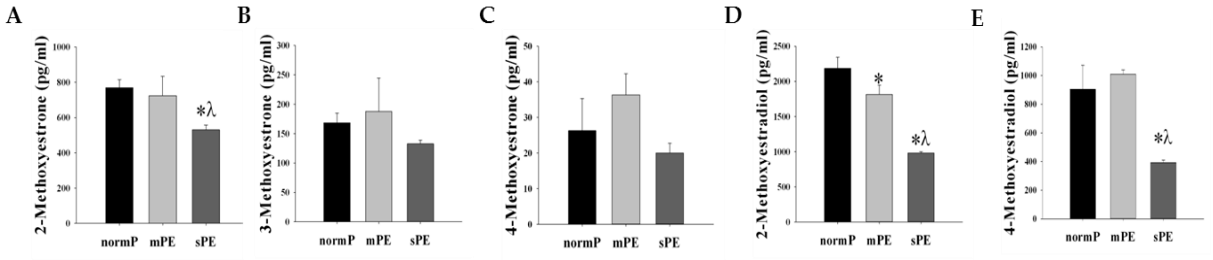


Figure 7.4: Plasma levels of 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol in normotensive pregnant control women (normP; n=8), women with mild preeclampsia (mPE; n=8), and women with severe preeclampsia (sPE; n=8). *Significantly different ($P< 0.001$) in levels compared to normP. λ Significantly different ($P< 0.001$) in levels compared to mPE.

Figure 7.5

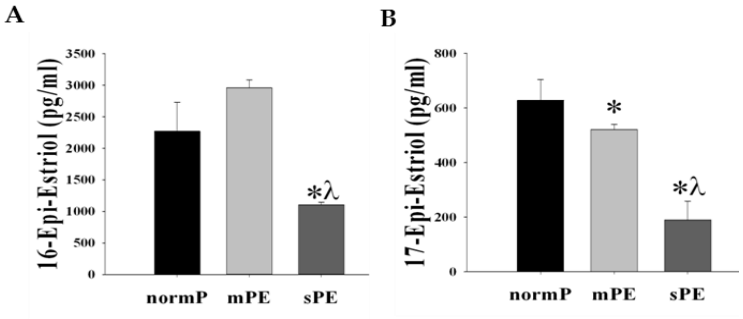


Figure 7.5: Plasma levels of 16-epi-estriol and 17-epi-estriol in normotensive pregnant control women (normP; n=8), women with mild preeclampsia (mPE; n=8), and women with severe preeclampsia (sPE; n=8). *Significantly different ($P< 0.001$) in levels compared to normP. λ Significantly different ($P< 0.001$) in levels compared to mPE.

Figure 7.6

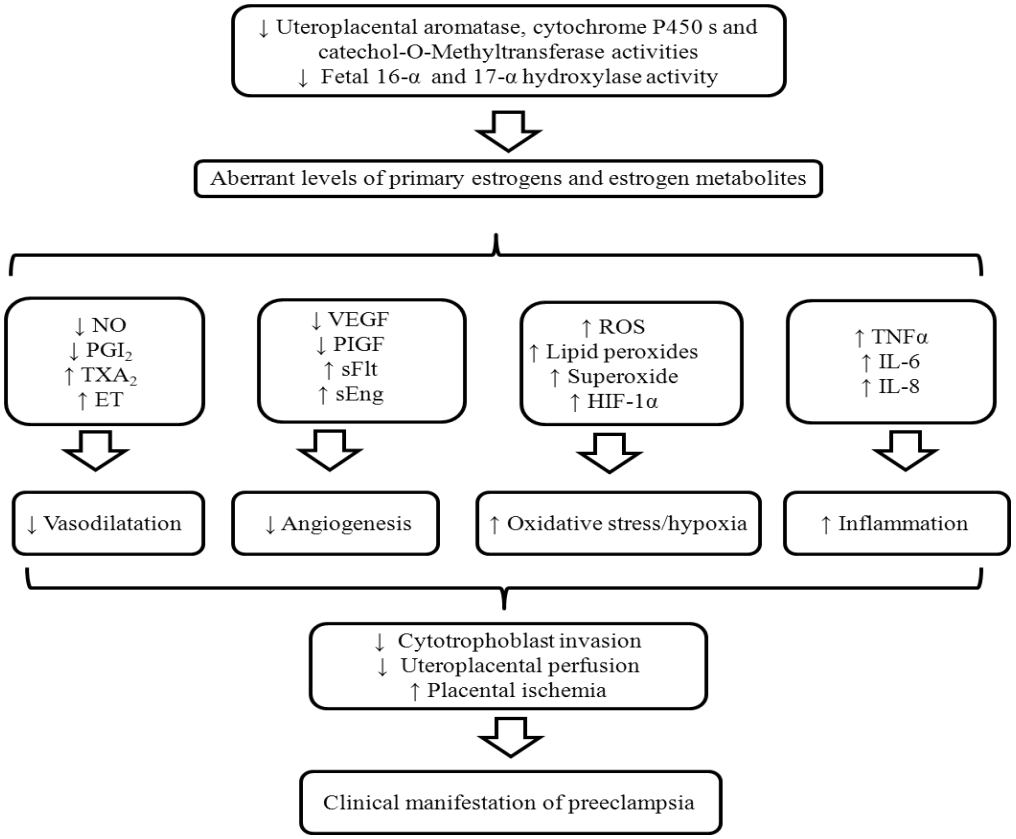
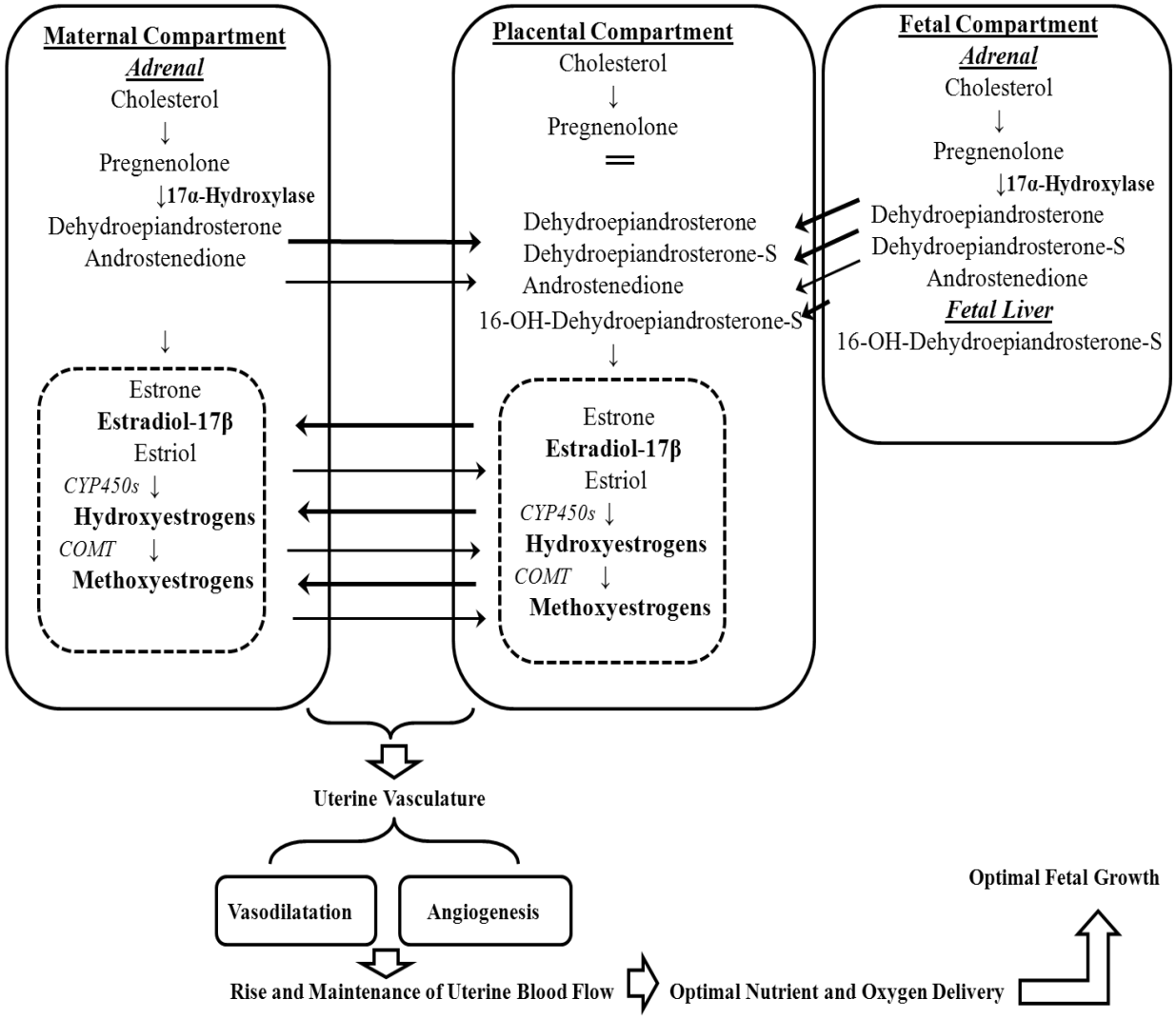


Figure 7.6: We propose a working hypothesis that reduced activities of uteroplacental aromatase, cytochrome P450s, catechol-*O*-methyltransferase and fetal 16-α and 17-α hydroxylase activities leading to low levels of primary estrogens and estrogen metabolites causes low activities of several local molecular mediators of numerous normal uteroplacental adaptations during pregnancy thereby triggering some of the pathophysiology of the clinical manifestations of preeclampsia. NO, nitric oxide; PGI_2, prostacyclin; TXA_2, thromboxane; ET, Endothelin; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; sFlt1, soluble Fms-like tyrosine kinase 1; sEng, soluble endoglin; ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor 1; TNFα, tumor necrosis factor-alpha; IL-6, interleukin 6; IL-8, interleukin 8

CHAPTER 8:

GENERAL DISCUSSION



We propose a working hypothesis that circulating and/or locally produced estrogens and/or estrogen metabolites regulate maternal uterine vascular adaptations during pregnancy including angiogenesis and vasodilatation. We believe that the multiple and diverse pathways by which primary estrogen is converted to multiple metabolites may contribute to numerous cardiovascular adaptations attributed to the parent substrate in the uterine vascular bed during pregnancy. While not exhaustive and comprehensive, this model is built upon data we collected in this study and integrates numerous findings.

Systemic and Local Uterine Plasma Concentrations of Estrogen and Estrogen Metabolites during Pregnancy

Although research on the metabolism of estrogens by target tissues has been pursued during the past 20 years, and reviews on certain aspects of this topic have appeared, the lack of simple, specific and sensitive assays capable of simultaneously quantitating the levels of estrogens and estrogen metabolites from biological fluids has hindered research aimed at elucidating the role of these steroids in health and disease. Even though to-date, many assays have been published that measure estrogens, there is no published assays that has s separate and simultaneously measured estrogens and estrogen metabolites in the uterine and systemic circulation whilst focused on pregnancy vascular adaptation. For the purposes of this thesis and for our understanding of the numerous cardiovascular and other physiologies of estrogens and estrogen metabolites in pregnancy, it was necessary to first establish the plasma concentrations of estrogens and estrogen metabolites. With respect to this first study, it is evident that the dynamics of synthesis and metabolism of estrogens and estrogen metabolites differs significantly in pregnancy compared to the nonpregnant state. The plasma concentrations of the estrogens and estrogen metabolites measured in this study suggests that pregnancy is characterized by specific synthesis and metabolism of estrogens and estrogen metabolites leading to distinct and increased plasma accumulation of these steroids in the local uterine and systemic circulations. These findings have numerous implications in our understanding of placental steroid biosynthesis during pregnancy. It is well established that during normal pregnancy, plasma levels of estrone, estradiol-17β and estriol are increased and parallel some of the increases in uteroplacental blood flow as well as development of an extensive uteroplacental vascular bed.[1, 214] During this time, the placenta is the main source of these estrogens using circulating steroid precursors from the

maternal uterine compartment, adrenal glands as well as fetal adrenal glands.[1] The placental biosynthesis of primary estrogens during pregnancy is complex and results from interaction and interdependence of separate maternal, placental and fetal systems that individually do not possess the necessary enzymatic capabilities to make these critical estrogens.[214] Because the placenta does not express 17α-hydroxylase, the obligatory synthesis of C19-steroid precursors is not possible for estrogen synthesis.[214] Therefore, the primary classical estrogens are synthesized from dehydroepiandrosterone and dehydroepiandrosterone-sulfate precursors synthesized from the maternal and fetal adrenal glands.[270] It follows thus that any discussion of the role of estrogens in pregnancy fundamentally stops at the classical biosynthesis pathways and neglects for the most part the complex metabolism of the primary estrogens and how this may play a role in the bioavailability of these steroids. In addition, for ethical reasons, investigation of maternal uteroplacental steroid biosynthesis in pregnant women has been restricted by the inability to utilize invasive experimental procedures. Therefore, animal models such as the ovine become critical in our understanding of steroid biosynthesis and metabolism during pregnancy. We have thus demonstrated that placental steroid biosynthesis does not stop at the synthesis of the primary estrogens but that these classical steroids follow numerous, diverse and multiple pathways that convert them to a multitude of other metabolites with physiological function. Therefore, the take home message from this aim is simple: that when it comes to placental biosynthesis, the concept that all the synthesis and/or functional relevance stops at the creation of primary estrogenic steroids may not be the whole story. We thus propose that a better understanding of the metabolism of these classical steroids following primary synthesis is essential to our understanding of their comprehensive functions in pregnancy-induced cardiovascular adaptations.

The Effects of Estradiol-17β and Estradiol Metabolites on Uterine Endothelial Prostacyclin Production: Role of ER-α versus ER-β

There is substantial evidence that endothelial-derived vasodilators such as nitric oxide and prostacyclin produced by the uteroplacental unit plays an important role in the regulation of vascular tone and ultimately blood flow during pregnancy. Consequently, it was critical to examine the effects of estrogens and estrogen metabolites on this pivotal process on the maternal uterine endothelial cells. Although prostacyclin is a potent vasodilator, its role in pregnancy vascular adaptations is not as well understood as that of nitric oxide, which has been shown to have a definitive role in partly regulating uteroplacental blood flow during gestation especially estrogen-induced effects. Therefore, we opted here to elucidate the effects of estrogens and estrogens metabolites on prostacyclin production in the uterine artery endothelial cells in the pregnant versus the nonpregnant state. Estrogen is well established as vasoactive hormone that can initiate both rapid and chronic vasodilation in both local uterine and systemic vasuclar beds.[87, 221, 281] In addition, the studies of Rosenfeld and Jackson[195] as well as Stice and coworkers[196], laid the foundation that estrogen metabolites especially the catecholestrogens also play important roles in the regulation of blood flow during pregnancy via vasodilatory actions. Interestingly, in the studies of Rosenfeld and Jackson[195] , the authors concluded using Lineweaver-Burk plots that since the y-intercept was similar between estrogen and the catecholestrogens that these steroids maybe acting through the same receptor. In this regard, our data that the estrogen metabolites including the catecholestrogens stimulate prostacyclin production independent of the estrogen receptors contradicts the findings of Rosenfeld and Jackson[195]. We believe that Rosenfeld and Jackson[195] may have overlooked the complexity of estrogen metabolites mechanims of actions since Lineweaver-Burk plots are only able to explain

the receptor kinetics of compounds as competing for the same receptor but unable to identify the types of receptor mediating an action. Indeed, catecholestrogens have been known to bind to estrogen receptors, albeit with lower affinity compared to the parent estrogen.[176] Nevertheless, it is important to note that a physiological cause and effect relationship has not been established between increases in uterine blood flow and PGI_2. Magness et al (1992) have shown that local infusion of the nonspecific COX-1/COX-2 inhibitor indomethacin does not significantly drop basal uterine blood flow in pregnant sheep.[69] These observations support the conclusion that during late ovine gestation, basal uterine blood flow may not be primarily dependent on maintaining basal production of PGI_2. [69] However, those studies have still not ruled out the possibility that PGI_2 may play important roles in the maintenance of uterine blood flow in pregnancy. One possible explanation is that since multiple vasodilators regulate vascular tone during pregnancy, that inhibiting one of them is not enough to completely inhibit rises in uterine blood flow and that rises in uterine blood flow may be controlled by several mechanisms. Because how does one explain then why low PGI_2 is implicated in all hypertensive disorders of pregnancy including preeclmapsia which are characterized by high vascular resistance, aberrant vasuclar tone as well as low uteroplacental blood flow. Also what would then be the physiologic relevance of the uterine vasculature exhibiting increased expression of the prostanoid system enzymes in ovine uterine arteries if they are not needed? Nevertheless, our findings under this aim suggest that estrogens and estrogen metabolites influence a higher de novo endothelial biosynthesis of prostacyclin in cells derived from pregnancy compared to the nonpregnant state, which may be important in the regulation of vascular tone during pregnancy. Herein, we demonstrate novel and compelling evidence that the vasodilatory effects of $E_2\beta$ during pregnancy may also involve its sequential conversion to catecholestradiols and methoxyestradiols which are

capable of stimulating ER-independent endothelial PGI_2 synthesis. Additional studies are required to understand whether the ER-independent induction of endothelial PGI_2 synthesis by estrogen metabolites within uterine vasculature represents unappreciated signaling complexity of estrogens or just simply an evolutionary functional redundancy. Nevertheless, our findings necessitate the evaluation of catecholestradiols and methoxyestradiols in the regulation of vascular tone in physiology via endothelial-derived relaxing factors as well as dysregulation in the pathophysiology of vascular diseases such as hypertension, atherosclerosis and gestational vascular diseases such as preeclampsia.

The Effects of Estradiol-17β and Estradiol Metabolites on Uterine Endothelial Proliferation: Role of ER-α versus ER-β

Adequate uteroplacental angiogenesis is critical for the establishment an intricate uteroplacental circulation and thus crucial for normal fetal growth and development.[75, 78] The patterns of uteroplacental angiogenesis coincide with the dramatic increases that have been reported for uteroplacental blood flows during gestation.[78, 282] Multiple lines of evidence suggest that estrogen directly modulates angiogenesis via effects on endothelial cells. In addition, under physiological conditions, angiogenesis is routinely observed in the uterus in association with fluctuations in the levels of circulating estradiol and other sex steroids. $E_2\beta$ induces endothelial proliferation and migration mediated by the classic estrogen receptor, which is expressed by endothelial cells from several vascular beds including the uterine vasculature. In this regard, our findings under this aim support several lines of evidence that $E_2\beta$ mediated pregnancy-induced angiogenesis supporting the notion that estrogen regulates uterine blood flow via angiogenic mechanisms. Demonstrating a role for ER-α and/or ER-β, ICI abrogated $E_2\beta$-induced P-UAEC proliferation, supporting previous observations that ICI blocks $E_2\beta$-induced P-UAEC [H^3]-

thymidine incorporation and $E_2\beta$-induced vascular endothelial growth factor–mediated proliferation Antagonism of ER-β with PHTPP abrogated $E_2\beta$-induced P-UAEC proliferation and ER-β activation with DPN-induced proliferation demonstrating an ER-β only effect. Therefore, the differences in P-UAEC proliferation in response to DPN, PPT, or $E_2\beta$ may be because of their distinct differences in affinity for ERs in association with the complex nature of ER-ligand complexes. Nevertheless, PHTPP inhibition of $E_2\beta$- and DPN-induced P-UAEC proliferation validates that these $E_2\beta$ effects are solely ER-β mediated and independent of ER-α. The differences and receptor selectivity in response may be due to the differences in the structure-function characteristic of each receptor; therefore, functional heterogeneity of the receptor subtypes, in turn, allows for the induction of a multitude of diverse biological outcomes by estrogen on the uterine vasculature during pregnancy. Our findings under this aim that estrogen metabolites with the exception of 2-methoxyestradiol stimulate significant proliferation of P-UAECs and not NP-UAECs supports our hypothesis that these metabolites also may play important roles in the angiogenesis and thus increases and/or maintenance of uterine blood flow. However, in contrast to estrogen's actions, the metabolites induce these actions independent of the estrogen receptors and points to the notions that estrogen metabolites possess several actions independent of the parent substrate. We do not know whether the action of the estrogen metabolites on uterine vascular endothelial cells represents unappreciated signaling complexity of estrogen metabolites or just simply an evolutionary functional redundancy to control angiogenesis at multiple levels. Nevertheless, our findings implicate estrogen metabolites in the regulation of angiogenesis via actions on endothelial cell proliferation and point to the possibility that metabolism of $E_2\beta$ by CYP450s and COMT may partly define the maternal cardiovascular responses to estradiol during pregnancy. Although the *in vivo* physiological effects of

catecholestradiols and methoxyestradiols in the uterine compartment is presently not completely understood and is essentially nonexistent in standard textbooks of pharmacology and endocrinology, this aim necessitates a serious rethinking of investigating the angiogenic and other actions of estrogen by itself on the uterine vasculature during pregnancy without concurrently evaluating the effects of it metabolites.

The Effects of Catecholamines and Adrenergic Receptors on Estradiol-17β and Estradiol Metabolites-Induced Uterine Endothelial Proliferation

Our observations that the estrogen metabolites, 2-hydroxyestradiol, 4-hydroxyestradiol and 4-methoxyestradiol stimulated significant proliferation of P-UAECs but not NP-UAECs via estrogen receptor independent mechanims presented an interesting conundrum. Consequently, we developed an aim to attempt to clarify whether estrogen metabolites induce proliferation via other receptor-mediated mechanisms outside the estrogen receptors. Our investigation and review of earlier literature showed very significant observations by several investigators about the biology of compounds with "catechol" structures. Owing to a shared "catechol" structure, catecholestrogens have been shown in vitro and in vivo to interact with the biosynthesis, metabolism and mechanisms of action of catecholamines, a property not shared by the parent $E_2\beta$.[186, 236] These include inhibition of the enzymes tyrosine hydroxylase and catechol-O-methyltransferase as well as blockade of accumulation of dopamine and norepinephrine in rat brain synaptosomes.[194, 283, 284] Importantly, in all these studies, changes in the D ring and/or amine structures of the catecholestrogens and catecholamines respectively, does not appear to markedly affect the responses.[284, 285] Catecholestradiols also 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.[240, 284-286] 3D structural and

functional analyses demonstrate that the catecholamine catechol moiety is functionally important in AR activation.[240] Collectively, these observations led us to hypothesis that the estrogen receptor–independent mitogenic effects of 2-OHE_2 and 4-OHE_2 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. We describe in this aim 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 model by which estrogen metabolites function as potential circulating β-AR mimetic agonists. Therefore, modifying the phenolic A ring of estrogens to catechol moieties appears to generate an endogenous β-mimetic agent with angiogenic and possibly other cardiovascular capabilities. Overall, the present aim indicates that actions of catecholestradiols and catecholamines via endothelial ARs represent an evolutionary conserved and highly versatile signaling mechanism for regulating endothelial proliferation. Since maternal uterine perfusion is maintained 1- to 2-fold in excess of the needs of the parallel, but separate, fetoplacental circulation during flight or fight, Magness et al[1, 254] have shown previously that, during an acute gestational flight or fight response, 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. This aim 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. We believe that the findings in this aim also points to the potential relevance of the convergence of the sympathetic catecholamine system and estrogen metabolism systems in the regulation of vascular responsiveness and growth during pregnancy

The Role of Signaling Pathways in Estradiol-17β and Estradiol Metabolites-Induced Uterine Endothelial Cell Proliferation

The results in this aim show that even with the absence of common receptor-mediated routes, estrogen and its metabolites stimulate signaling pathways in uterine artery endothelia cells that converge at the level of mitogen-activated protein kinases including p42/44, p38 and JNK. Even though this aim was done to evaluate the signaling pathways in angiogenic properties of the uterine artery endothelial cells, we cannot rule out the notion that similar pathways maybe implicated in vasodilatory events.[287] Highly conserved signaling pathways that activate the mitogen-activated protein kinases are involved in relaying extracellular stimuli to intracellular responses. Endothelial cells recognize and respond to extracellular stimuli by engaging specific intracellular programs, such as the signaling cascade that leads to activation of the mitogen-activated protein kinases.[288] Although each MAPK named above has unique characteristics, a number of features are shared by the MAPK pathways studied to date. Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK).[289, 290] The MAPKKKs, which are serine/threonine kinases, are often activated through phosphorylation and/or as a result of their interaction with the Ras family. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on

threonine and tyrosine residues. For these reasons, we believe that our observations of a convergence of the actions of estrogen versus its metabolites is not surprising given the highly conservative nature of MARKs in mediating cellular growth processes such as proliferation.

The Plasma Profile of Estrogens and Estrogen Metabolites in Preeclampsia

Evidence including our own data is increasing that the multiple and diverse pathways by which primary estrogens are converted to multiple metabolites may contribute to numerous cardiovascular functions attributed to these estrogens in several vascular beds. The connection between COMT and preeclampsia was first noted in 1988, when COMT activity was found to be lower in the placentas of patients with severe preeclampsia.[291] To better elucidate the role of COMT in preeclampsia, a genetic COMT knockout ($COMT^{-/-}$) mouse model was recently developed by Kanasaki and coworkers.[231] Interestingly, pregnant $COMT^{-/-}$ mice developed a preeclampsia-like phenotype characterized by proteinuria, increased blood pressure, fetal wastage and histopathological changes in the placenta and kidney.[231] This phenotype was accompanied by lower plasma concentrations of 2-ME_2 and higher placental protein levels of HIF-1α.[231] Infusion of 2-ME_2 in $COMT^{-/-}$ mice decreased HIF-1α and ameliorated the preeclamptic phenotype without toxicity. Together, these results suggest that COMT and 2-ME_2 deficiency may play a significant role in the development of preeclampsia. However, the precise means by which 2-ME_2 prevents the emergence of preeclampsia remain unknown and not completely studied. 2-ME_2 was found to suppress HIF-1α and sFlt1, recent experiments suggest that there might be several other mechanisms through which 2-ME_2 promotes vascular health.[231] In addition to preventing cerebral vasospasm, 2-ME_2has been shown to inhibit smooth muscle cell growth and block the synthesis of the vasoconstrictor protein endothelin.[206, 292] Other experiments have suggested that 2-ME also has cardioprotective and anti-inflammatory

properties. More recently, studies by Pérez-Sepúlveda and coworkers have shown that during the first trimester of pregnancy, women who would develop preeclampsia have lower plasma levels of 2-ME_2 compared with normal pregnant controls.[293] All being said and as per our studies under this aim, the diverse pathways that extensively convert estrogens to multiple metabolites are extremely complex. This necessitates/necessitated the importance to investigate a plethora of other functional estrogen metabolites to achieve a more comprehensive knowledge of their contributions, if any, in preeclampsia and other hypertensive diseases. We show herein under this aim that aberrant synthesis and metabolism of a plethora of estrogens and estrogen metabolites in preeclampsia which suggests a connection and provides insight into several previously unknown and underappreciated critical links between the clinical pathophysiology of the disease with the cardiovascular and other functional physiology of these estrogens and estrogen metabolites. We show herein that apart from 2-ME_2, other physiologically relevant estrogen metabolites including are also decreased in preeclampsia suggesting that the role of estrogen metabolites in preeclmapsia is more complex than previously thought. We believe that these findings necessitate the need for prospective longitudinal studies covering pre-pregnancy, early, mid and late pregnancies to further assess and validate whether these estrogens and estrogen metabolites can be useful biomarkers as well as aid in our understanding of the pathophysiology of preeclampsia, early prediction and identification.

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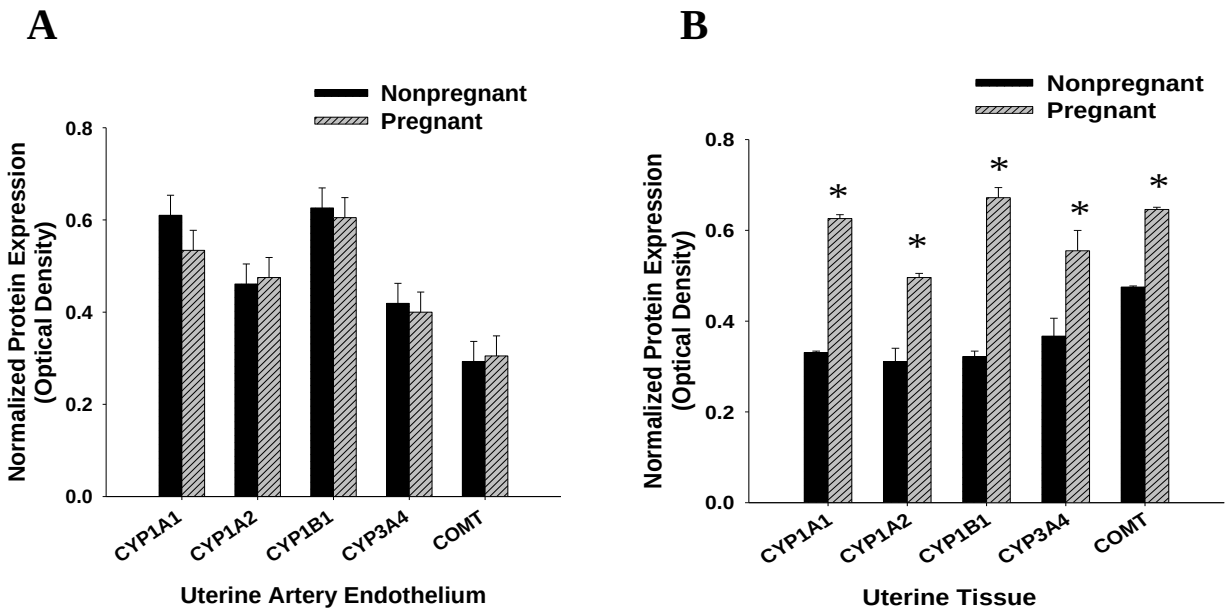
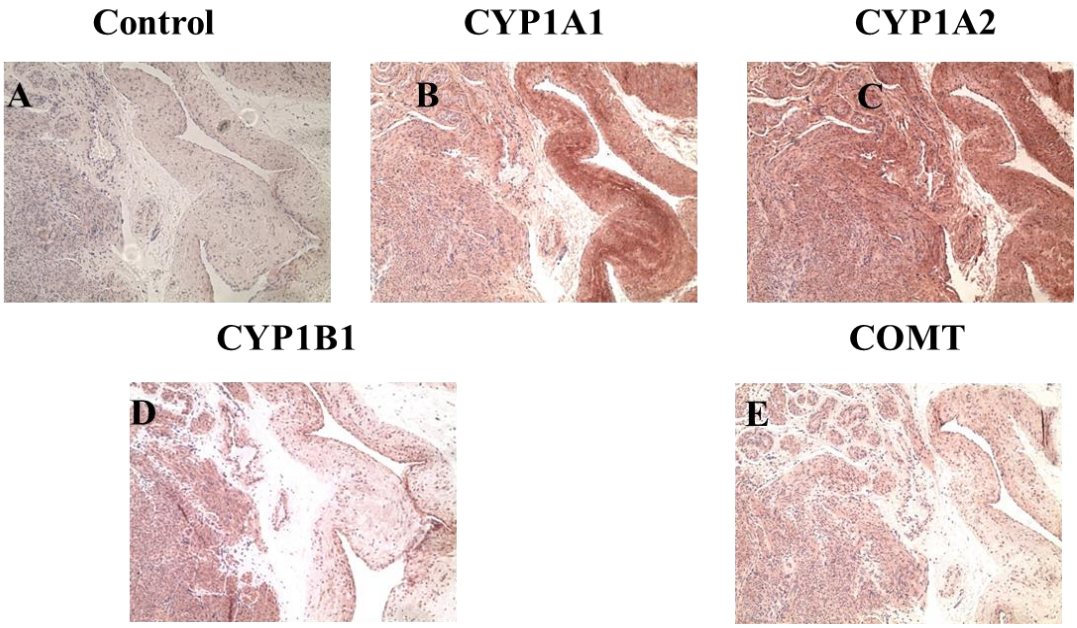
APPENDIX

Figure A.1



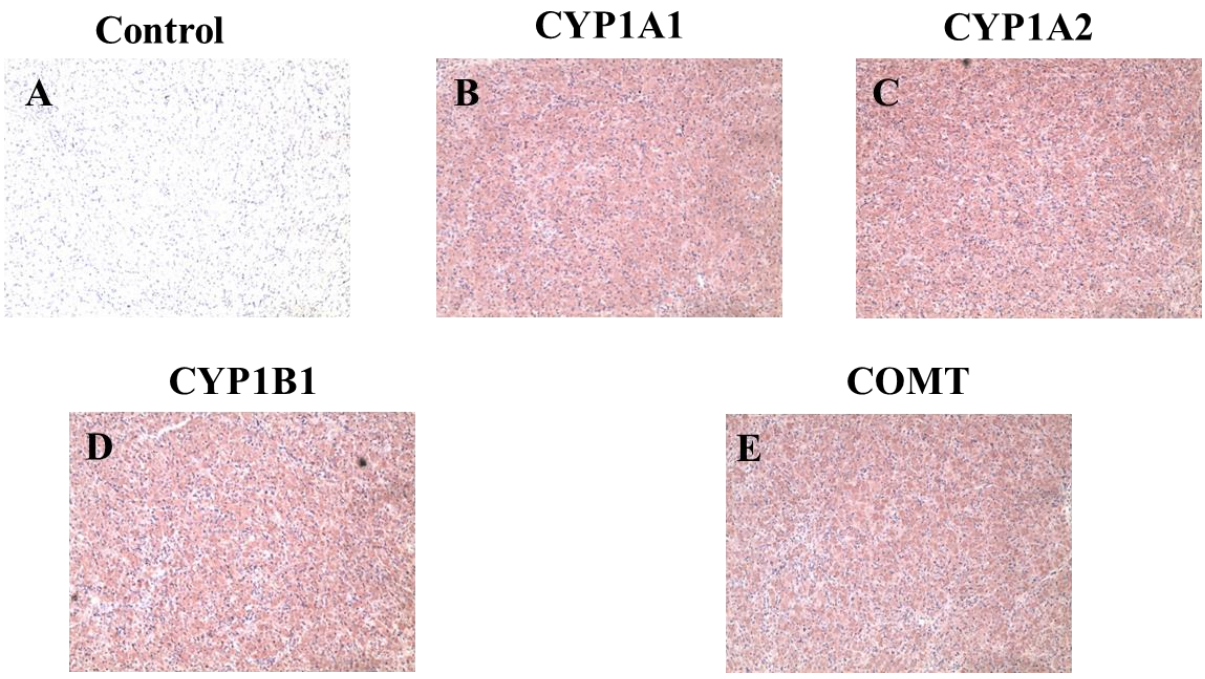
(A) Expressions of CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT in NP-UAECs versus P-UAECs. Densitometric analyses (relative protein expression=enzyme expression optical density/GAPDH optical density) showed no difference between NP-UAECs (n=6) and P-UAECs (n=6). P = 0.949, 2-way ANOVA. (B) Expressions of CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT in Nonpregnant versus Pregnant uterine tissue. Densitometric analyses (relative protein expression=enzyme expression optical density/GAPDH optical density) showed a significant difference between NP- (n=4) and P (n=4). * Increase in protein expression compared to respective nonpregnant group; $P < 0.05$, 2-way ANOVA.

Figure A.2



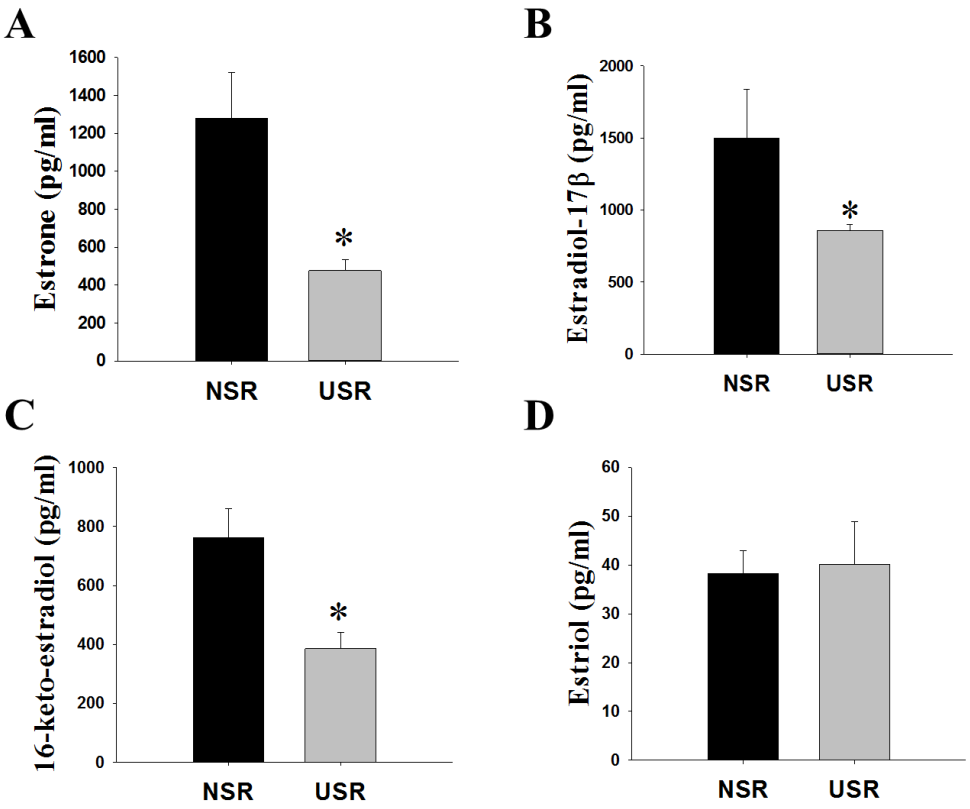
Hematoxylin–eosin (H&E) staining and immunohistochemical localization of CYP1A1, CYP1A2, CYP1B1 and COMT in caruncular section of pregnant sheep placentae.

Figure A.3



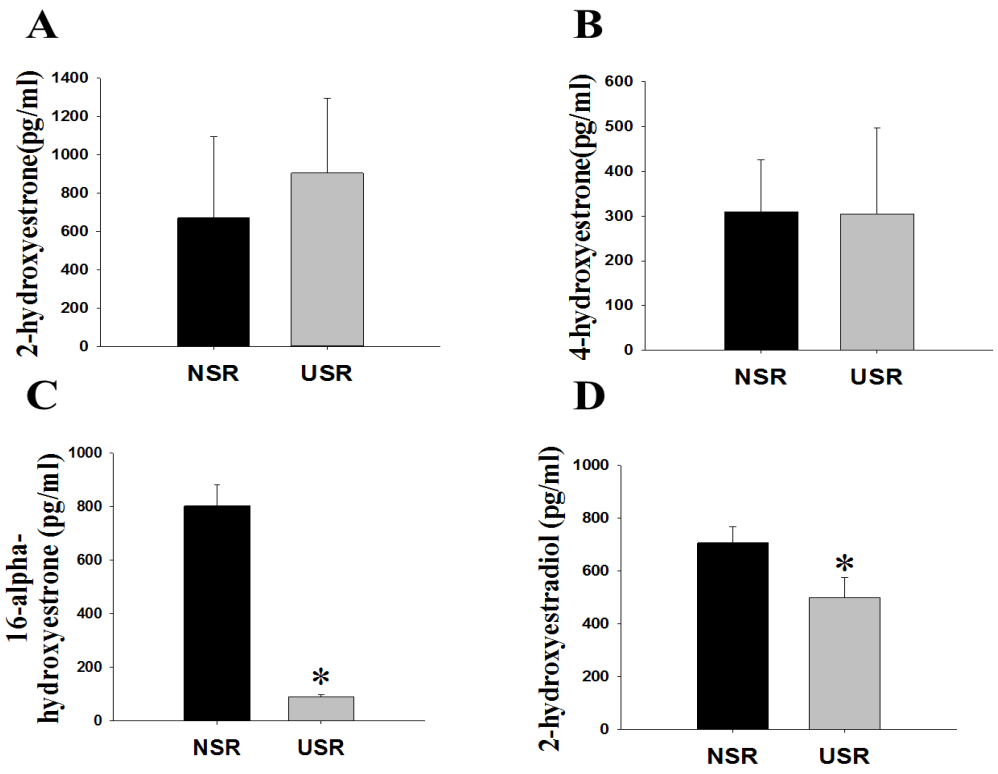
Hematoxylin–eosin (H&E) staining and immunohistochemical localization of CYP1A1, CYP1A2, CYP1B1 and COMT in corpus luteum of pregnant sheep.

Figure A.4



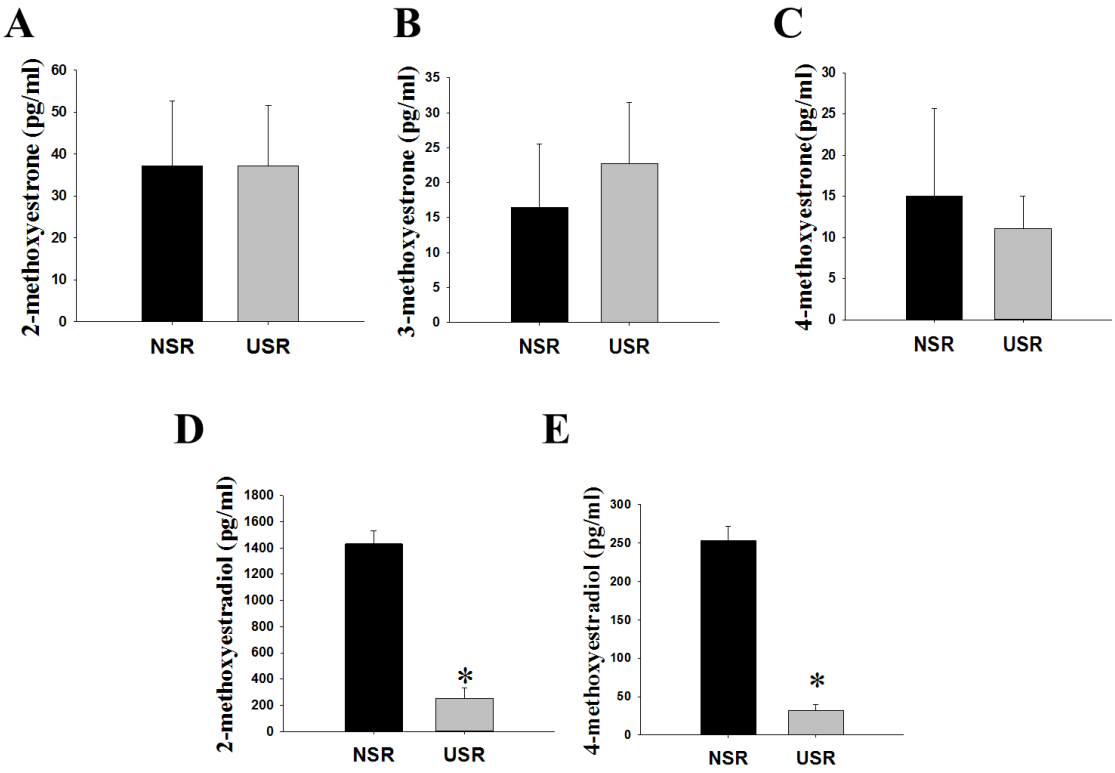
Products of primary synthesis: plasma levels of estrone, estradiol-17β, 16-keto-estradiol-17β, and estriol in unilateral space restricted (USR; n = 3) and control non-space restricted (NSR; n = 3). * Significantly different (P<0.001) in levels compared with unilateral space restricted (USR; n = 3).

Figure A.5



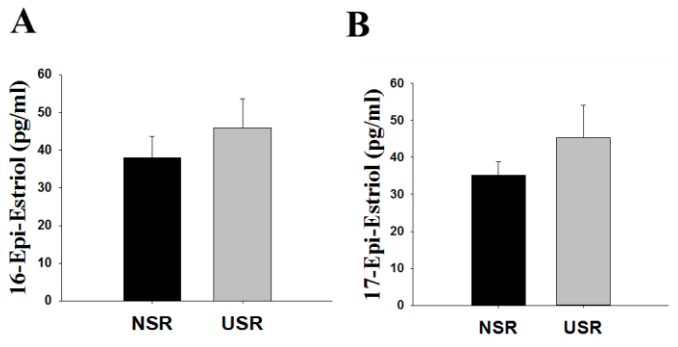
Products of hydroxylation: plasma levels of 2-hydroxyestrone, 4-hydroxyestrone, 16-α-hydroxyestrone and 2-hydroxyestradiol in unilateral space restricted (USR; n = 3) and control non-space restricted (NSR; n = 3). * Significantly different (P<0.001) in levels compared with unilateral space restricted (USR; n = 3).

Figure A.6



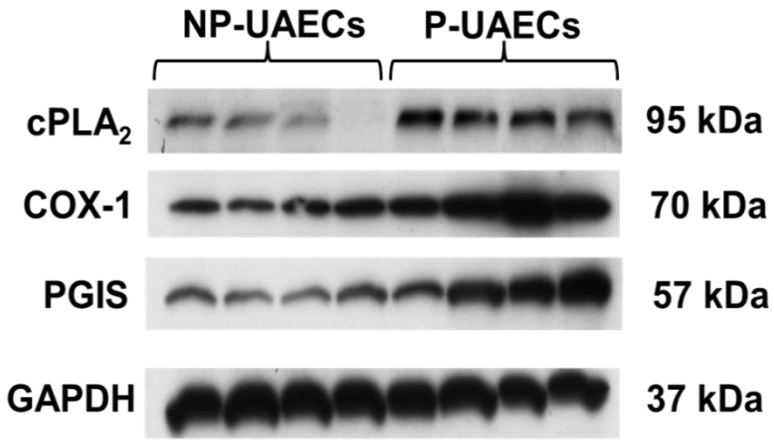
Products of *O*-methylation: plasma levels of 2-methoxyestrone, 3-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol in unilateral space restricted (USR; n = 3) and control non-space restricted (NSR; n = 3). * Significantly different (P<0.001) in levels compared with unilateral space restricted (USR; n = 3).

Figure A.7



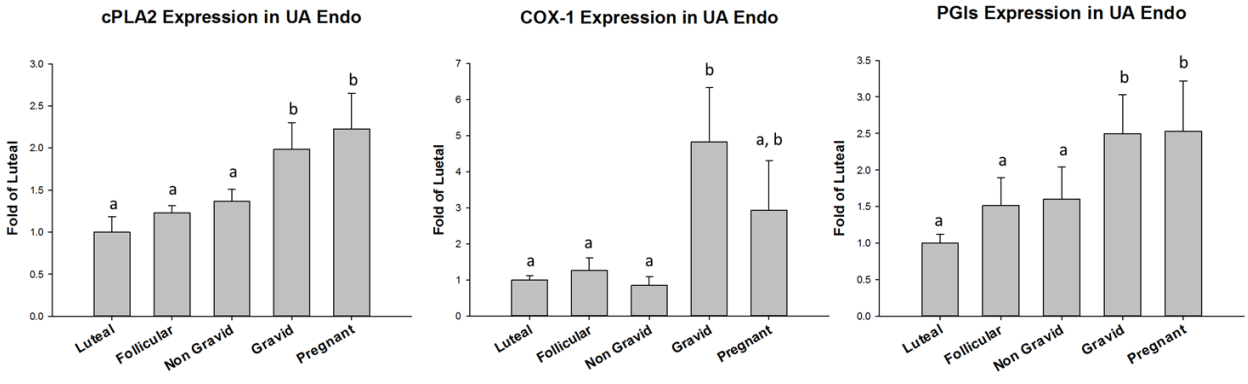
Products of epimerization: plasma levels of 16-epi-estriol and 17-epi-estriol in unilateral space restricted (USR; n = 3) and control non-space restricted (NSR; n = 3). * Significantly different (P<0.001) in levels compared with unilateral space restricted (USR; n = 3).

Figure A.8



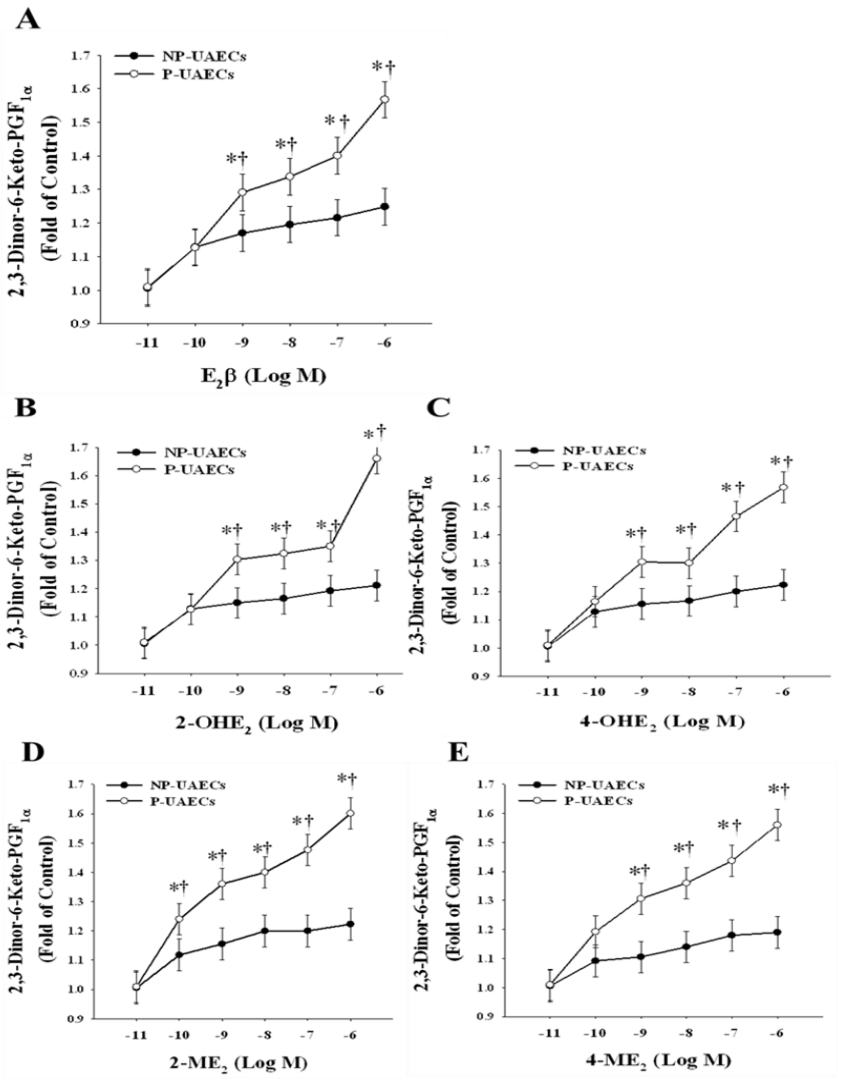
Expression of the prostanoid system enzymes in NP-UAECs and P-UAECs: Western blots demonstrating expression of $cPLA_2$, COX-1 and PGIS in NP-UAECs and P-UAECs.

Figure A.9



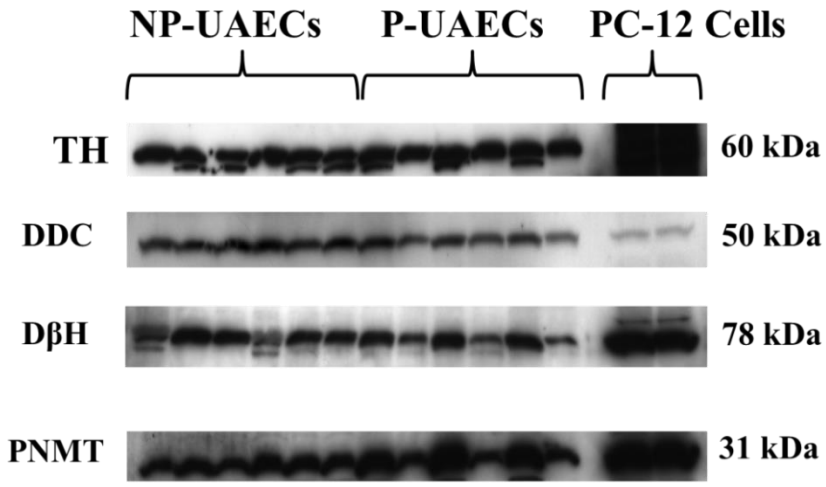
Comparison of COX-1, cPLA2 and PGIS protein levels in uterine artery endothelial-isolated protein (UA endo) from Luteal, Follicular, Non-Gravid, Gravid and Pregnant ewes. Western immunoblot analysis was performed with 20 µg per lane of UA endo protein from each ewe (L, F, P), and each side (NG and G) for Unilateral Pregnant ewes.

Figure A.10



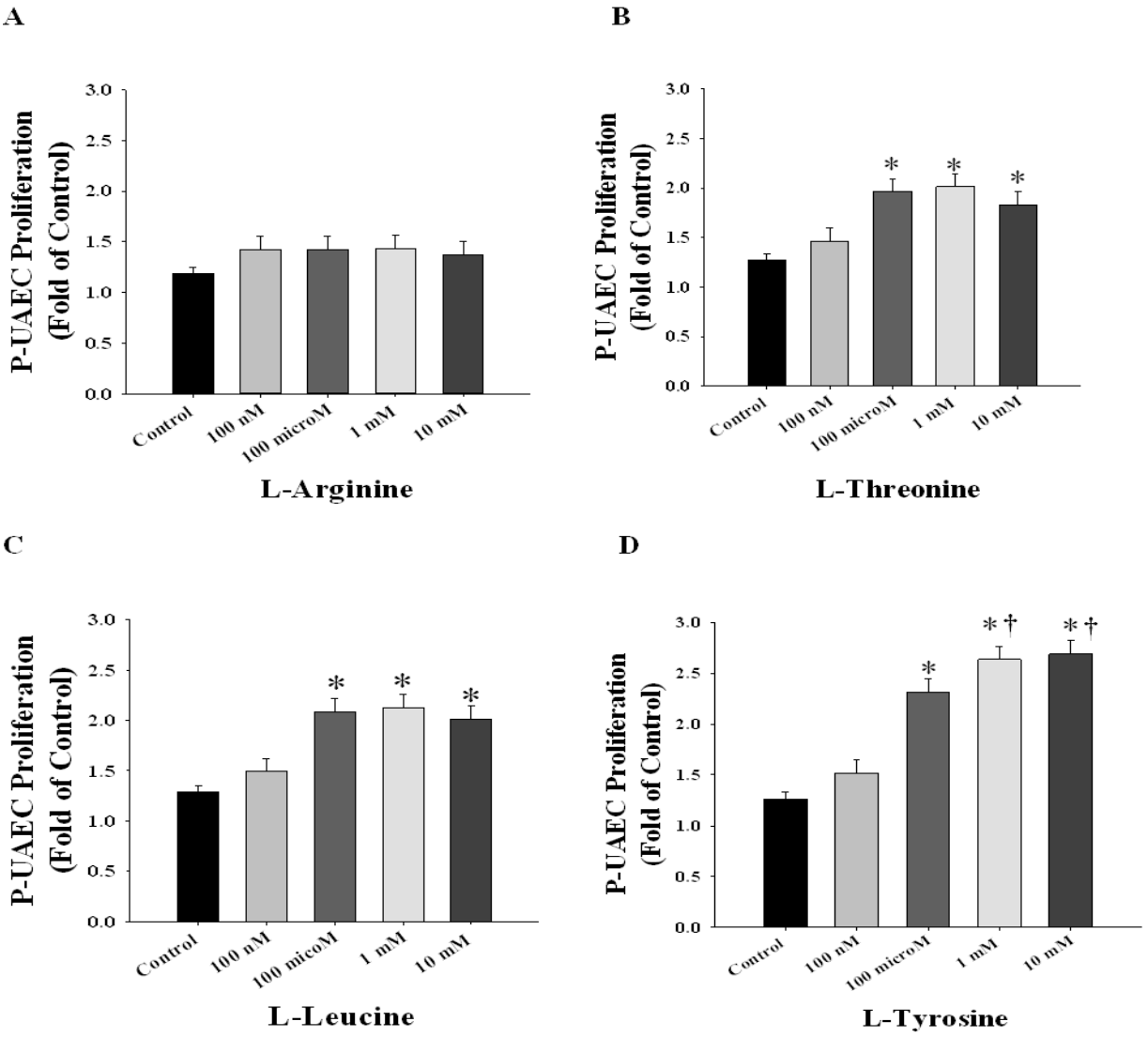
Concentration-dependent cell 2, 3-Dinor-6-Keto-PGF1α production by NP-UAECs and P-UAECs to (A) $E_2\beta$, (B) 2-OHE_2, (C) 4-OHE_2, (D) 2-ME_2, and (E) 4-ME_2. Concentration-dependent responses at 12 hrs were observed in P-UAECs in response to $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2; NP-UAECs exhibited lower 2,3-Dinor-6-Keto-PGF1α production. *Increase (P<0.05; n=4) in UAEC 2, 3-Dinor-6-Keto-PGF1α production compared with untreated control. † Increase in P-UAEC 2, 3-Dinor-6-Keto-PGF1α production compared to respective NP-UAEC (P<0.05; n=4) group at specified concentration.

Figure A.11



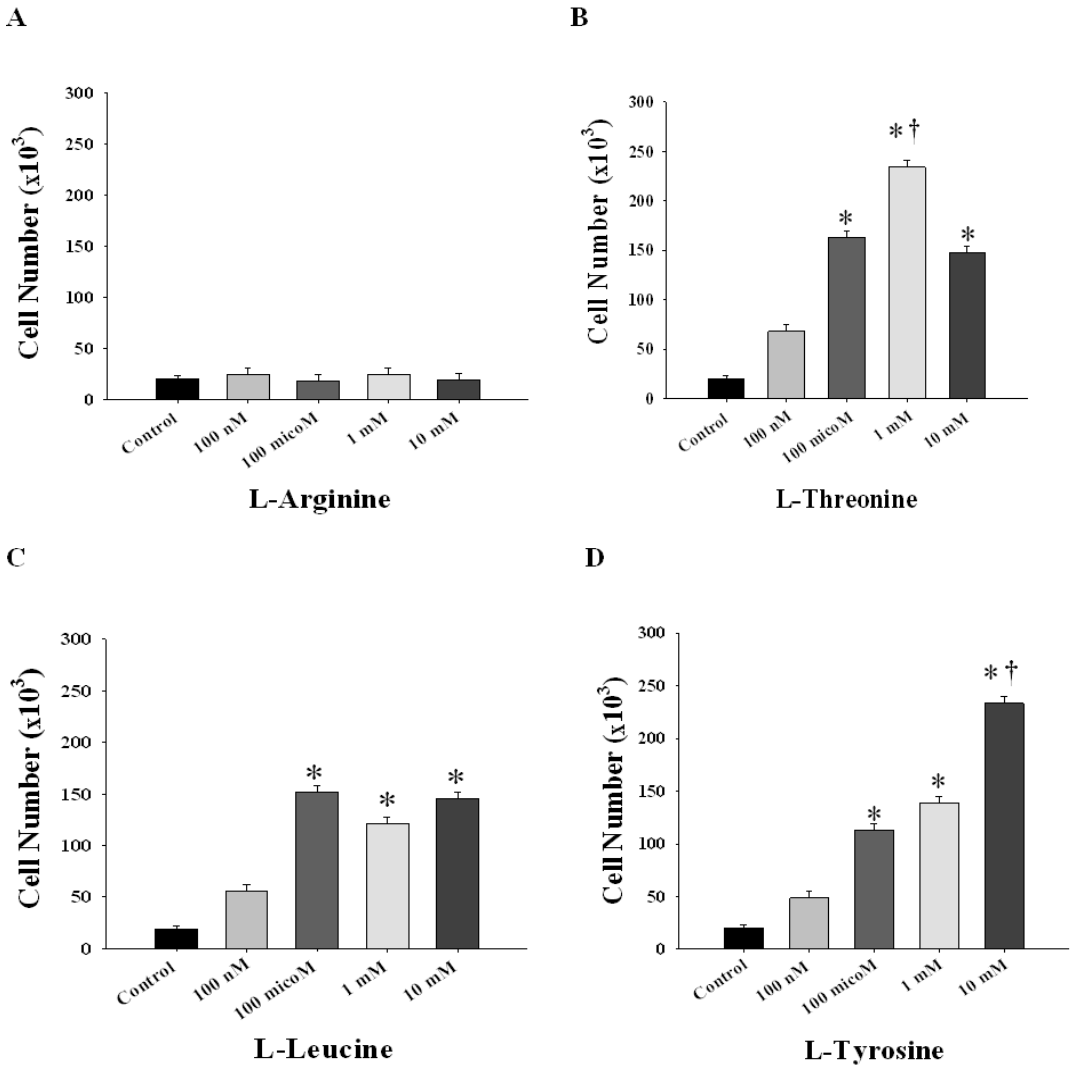
Western Immunoblotting provides evidence for the presence of catecholamine biosynthetic enzymes tyrosine hydroxylase, dopamine decarboxylase, dopamine β decarboxylase, and phenylethanolamine-*N*-methyltransferase in P-UAECs. The positive control lanes show protein expression of catecholamine biosynthetic enzymes in pheochromocytoma cells (PC-12), thus validating the presence of catecholamine biosynthetic enzymes in P-UAECs.

Figure A.12



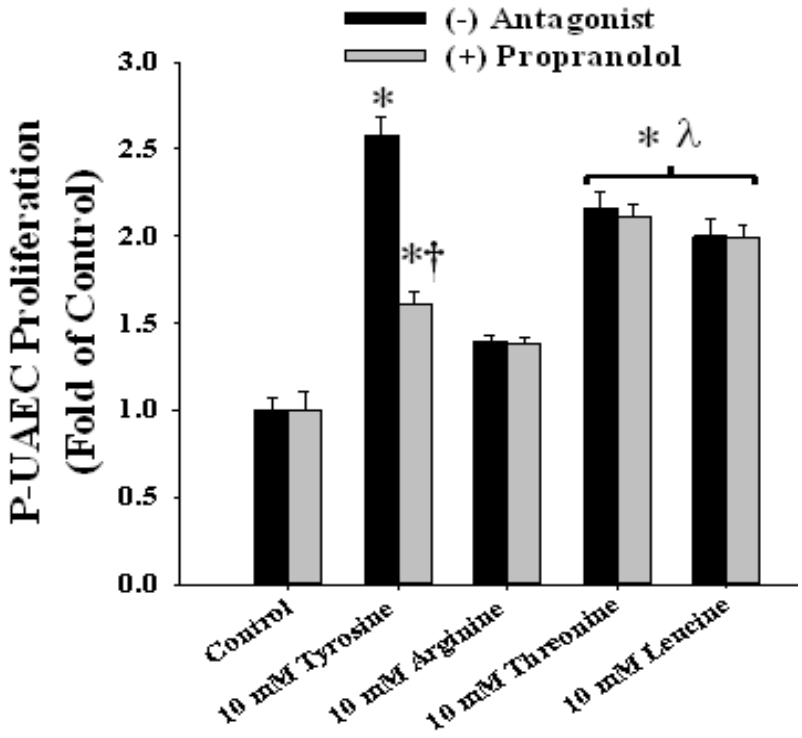
(A)P-UAECs did not have a dose dependent response to L-Arginine. (B) Proliferation of P-UAECs to L-Leucine treatment groups plateaus after a dose of 100microM. (C) Proliferation induced by L-Threonine treatment plateaus after a dose of 100microM. (D) Proliferation is dose dependent in response to L-tyrosine. As the dose is increased, there is a significant difference between the proliferations seen in each group beyond 100nM. * Significantly different compared with control. † Significantly different from the 100mM concentration.

Figure A.13



(A)P-UAECs did not have a dose dependent response to L-Arginine. (B) Cell number increases P-UAECs to L-Leucine treatment group plateaus after a dose of 100microM. (C) Cell number increase induced by L-Threonine treatment plateaus after a dose of 100microM. (D) Cell number increase is dose dependent in response to L-tyrosine. As the dose is increased, there is a significant difference between the cell numbers seen in each group beyond 100nM. * Significantly different compared with control. † Significantly different from the 100mM concentration

Figure A.14



P-UAECs treated with L-Tyrosine and propranolol is shown exhibit significantly lower proliferation than P-UAECs treated with L-Tyrosine alone. However, this proliferation is also significantly higher than P-UAECs in the control. This suggests that catecholamines may only partially regulated P-UAEC proliferation. * Significantly different from control. † Significantly different from 10mM L-Tyrosine group. λ Significantly different from all other groups