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Estrogen Receptor-β Mediates Estradiol-Induced Pregnancy-Specific Uterine Artery Endothelial Cell Angiotensin Type-2 Receptor Expression

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ERβ mediates estradiol-induced pregnancy specific uterine artery endothelial cell AT_2R expression

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Abstract

The pregnancy-augmented uterine vasodilation is linked to increased angiotensin type-2 receptor (AT_2R) that mediates the vasodilatory effects of angiotensin II. However, the mechanisms controlling AT_2R expression during pregnancy remain unclear. Estrogens are known to play a role in vascular adaptations during pregnancy. We hypothesized that estrogen stimulates uterine artery AT_2R expression via ERβ-dependent transcription in a pregnancy-specific endothelium-dependent manner. Plasma estradiol levels increased and peaked in late pregnancy, and returned to pre-pregnant levels postpartum, correlating with uterine artery AT_2R and ERβ upregulation. Estradiol stimulated AT_2R mRNA expression in endothelium-intact but not –denuded late pregnant and non-pregnant rat uterine artery exvivo. Consistently, estradiol stimulated AT_2R mRNA expression in late pregnant but not non-pregnant primary human uterine artery endothelial cells (hUAECs) invitro, which was abolished by ER-antagonist ICI-182,780. Higher ERα protein bound to ER-responsive elements in *AT_2R* promoter in the nonpregnant arteries whereas higher ERβ bound in the pregnant state. Similar ERα but higher ERβ protein expressed in pregnant versus nonpregnant hUAECs. Estradiol stimulation recruited ERα to the AT_2R promoter in the nonpregnant state and ERβ to the AT_2R promoter in pregnancy; however, only ERβ recruitment mediated *trans*-activation of the *AT_2R* reporter gene in pregnant hUAECs. Estradiol-induced AT_2R expression was abolished by the specific ERβ (not ERα) antagonist PHTPP and mimicked by the specific ERβ (not ERα) agonist DPN in pregnant hUAECs invitro. This study demonstrates a novel role of pregnancy-augmented ERβ in AT2R upregulation in the uterine artery and provides new insights into the mechanisms underlying uterine vascular adaptation to pregnancy.

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Summary

The pregnancy-augmented uterine artery AT_2R expression is stimulated by estrogens via endothelium-dependent gene transcription involving the enhanced expression of ERβ and its direct interaction with βEREs in AT_2R promoter. This mechanism sheds new light on understanding the complex interactions of elevated estrogens and angiotensin II in pregnancy-associated uterine vasodilation. It remains to be determined whether uterine artery ERβ expression is reduced in preeclampsia and if drugs that upregulate endothelial ERβ could be valuable therapeutic options to increase uterine artery AT_2R and UBF.

Keywords

Estradiol; Estrogen Receptor; Pregnancy; Uterine Artery; Endothelial Cell; Angiotensin Receptor

INTRODUCTION

Normal pregnancy is associated with up to 60-fold rise in uterine blood flow (UBF) that provides the sole source of nutrients for fetoplacental growth and the route for mother-fetal gas (i.e., O_2 and CO_2) exchanges.[1] The adequate rise in UBF maintains the well-being of the fetus and mother during pregnancy. An insufficient rise in UBF during pregnancy often leads to preeclampsia and intrauterine growth restriction, which are major causes of perinatal morbidity and mortality[2, 3] and also predispose the mother and her offspring at higher risk of developing metabolic diseases such as diabetes and cardiovascular diseases later in life.[4] The molecular mechanism underlying pregnancy-associated rise in UBF is incompletely understood; however, this is known to be associated with elevated circulating estrogens[5, 6] and angiotensin II levels (Ang II).[7] Estrogens are potent vasodilators that induce rapid and sustained dilation of selected vascular beds in the body with the most significant response in the uterine vasculature.[8, 9] In contrast, Ang II is a potent vasoconstrictor;[10] yet, uterine artery displays refractoriness to Ang II-induced vasoconstriction than systemic circulations during pregnancy.[11, 12] Ang II mainly mediates its effect through vasoconstrictive angiotensin type-1 receptor (AT_1R) and vasodilatory angiotensin type-2 receptor (AT_2R).[13] Radiolabeled ligand binding studies have convincingly shown an increased density of AT_2R but unchanged AT_1R in pregnant versus nonpregnant uterine artery in rodents, sheep, and women.[14–16] Pregnancy-specific AT_2R regulation provides an attractive target for developing treatments of hypertension-related pregnancy disorders such as preeclampsia.[17] However, this has been hindered by a knowledge gap regarding how uterine artery AT_2R is specifically regulated in pregnancy.

We have recently shown that uterine artery AT_2R mRNA and protein are 6-fold greater in pregnant versus nonpregnant rats and that the pregnancy-augmented AT_2R contributes to enhanced vasodilation and rise in UBF.[18] We further showed that pregnancy-augmented AT_2R in the rat uterine arteries is induced by estradiol.[18] Estrogen stimulation of vascular AT_2R expression seems to be vascular bed-specific and pregnancy-dependent since ovariectomy, and estradiol supplementation does not alter mesenteric artery AT_2R expression in nonpregnant rats.[19] Also, AT_2R is expressed in both vascular smooth muscle

and endothelium.[20, 21] However, the underlying mechanisms of how estrogen stimulates pregnancy-specific uterine artery AT_2R expression are unknown.

Local infusion of a specific estrogen receptor (ER) antagonist ICI 182,780 partially inhibits estrogen-induced and pregnancy-associated rises in UBF in sheep, demonstrating specific ER-dependent mechanisms.[22] Estrogen stimulates endothelial expression of vasodilatory genes such as endothelial nitric oxide synthase (eNOS)[23] and prostacyclin synthase,[24] via nuclear specific ER (i.e., ERα and ERβ) interactions with estrogen response elements (EREs) in their promoters.[25, 26] Of note, ERα and ERβ may play different and even opposite roles in regulating cellular responses to estrogens in cells expressing both ERs.[27] ERα and ERβ are expressed in uterine artery endothelium, and smooth muscle[28] and pregnancy preferentially upregulates uterine artery ERβ.[29] However, the specific roles of ERβ in estrogen stimulation of vascular AT_2R expression are unknown.

The present study aimed to examine the mechanism underlying estrogen stimulation of uterine artery AT_2R expression. We hypothesized that estrogen stimulates uterine artery AT_2R expression via ERβ dependent transcription in a pregnancy-specific and endothelium-dependent manner. The objectives were to determine: 1) gestational changes and relationships in circulating estradiol and uterine artery ERs and AT_2R expression; 2) whether estradiol stimulates pregnancy-dependent endothelial AT_2R expression in isolated rat uterine arteries ex vivo and uterine artery endothelial cells in vitro; 3) whether estradiol stimulation of pregnancy-dependent endothelial AT_2R expression is mediated by specific ER; and 4) whether ERβ regulates estradiol-stimulated AT_2R transcription.

METHODS

The authors declare that all supporting data are available within the article (and in the online-only Data Supplement). All procedures of animal care and use were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Institutional Animal Care and Use Committee at the University of Wisconsin at Madison. Twelve-week-old non-pregnant and timed-pregnant (positive plug = gestation day (GD) 1) Sprague-Dawley rats were purchased from Envigo laboratories and were maintained on 12L/12D cycles in a temperature-controlled room (23°C) and provided with food (Teklad global rodent diet # 2020X) and water ad libitum. Non-pregnant (diestrus), pregnant (different GDs) and postpartum day 4 rats were sacrificed to collect plasma and isolate uterine arteries. Plasma was used to measure estradiol levels, and uterine arteries were used to measure the mRNA and/or protein expression of ERα, ERβ, and AT_2R. Also, uterine arteries from non-pregnant (diestrus) and GD20 pregnant rats, and human uterine artery endothelial cells (hUAECs) isolated from non-pregnant (proliferative phase) and pregnant (35 to 36 weeks of gestation) women were used for ex vivo and in vitro studies, respectively.

The main uterine arteries of pregnant and nonpregnant women (n = 5/group) were collected in the event of hysterectomy at the University of California Irvine Medical Center. Written consent was obtained from all subjects, and ethical approval (HS#2013–9763) was granted by the Institutional Review Board at the University of California-Irvine.[30] An expanded

Methods section is available in online-only Data Supplement, including characteristics of the patients and procedures of isolating primary hUAECs and culture conditions, plasma estradiol assay, RNA isolation and quantitative real-time PCR, immunoblotting, cell culture, ex vivo and in vitro treatments, bioinformatics and ChIP analysis, cell transfection, luciferase assay, and statistical analysis.

RESULTS

Plasma estradiol levels and uterine arterial AT_2R expression in rats

Plasma estradiol levels were steady at pre-pregnancy levels up to GD6 in early-pregnancy, increased 2-fold ($p<0.05$) on GD14 in mid-pregnancy and further increased as pregnancy advanced, reaching a peak in late pregnancy on GD20–21 with a 500% maximum rise compared to pre-pregnant baseline. On day 4 postpartum, estradiol levels returned to pre-pregnancy baseline (Figure 1A).

Consistent with our previous reports on pregnancy-specific upregulation of uterine artery AT_2R expression,[18] uterine artery AT_2R mRNA was unchanged until mid-gestation on GD14, significantly ($p < 0.05$) increased on GD18, and increased approximately six-fold pre-pregnancy baseline on GD20–21, returned to pre-pregnancy baseline on day 4 postpartum (Figure S1).

Uterine artery ERα and ERβ during rat pregnancy

Uterine artery ERα mRNA did not differ among pre-pregnant, pregnant, and postpartum states (Figure 1B). ERβ mRNA began to increase significantly ($P < 0.05$) on GD6, reached approximately five-fold pre-pregnancy baseline on GD20–21 and returned to baseline on day 4 postpartum (Figure 1B). Compared to the pre-pregnant state, GD20 pregnant uterine artery ERα protein was unchanged, but ERβ protein was significantly higher ($p < 0.05$) (Figure 1C).

Estradiol on AT_2R expression in isolated rat uterine arteries ex vivo

Circulating estradiol increases in parallel with an increase in uterine artery AT_2R mRNA during rat pregnancy in vivo (Figure 1 & S1).[18] We determined whether estradiol contributes to pregnancy-augmented AT_2R expression and whether this is endothelium-dependent using ex vivo cultures of isolated uterine arterial rings from nonpregnant and GD20 pregnant rats. Estradiol treatment, at all doses tested at 24 h, did not alter AT_2R mRNA in endothelium-intact and endothelium-denuded uterine arteries isolated from nonpregnant rats (Figure 2A). In contrast, estradiol dose-dependently increased AT_2R mRNA ($P < 0.05$) in endothelium-intact uterine arteries at 24 h (Figure 2B). The maximal response was achieved with 100 nM estradiol, which was abolished by ICI 182,780 (Figure 2B). Estradiol failed to stimulate AT_2R expression in endothelium-denuded pregnant uterine arteries (Figure 2C).

Estradiol on ER expression in hUAECs in vitro

In hUAECs, ERα mRNA did not differ between pregnant and nonpregnant cells, and this was not altered by estradiol treatment. However, baseline ERβ mRNA was significantly

higher in pregnant cells, which was further stimulated by estradiol only in pregnant cells (Figure S2).

Estradiol on AT_2R expression in hUAECs in vitro

Since estradiol stimulation of uterine artery AT_2R mRNA is pregnancy-specific and endothelium-dependent (Figure 2A–C), we further tested whether estradiol directly stimulates pregnancy-dependent uterine artery endothelial AT_2R mRNA expression using primary hUAECs isolated from pregnant and nonpregnant women. Estradiol did not alter AT_2R mRNA in nonpregnant cells (Figure 2D). In contrast, estradiol, at physiologically relevant concentrations (0.1 – 10 nM) as seen in pregnant women,[5, 6] increased AT_2R mRNA in pregnant cells; the maximal response was achieved with 100 nM estradiol, which was attenuated by ICI 182,780 (Figure 2E).

ERα and ERβ binding sites in rat *AT_2R* gene promoter in vivo

Ovine uterine artery endothelium express both ERα and ERβ [28] and pregnancy preferentially upregulates ERβ.[28] We searched for the potential ER binding motifs on the full-length 4.2 kb promoter of rat *AT_2R* gene (Gene ID: 24182) by using LASAGNA motif search tool (https://biogrid-lasagna.engr.uconn.edu/lasagnasearch/index.php). There are seven putative EREs (designated as αERE) for binding ERα and four putative EREs (designated as βERE) for binding ERβ (Figure 3A). We used freshly isolated uterine arteries from nonpregnant and pregnant rats to investigate pregnancy-dependent interactions of ERα and ERβ with these *AT_2R* EREs in vivo by ChIP-PCR analyses using specific ERα and ERβ antibodies. There was significantly ($p < 0.05$) higher binding of ERα to αERE2, αERE3, αERE5 and αERE7 (3.9-, 7.7-, 2.1- and 4.1–fold enrichment, respectively) in nonpregnant versus pregnant uterine arteries (Figure 3B). In contrast, there were significantly ($p < 0.05$) higher binding of ERβ to βERE1, βERE2, and βERE4 (8.3-, 7.7- and 3.5-fold enrichment, respectively) to *AT_2R* promoter in pregnant versus nonpregnant uterine arteries (Figure 3C).

Functional ERα and ERβ binding sites in human *AT_2R* promoter in vitro

In hUAECs, ChIP-PCR analyses identified four putative ERE binding sites for each of ERα and ERβ in the full-length 2.5 kb promoter of human *AT_2R* gene (Gene ID: 186) (Figure 4A). The ERα and ERβ binding signals were readily detectable in the absence of the hormone in both pregnant and nonpregnant cells. Estradiol treatment resulted in significantly ($p < 0.05$) increased ERα recruitment to αERE2 (3.4 to 3.9 fold at 10–100 nM estradiol) and αERE4 (1.9 to 2.01 fold at 10–100 nM estradiol) on human *AT_2R* promoter in nonpregnant but not pregnant cells (Figure 4B). In contrast, estradiol treatment resulted in significantly ($p < 0.05$) increased ERβ recruitment to βERE2 (2.2 to 3.0 fold at 10–100 nM estradiol), βERE3 (2.5 to 3.5 fold at 10–100 nM estradiol), and βERE4 (2.4 to 4.9 at 1–100 nM estradiol), respectively) on human *AT_2R* promoter in pregnant but not nonpregnant cells (Figure 4C).

To determine whether the ERα-αERE and ERβ-βERE binding could result in activation of human *AT_2R* promoter, we made human *AT_2R* luciferase reporter constructs containing either αEREs or βEREs. When transfected into hUAECs, estradiol at all concentrations (1, 10, and 100 nM) failed to activate αERE-human *AT_2R* promoter-luciferase reporter gene in

pregnant and nonpregnant cells (Figure 4D). Estradiol at all doses also failed to activate βERE-human *AT_2R* promoter-luciferase reporter gene in nonpregnant cells. Estradiol dose-dependently activated βERE-human *AT_2R*-luciferase reporter gene in pregnant cells; 1 nM estradiol was effective to provoke a significant ($p<0.05$) 2-fold activation and 10–100 nM provoked 5.0 to 5.5-fold ($p<0.05$) activation of βERE-human *AT_2R* reporter-luciferase reporter gene in pregnant cells (Figure 4E).

ERα and ERβ in estradiol-stimulated endothelial AT_2R expressions

We determined the specific roles of ERα and ERβ in estradiol-stimulated endothelial AT_2R expression in hUAECs using ER isoform-specific antagonists or agonists. In nonpregnant cells, estradiol, in the presence or absence of antagonists of either ERα (MPP) or ERβ (PHTPP), did not alter AT_2R mRNA (upper panel) and protein (lower panel) expression (Figure 5A). In pregnant cells, estradiol significantly ($P < 0.05$) increased AT_2R mRNA (upper panel) and protein (lower panel) expression, which was unaffected by MPP but completely attenuated by PHTPP (Figure 5B). In nonpregnant cells, an agonist of neither ERα (PPT) nor ERβ (DPN) was able to affect AT_2R mRNA (upper panel) and protein (lower panel) expression (Figure 6A). In pregnant cells, PPT did not alter AT_2R mRNA (upper panel) and protein (lower panel) expression (Figure 4A); however, DPN was able to significantly ($p < 0.05$) increase AT_2R mRNA (upper panel) and protein (lower panel) expression (Figure 6B).

DISCUSSION

The enhanced density of AT_2R has long been identified in conjunction with uterine artery refractoriness to Ang II-induced vasoconstriction than systemic circulations during pregnancy.[18, 31–34] It has been shown that AT_2R promotes vasodilation through the release of bradykinin,[35] nitric oxide,[36] EDHF,[37] and prostacyclin.[38] We have recently shown that pregnancy-augmented of AT_2R mediates enhanced vasodilation and UBF, correlating with an increase in and rapid fetal growth (approximately 1 g day^{-1}) in the last one-third of rat pregnancy.[18] We further showed that enhanced AT_2R expression is induced by estradiol through an ER-dependent mechanism.[18] Our present study provides compelling evidence further demonstrating that estrogen stimulation of uterine artery AT_2R expression is endothelium-dependent and that estradiol stimulates uterine artery endothelial AT_2R expression is mediated by transcriptional mechanisms involving ERβ interaction with functional βEREs in *AT_2R* promoter.

In parallel with the gestation-dependent increase in circulating estradiol levels ([5, 6], current study), uterine artery AT_2R expression significantly increases during pregnancy ([18], current study). These positive correlations suggest that elevated circulating estrogens function upstream of pregnancy-augmented uterine artery AT_2R expression. This idea is further strengthened by the fact that treatment with estradiol, at physiologically relevant concentrations seen during pregnancy,[5, 6] stimulates AT_2R expression only in pregnant but not nonpregnant rat uterine artery ring cultures ex vivo. Moreover, our data show that estrogen stimulation of pregnancy-augmented rat uterine artery AT_2R expression ex vivo is endothelium-dependent because treatment with estradiol only stimulates endothelium-intact

uterine artery rings from pregnant but not nonpregnant rats ex vivo. Our hUAECs maintain pregnancy-dependent differential baseline ERα and ERβ mRNA expression patterns and estrogen-responsiveness in ERβ but not ERα mRNA induction, providing an excellent model for dissecting ER-subtype (ERα versus ERβ) specific mechanisms controlling estrogen stimulation of pregnancy-augmented uterine artery endothelial AT_2R expression. Our in vitro studies further confirm that estrogen can stimulate AT_2R in endothelial cells isolated from pregnant but not nonpregnant women. This finding is, perhaps, the first evidence demonstrating that estrogens stimulate pregnancy-specific endothelium-dependent vascular AT_2R expression, which is essential for uncovering pregnancy-specific uterine artery refractoriness to Ang II-induced vasoconstriction.

Using the ER antagonist ICI 182, 1780, we have shown that estrogen stimulation of uterine artery endothelial AT_2R expression is mediated by specific ER in pregnant rat uterine artery cultures ex vivo. This effect is expected as the rat uterine artery express both ERα and ERβ, consistent with previous sheep studies.[27] However, pregnancy preferentially upregulates uterine artery ERβ (no change in ERα) in parallel with estradiol levels with advancing gestation age ([27], current study), suggesting that ERβ may emerge as an important receptor for mediating estrogen's effect during pregnancy.

ERα and ERβ are transcription factors that play synergistic or opposite roles in gene transcription in target cells expressing both ERs.[39, 40] *In silico* analysis of the entire upstream sequences of the *AT_2R* transcription start codon revealed multiple αEREs and βEREs within rat and human *AT_2R* promoters, which preferentially bind to either ERα or ERβ, respectively. Bioinformatics comparisons of these EREs identified distinct ERα and ERβ binding sites. ChIP-PCR analysis using specific ERα and ERβ antibodies show that ERα is only recruited to αEREs in nonpregnant uterine artery, whereas ERβ is only recruited to βEREs in the pregnant uterine artery in the rat *AT_2R* promoter. The pregnancy-dependent baseline ERα-αEREs and ERβ-βEREs interactions in human *AT_2R* promoter are also evident in pregnant versus nonpregnant hUAECs in vitro. These data provide the first line of evidence regarding the dominant role of pregnancy enhanced ERβ in mediating estrogen stimulation of uterine artery endothelial AT_2R expression. Indeed, this premise is supported by that interaction of ERβ-βEREs but not ERα-αEREs mediates *trans*-activation of human *AT_2R* promoter-driven luciferase reporter gene by estradiol in pregnant but not nonpregnant hUAECs in vitro. Among the four βERE sites, three are required for estrogen-stimulated trans-activation of AT_2R promoter. Future studies are needed to determine which one or more of them play a critical role. However, the primary role of ERβ mediating estradiol's pregnancy-specific effect on AT_2R expression is further strengthened by our in vitro data in hUAECs. This is because estradiol only stimulates AT_2R expression in hUAECs from pregnant but not nonpregnant women and the response can be attenuated by the specific ERβ antagonist PHTPP but not ERα antagonist MPP and mimicked by the specific ERβ agonist DPN but not ERα agonist PPT. Our current study does not rule out other mechanisms that may be involved in the differential roles of ERα and ERβ in *AT_2R* transcription as transcription of ERE-containing genes can be regulated by ER interactions with co-activators or co-repressors,[41] a notion that needs to be further examined. Also, future studies should dissect the mechanisms that contribute to the pregnancy-specific augmentation of ERβ in the uterine artery endothelial cells.

In conclusion, our current study show for the first time that elevated circulating estrogens stimulate pregnancy-augmented uterine artery AT_2R expression via endothelium-dependent gene transcription involving enhanced ERβ expression and ERβ-βERE interaction. This important mechanism sheds new light on the understanding of uterine artery refractoriness to Ang II-induced vasoconstriction than systemic circulations during pregnancy,[18, 31–34] contributing to the pregnancy-associated rise in UBF.

PERSPECTIVES

The present study provides new evidence that pregnancy-augmented uterine artery AT_2R expression is stimulated by estrogens via endothelium-dependent gene transcription involving the enhanced expression of ERβ and its direct interaction with βEREs in AT_2R promoter. This mechanism is important for mediating the complex interactions of elevated circulating estrogens and Ang II in raising UBF during pregnancy.[18] Hypertension-related pregnancy disorders such as preeclampsia are associated with significantly lower circulating estrogens and vascular AT_2R receptor expression.[42, 43] Since estrogen augments vasoconstriction in blood vessels from ERβ deficient mice,[44] it remains to be determined whether uterine artery ERβ expression is reduced in preeclampsia and if drugs that upregulate endothelial ERβ could be valuable therapeutic options to increase uterine artery AT_2R and UBF. Based on our current study, we propose a working hypothesis that reduced estrogens/ERβ decreases endothelial AT_2R expression, which in turn compromises endothelial relaxation and exaggerates vasoconstriction to decrease UBF thereby triggering some of the pathophysiology and clinical manifestations of preeclampsia and IUGR (Figure S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What Is New?

- Circulating estradiol and uterine artery ERβ correlate positively with uterine artery AT_2R expression during pregnancy.
- Estradiol stimulates AT_2R mRNA expression in a pregnancy-specific endothelium-dependent manner.
- Higher ERα is recruited to αEREs in the nonpregnant state, whereas higher ERβ is recruited to βEREs in *AT_2R* promoter during pregnancy.
- ERα-αEREs interaction does not activate AT_2R promoter, but ERβ-βERE interaction induces pregnancy-specific *trans*-activation of *AT_2R* promoter in human uterine artery endothelial cells.
- Pregnancy-augmented ERβ mediates estrogen stimulation of uterine artery endothelail AT_2R expression.

What Is Relevant?

- The study identifies a novel mechanism of pregnancy-augmented uterine artery endothelial ERβ targeting specific ER-response elements to upregulate vasodilatory AT_2R expression, providing new insights on the mechanisms controlling normal uterine blood flow during gestation, which may be dysfunctional in preeclampsia.

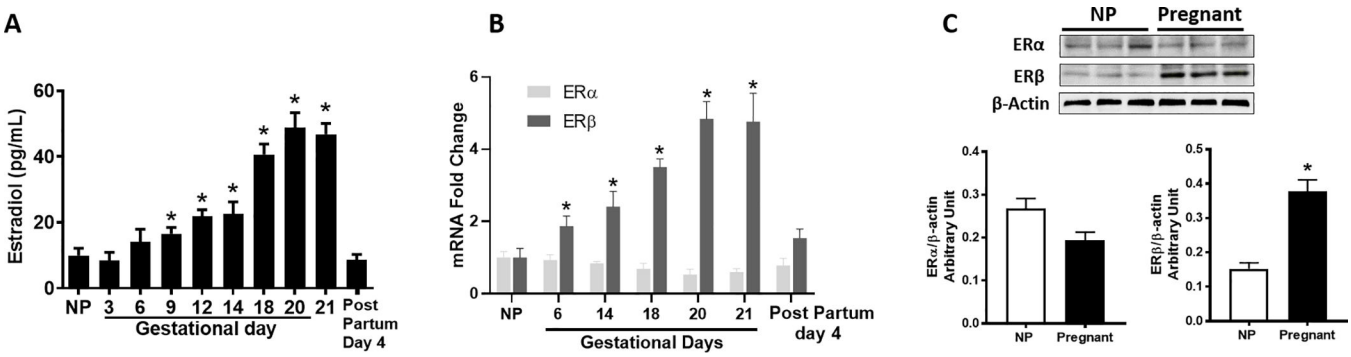

Figure 1.

Changes in plasma estradiol levels and ER mRNA levels in the uterine arteries across pregnancy. Plasma and uterine arteries were collected from non-pregnant (NP), different days of gestation and post-partum (day 4) rats. (**A**) Estradiol levels were quantified by ELISA. (**B**) Real-time qPCR analysis of temporal mRNA levels of ERα and ERβ normalized to β-actin in non-pregnant (NP), pregnant and postpartum rats. (**C**) Representative Western blots for ERα and ERβ and β-actin are shown at top; blot density obtained from densitometric scanning of estrogen receptors normalized to β-actin is shown at the bottom. n=6 each, *P<0.05 versus NP.

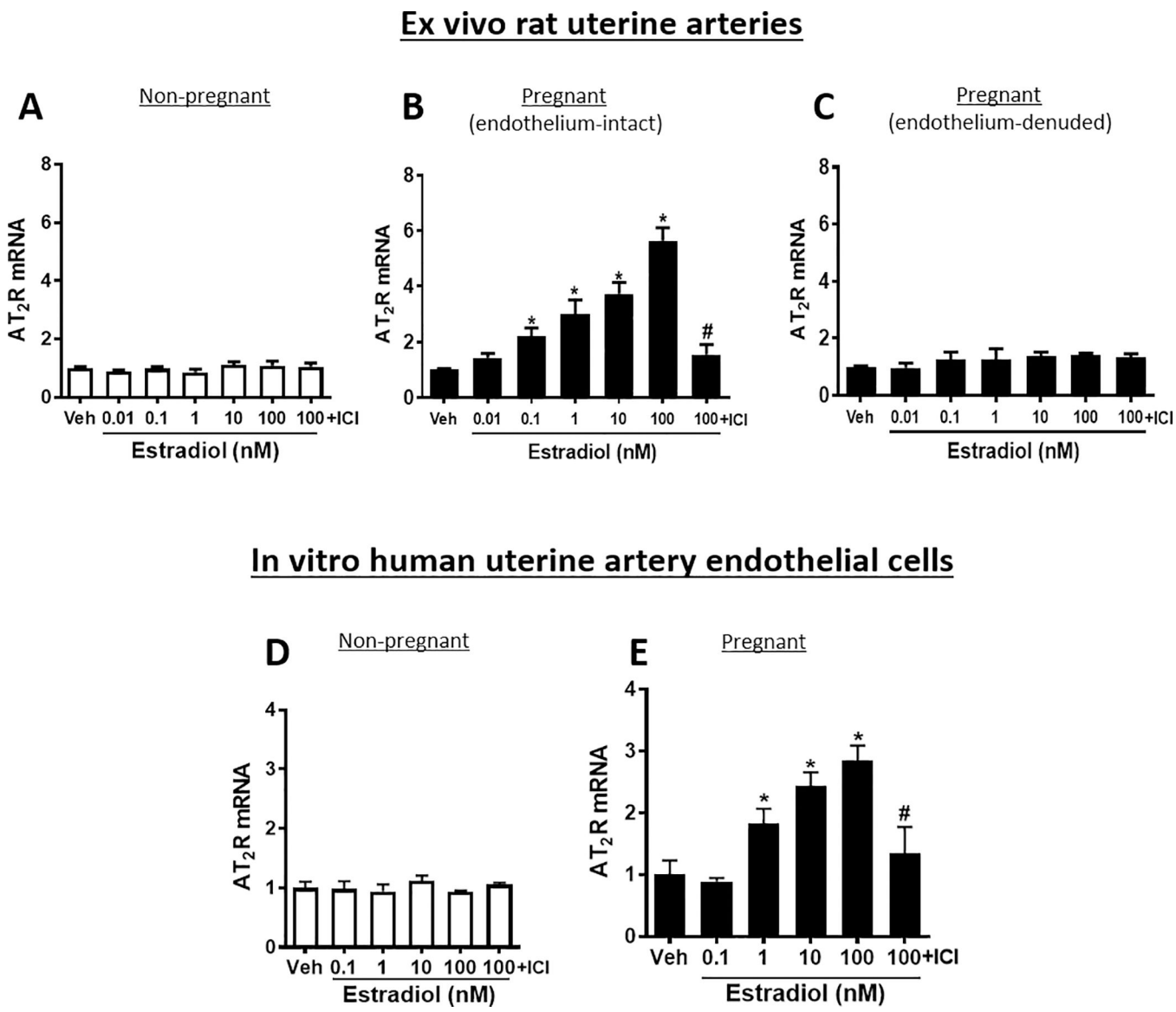

Figure 2.

Effect of estradiol on AT_2R mRNA expression in isolated uterine arteries and cultured endothelial cells. Endothelium-intact and -denuded uterine arteries from non-pregnant and pregnant (day 20) rats and primary uterine artery endothelial cells from non-pregnant and pregnant women were treated with estradiol for 24 hours, and then AT_2R mRNA was measured using quantitative real-time PCR. Estradiol (**A**) did not alter AT_2R transcription in endothelium-intact non-pregnant uterine arteries, but (**B**) dose-dependently upregulated AT_2R transcription in endothelium-intact pregnant uterine arteries. (**C**) Estradiol did not alter AT_2R transcription in endothelium-denuded pregnant uterine arteries. In uterine artery endothelial cell estradiol (**D**) did not alter AT_2R expression in non-pregnant endothelial cells, but (**E**) upregulated AT2R in pregnant endothelial cells n=4 independent experiments. *P<0.05 versus vehicle (Veh).

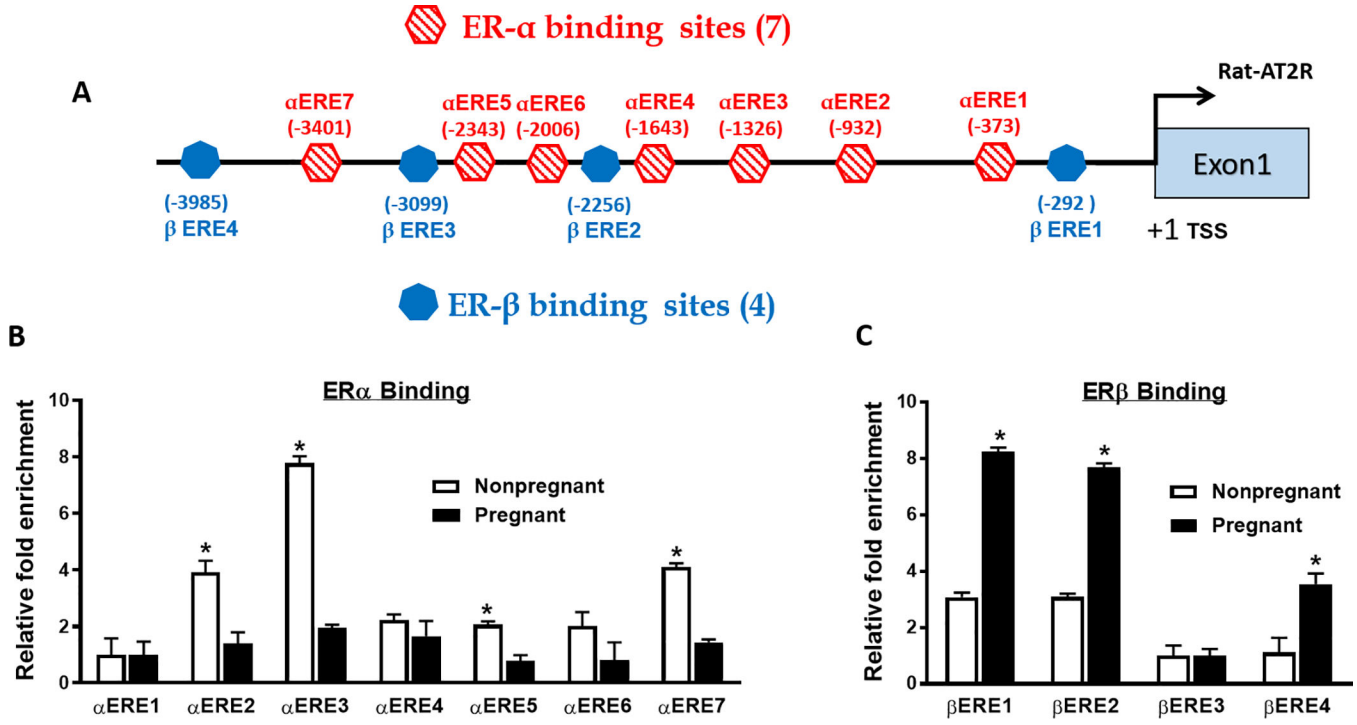

Figure 3.

Relative binding of ERα and ERβ to EREs in the rat *AT_2R* promoter. (**A**) Bioinformatic prediction showed seven ERα sites (designated as αERE) and four ERβ sites (designated as βERE) in the promoter of the rat *AT_2R* gene. ChIP assay using isolated uterine arteries from non-pregnant and pregnant rats revealed (**B**) increased binding of ERα to EREs in the non-pregnant state, $*P < 0.05$ versus pregnant, and (**C**) increased binding of ERβ to EREs during pregnancy, $*P < 0.05$ versus non-pregnant. n=4 independent experiments.

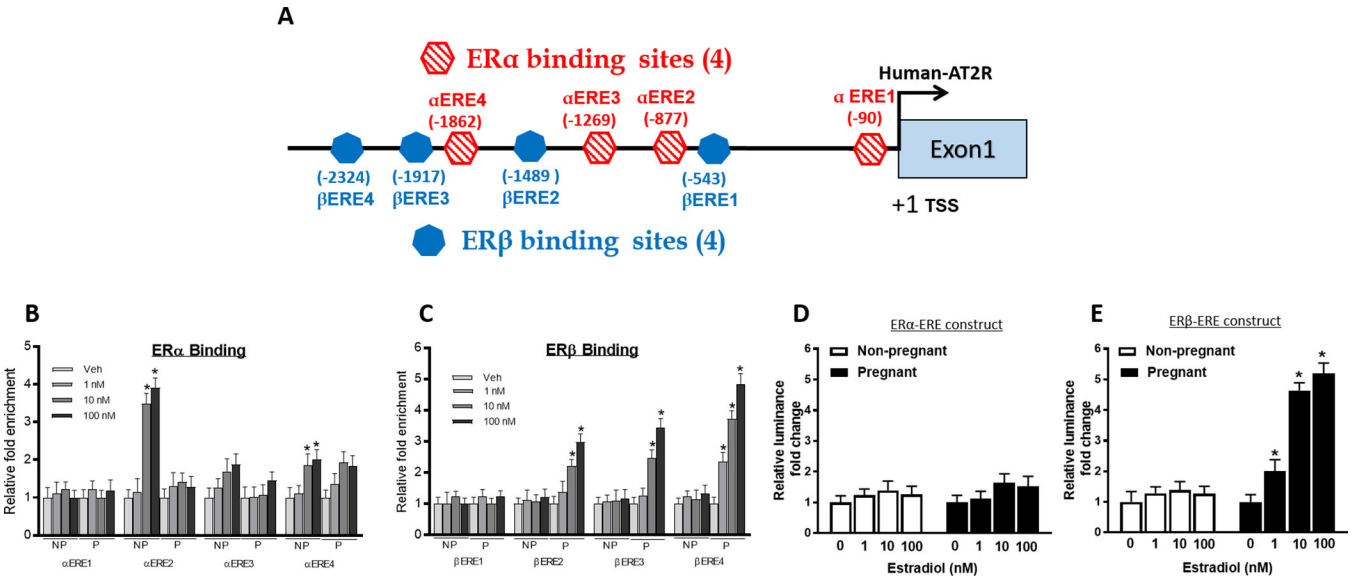

Figure 4.

Differential estradiol-induced recruitment of ERα and ERβ to EREs in pregnant versus non-pregnant endothelial cells and functional effect of ERs binding to ERE in enhancing AT_2R gene expression. (**A**) Bioinformatic prediction showed four ERα sites (designated as αERE) and four ERβ sites (designated as βERE) in the promoter of human AT_2R gene. Estradiol recruited (**B**) higher ERα on to AT_2R promoter in the non-pregnant state, $^*P < 0.05$ versus pregnant, and (**C**) higher ERβ on to AT_2R promoter during pregnancy, $^*P < 0.05$ versus non-pregnant. n=4 independent experiments. Reporter assay showing luciferase activity in non-pregnant and pregnant hUAECs transfected with a reporter plasmid containing ERα or ERβ binding EREs when incubated with estradiol. Construct that contained (**D**) ERα-ERE binding sites does not induce luciferase activity in both non-pregnant and pregnant endothelial cells, and (**E**) ERβ-ERE binding sites show dose-dependent increase luciferase activity in pregnant, but not non-pregnant, endothelial cells. n=3 independent experiments, $^*P < 0.05$ versus vehicle (Veh).

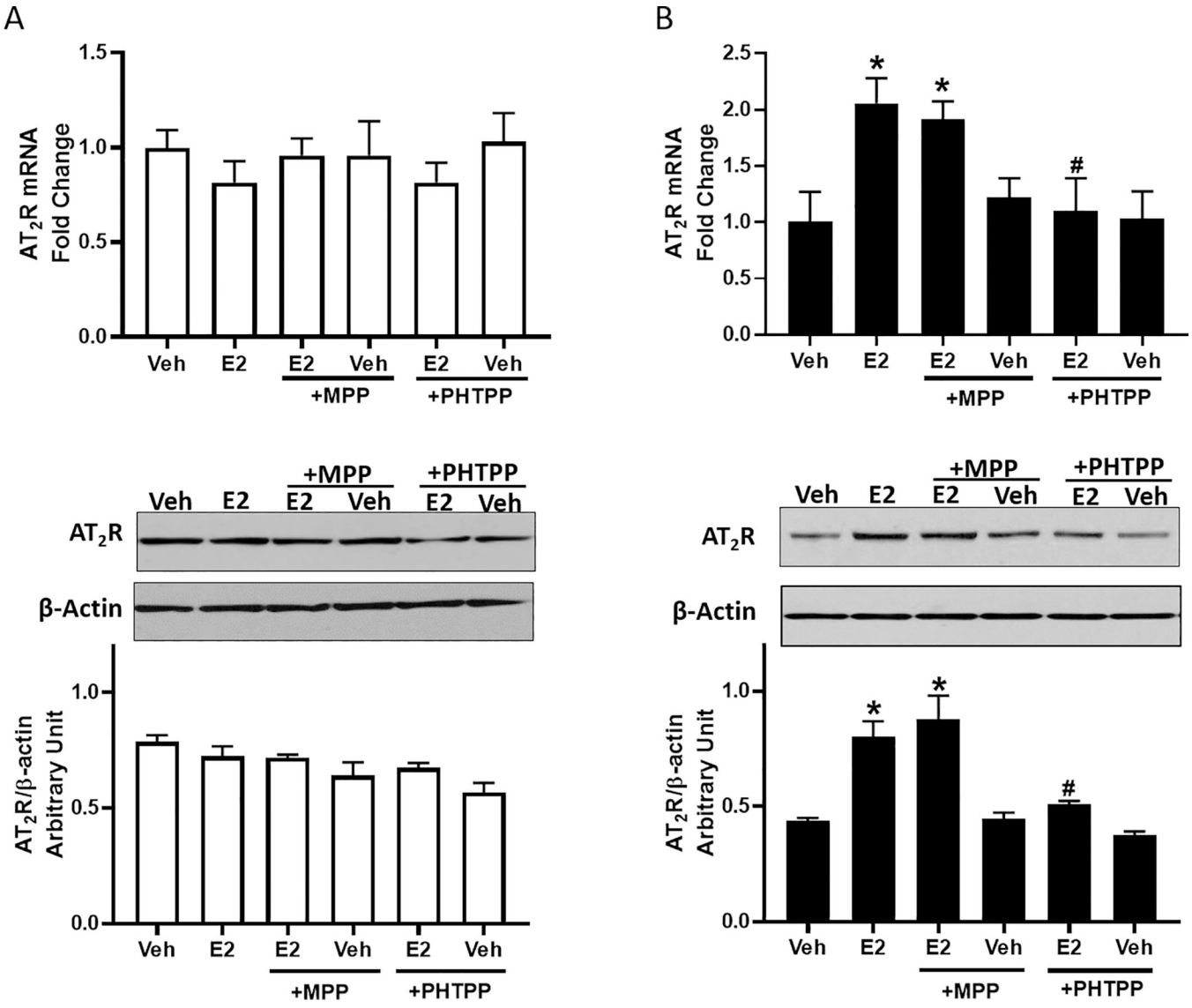

Figure 5.

Effect of ERα and ERβ on estradiol (E2)-stimulated AT_2R expression. Primary uterine artery endothelial cells from non-pregnant and pregnant women were treated with estradiol (10 nM) with or without 1 μM MPP (ERα antagonist), PHTPP (ERβ antagonist) for 24 h, and then AT_2R mRNA (upper panel) and protein (lower panel) were measured. (**A**) In non-pregnant endothelial cells, estradiol treatment in the presence or absence of ER antagonists did not alter AT2R mRNA and protein levels. (**B**) In pregnant endothelial cells, estradiol-stimulated AT_2R mRNA and protein were unaffected by ERα antagonist MPP but completely attenuated by ERβ antagonist PHTPP. n=4 independent experiments. *P<0.05 versus vehicle (Veh), #P<.05 versus E2 in the absence of the antagonist.

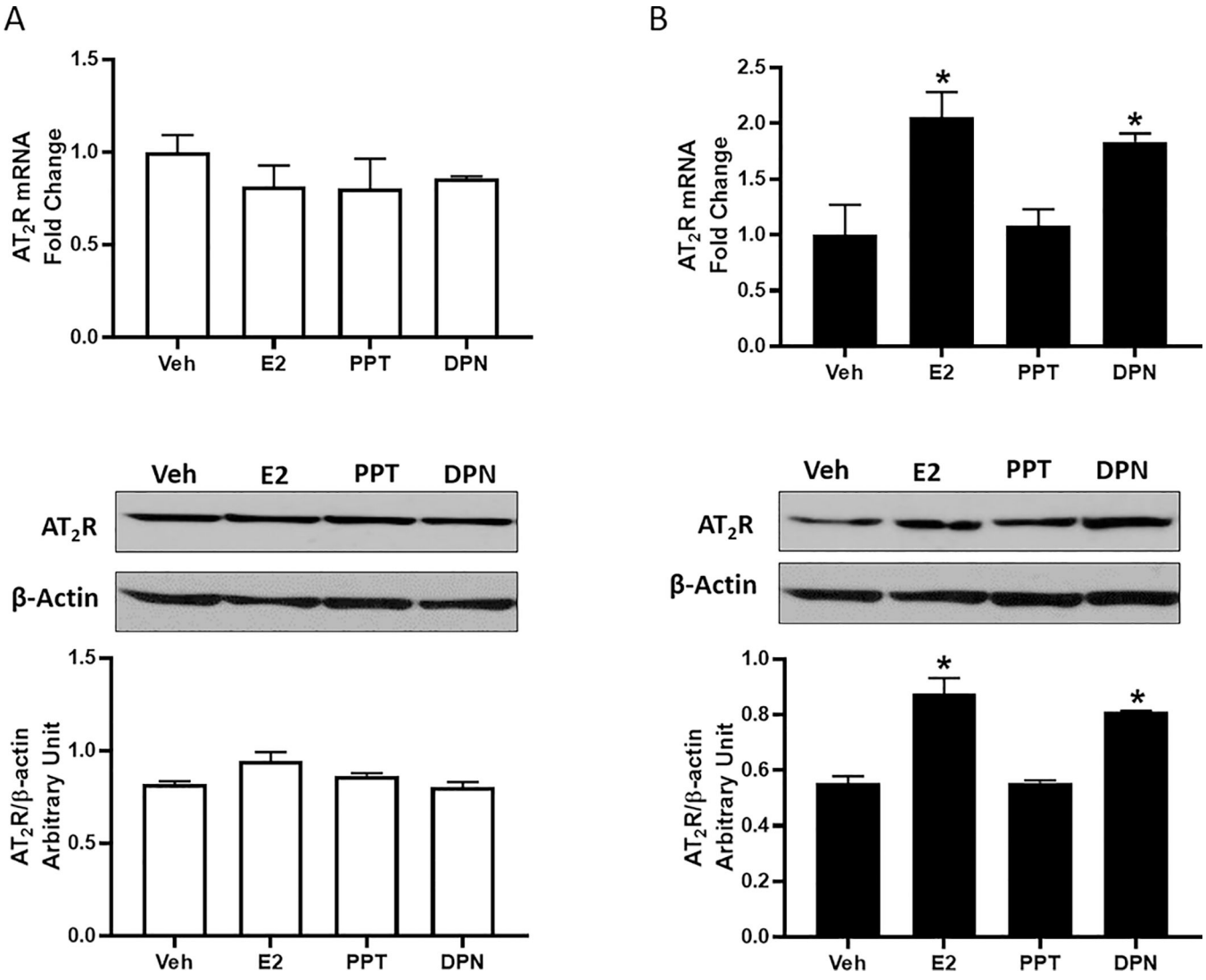

Figure 6.

Effect of ERα and ERβ agonists on AT2R expression. Primary uterine artery endothelial cells isolated from non-pregnant and pregnant women were treated with 10 nM of estradiol, PPT (ERα agonist) or DPN (ERβ agonist) for 24 h, and then AT2R mRNA (upper panel) and protein (lower panel) were measured. (**A**) In non-pregnant endothelial cells, ERα and ERβ agonists did not affect AT_2R mRNA and protein levels. (**B**) In pregnant endothelial cells, ERα agonist PPT did not alter AT_2R expression, but ERβ agonist DPN induced AT_2R mRNA and protein levels. n=4 independent experiments. *P<0.05 versus vehicle (Veh).