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Title: Protein Restriction to Pregnant Rats Increases the Plasma Levels of Angiotensin II and Expression of Angiotensin II Receptors in Uterine Arteries [1]

Short title: Low protein diet alters RAS in uterine arteries

Summary sentence: Gestational protein restriction increases plasma levels of angiotensin II and AGTR1 and AGTR2 protein levels in late pregnancy. Estrogen plays a role in regulating the expression of angiotensin II receptors in rat uterine artery.

Key words and topic category: gestational protein restriction, angiotensin II, angiotensin II receptor, angiotensin I converting enzyme, uterine artery, pregnancy, rat

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ABSTRACT

Whether gestational protein restriction affects renin-angiotensin system in uterine artery remains unknown. In this study, we hypothesized that gestational protein restriction (LP) alters the expression of RAS components in uterine artery. In study one, time-scheduled pregnant Sprague Dawley rats were fed a normal or low protein diet from Day 3 of pregnancy until sacrificed at Days 19 and 22. The uterine arteries were collected and used for gene expression of *Ace*, *Ace2*, *Agtr1a*, *Agtr1b*, *Agtr2*, *Esr1,* and *Esr2* by quantitative real-time PCR and/or Western blotting. LP increased plasma levels of angiotensin II in pregnant rats. In the uterine artery, the expression of *Agtr1a*, *Agtr1b,* and *Esr1* were increased by LP at Day 19 and 22 of pregnancy, while the abundance of AGTR1 and AGTR2 was increased by LP at Day 19 of pregnancy. The expression of *Ace2* was not detectable in rat uterine artery. In study two, virgin female rats were ovariectomized and implanted with either 17beta-estradiol (E2), progesterone (P4), both E2 and P4, or placebo pellets until sacrificed 7 days later. In rat uterine artery, E2 and P4 reduced the expression of *Agtr1a*, E2 increased the expression of *Agtr1b* and *Agtr2*, but neither E2 nor P4 regulated the expression of *Ace*. These results indicate that gestational protein restriction induces an increase in *Agtr1* expression in uterine artery and thus may exacerbate the vasoconstriction to elevated angiotensin II present in maternal circulation and that female sex hormones also play a role in this process.

INTRODUCTION

During normal pregnancy, the RAS (renin-angiotensin system) is activated and its different components demonstrate progressive changes [1-4]. Besides the critical role in regulating sodium and water homeostasis, recently, RAS particularly in kidney has been reported to play an important role in fetal programming of hypertension in response to hormonal manipulations [5;6]; dietary manipulation, such as maternal global nutrient restriction [7] or protein restriction [8]; and uteroplacental insufficiency [9] during gestation. Accumulating evidence indicates that RAS in hypertensive offspring affected by intrauterine growth restriction or maternal protein restriction was altered either systemically or locally [9;10;10-13]. However, to date, it is unclear whether and how RAS affects maternal cardiovascular function and metabolism in response to nutritional or hormonal disruption.

Uterine artery remodeling and corresponding utero-placental blood flow play a critical role in uterine, placental and fetal growth during pregnancy. Uterine blood flow is a determinant of fetal growth and survival [14]. Maternal vascular remodeling ensures adequate placental perfusion by transforming maternal arteries including uterine artery to low-resistance large-caliber vessels. Physiologically, the uterine artery dilation during pregnancy is achieved partly, if not all, by increased E2 level and local NO production and reduced vasoactive response to serotonin [15]. Global dietary restriction leads to remarkable reduction in uterine and placental blood flow[16], and protein restriction itself impairs vascular relaxation of mesenteric artery in pregnant rats [17], resulting in fetal growth retardation. In addition, limited studies demonstrate that RAS regulates the vasoconstriction in uterine artery. In mice, the contractile response in uterine artery to angiotensin II is elevated in mid and late pregnancy and vasoconstriction is mainly medicated by AGTR1 (angiotensin II receptor, type 1) and modulated by AGTR2 (angiotensin II receptor, type 2) [18;19].

Our laboratory and others have reported that maternal protein restriction, albeit to a different extent in the reduction of protein in diet, causes the development of hypertension in offspring [8;20-25]. Although the underlying mechanisms remain unclear, it is known that low protein diet alters endocrine status in pregnant rats including elevated plasma levels of testosterone and estradiol [26] and reduced plasma levels of progesterone [27;28]. The altered endocrine status may impact the maternal RAS function. For instance, estradiol, mainly via ESR1 (estrogen receptor 1), regulates the expression of RAS system in a tissue specific manner with reduced expression of *Ace* [angiotensin I converting enzyme (peptidyl-dipeptidase A) 1] and *Ace2* [angiotensin I converting enzyme (peptidyl-dipeptidase A) 2] in kidney, reduced expression of *Agtr1a* (angiotensin II receptor, type 1a) in hypothalamus and anterior pituitary, increased expression of *Agtr1a* and *Agtr2* in kidney and unaltered expression of *Ace*, *Ace2*, *Agtr1* and *Agtr2* in lung [29-33]. So far, it is unknown whether estrogen regulates expression of RAS in uterine artery, however, the presence and/or function of ESR1 [34-36], AGTR1 and AGTR2 [18;19;37;38] in the uterine artery support this possibility.

In this study, we hypothesized that gestational protein restriction and associated changes in female sex hormones may alter the expression of RAS in rat uterine artery. The objectives were to investigate the gene expression of *Ace*, *Ace2*, *Agtr1a*, *Agtr1b*, *Agtr2*, *Esr1* and *Esr2* [estrogen receptor 2 (ER beta)] in uterine artery in response to gestational protein restriction and assess if the expression of these genes is altered by estradiol and progesterone.

MATERIALS AND METHODS

Animal and Experimental Designs

All procedures were approved by the Animal Care and Use Committee at the University of Texas Medical Branch and were in accordance with those published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). In study one, timed pregnant Sprague-Dawley rats weighing between 175 and 225 g were purchased from Harlan (Houston, TX, USA). At Day 3 of pregnancy, rats were randomly divided into two dietary groups, housed

individually and fed a control (CT, 20% casein) or low protein (LP, 6% casein) diet until they were sacrificed on Days 19 or 22 of pregnancy (n = 5 rats/diet/day of pregnancy). The isocaloric low-protein and normal-protein diets were purchased from Harlan Teklad (Cat. TD.90016 and TD.91352, respectively; Madison, WI, USA). Fetal weights were recorded. The plasma from pregnant rats was collected for angiotensin II enzyme immunoassay. In study two, virgin female rats were ovariectomized, recovered for 5 days, then implanted with either 17β-estradiol [0.5 mg, 21-day release (OVX+E2 group)], progesterone [5 mg, 21-day release (OVX+P4 group)], both E2 and P4 (OVX+E2+P4 group), or placebo pellets (OVX group) (n = 5 rats/treatment). All animals were sacrificed 7 days after pellet insertion. The procedures of ovariectomy, hormonal replacement and efficacy of these procedures were described by Ross et al [39]. The plasma levels of E2 and P4 in this study were similar to those we reported previously using similar experimental design [39].

In both studies, the main uterine artery was separated carefully from surrounding tissues and snap frozen in liquid nitrogen and stored at -80°C until analyzed. The procedures for dissecting the uterine artery was described by Mandala et al. [40].

Angiotensin II enzyme immunoassay

Angiotensin II enzyme immunoassay kit (Cat. EK-002-12; Phoenix Pharmaceutical Inc., Buringame, CA) was used for measuring the concentration of angiotensin II in rat plasma. Fifty micro liter rat plasma in duplicate was used for this assay. All the procedures were conducted according to the instruction of the assay kit.

RNA extraction and RT-PCR

Total RNA from the whole piece of uterine artery (n = 4-5 rats/diet/gender/day of pregnancy) was extracted by Trizol reagent (Cat. 15596-018, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The possible genomic DNA in total RNAs was digested with RNA free DNase I (Cat. 79254, Qiagen Inc., Valencia, CA), followed by clean-up procedures using a Qiagen RNeasy minikit (Cat. 74104, Qiagen). All these procedures were completed by following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by RT in a total volume of 20 μl by using a MyCycler Thermal Cycler (Cat. 170-9703, Bio-Rad Laboratories, Hercules, CA) under the following conditions: One cycle at 28°C for 15 min, 42°C for 50 min, and 95°C for 5 min.

Quantitative real-time PCR

Quantitative real-time PCR detection was performed on a CFX96 Real-Time PCR Detection System (Cat. 184-5096, Bio-Rad). TaqMan Gene Expression Assays for rat *Ace* (Rn00561094_m1), *Ace2* (Rn01416289_m1), *Agtr1a* (Rn01435427_m1), *Agtr1b* (Rn02132799_s1), *Agtr2* (Rn00560677_s1), *Esr1* (R00664737_m1), *Esr2* (Rn00562610_m1) and supermix reagents were from Applied Biosystems (Carlsbad, CA). The reaction reagent mixture was incubated at 50°C for 2 min, heated to 95°C for 10 min, and cycled according to the following parameters: 95°C for 30s and 60°C for 1 min for a total of 40 cycles. *Rn18s* served as an internal control to standardize the amount of sample RNA added to a reaction. The primers for rat *Rn18s* are 5′-gctgagaagacggtcgaact-3′ (forward primer) and 5′-ttaatgatccttccgcaggt-3′ (reverse primer). Syber Green Supermix (Cat. 170-8882, Bio-Rad) was used for amplification of *Rn18s*. The mixture of reaction reagents was incubated at 95°C for 10 minutes and cycled according to the following parameters: 95°C for 30 seconds and 60°C for 1 minute for a total of 40 cycles. Negative control without cDNA was performed to test primer specificity. The relative gene expression was calculated by use of the threshold cycle (C_T) *Rn18s* /C_T target gene.

Protein preparation

Considering the heterogeneity of uterine artery [41], protein was extracted from the same uterine arteries where the total RNAs were prepared, as described previously [42]. Briefly, proteins were precipitated from organic phase in aforementioned RNA preparation procedure using Trizol reagent (Cat. 15596-018,

Invitrogen). All the procedures followed the manufacturer's protocol. The final protein pellets were dissolved in resuspension buffer (2% SDS, 10% glycerol and 50 mM Tris, pH 7.8).

Western blotting

Aliquots of 20 μg protein were added with 4X NuPAGE LDS Sample Buffer (Cat. NP0007, Invitrogen) and subjected to electrophoresis with NUPAGE 4-12% Bis-Tris Gel (Cat. NP0006; Invitrogen) and MOPS SDS Running Buffer (Cat. NP0001; Invitrogen). Briefly, the separated proteins in SDS-PAGE were transferred to a nitrocellulose membrane at 4ºC overnight. After blocking in 5% nonfat milk, a rabbit anti-ACE1 polyclonal IgG (Cat. PT344R; Panora Biotech, Sugarland, TX) at 1:1000 dilution, a goat anti-AGTR1 polyclonal IgG (Cat. Sc-31181; Santa Cruz) or a rabbit anti-AGTR2 polyclonal IgG (sc-9040; Santa Cruz) at 1:200 dilution was added to nitrocellulose membrane and incubated at room temperature for 2 hours. After washing, the blots were incubated with HRP-conjugated goat anti-rabbit IgG (Cat. 1030-05; SouthernBiotech, Birmingham, AL) or HRP-conjugated donkey anti-goat IgG (Cat. 6420-05; SouthernBiotech) at 1:5000 dilution at room temperature for 1 h. Proteins in blots were visualized with Pierce enhanced chemiluminescence detection (Cat. 32209; Thermo Scientific, Rockford, IL) and Blue Lite Autorad Film (Cat. F9024; BioExpress, Kaysville, UT) according to the manufacturers' recommendations. ACTB (beta-actin) was used as an internal control for Western blotting in this study. Primary antibody, mouse monoclonal antibody for ACTB (Cat. 3700; Cell Signaling, Danvers, MA), and secondary antibody, HRP-conjugated goat antimouse IgG (Cat. 1030-05; Southern Biotech, Birmingham, AL) were used at 1:10000 and 1:5000 dilution, respectively. Visualization of proteins in blots was the same as mentioned above. Blots were incubated with stripping buffer (Cat. 46430; Thermo Scientific) at room temperature for 15 minutes, followed by blocking in 5% nonfat milk for 1 h. The blots were incubated with a rabbit anti-ACE2 polyclonal IgG (Cat. ab87436; Abcam Inc., Cambridge, MA) at 1:1000 dilution at room temperature for 2 hours. The following procedures were the same as those for ACE analysis.

The signals in films representing the contents of the target proteins ACE, AGTR1, AGTR2 and the internal control protein ACTB were densitometrically quantified by using Fluorchem 8000 software (Cell Biosciences, Santa Clara, CA). The relative amount of target protein was calculated by the ratio of total densitometrical value of target protein to that of ACTB in each rat uterine artery analyzed by western blotting.

Statistical analysis

All quantitative data were subjected to least-squares analysis of variance (ANOVA) by using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Data on fetal-placental weight, plasma levels of angiotensin II and gene expression were analyzed for effects of day of pregnancy, diet treatment and their interaction. Data from steroid hormone supplementation were analyzed using preplanned orthogonal contrasts (OVX + P4 versus OVX, OVX + E2 versus OVX, and OVX + P4 + E2 versus OVX). A P value of 0.05 or less was considered significant, whereas a P value of 0.05 to 0.10 was considered to represent a trend toward significance. Data in tissue weights are presented as means with standard errors, while data in gene expression were presented as least-squares means (LSMs) with overall standard errors (SE).

RESULTS

Fetal growth was impaired by maternal protein restriction

Fetal weights were shown in Fig. 1. Weights of both male and female fetuses in LP were lower than those in CT at both Days 19 and 22 of pregnancy ($P < 0.05$).

Plasma levels of Angiotensin II in pregnant rats were increased by maternal protein restriction

At Days 19 and 22 of pregnancy, the plasma levels of angiotensin II in LP group were increased ($P < 0.05$) by 1.4-fold, and 1.5-fold compared to those in control group, respectively. In LP and control groups, the plasma levels of angiotensin II at Day 22 of pregnancy were increased by 1.9-fold and 2.1-fold, respectively, compared to those at Day 19 of pregnancy ($P < 0.05$, $P < 0.001$) (Figure 2).

Expression of Ace *in rat uterine artery*
mRNA levels of *Ace* in uterine artery was not changed by LPD at Day 19 of pregnancy, but increased 2.3-fold ($P < 0.05$) by LPD at Day 22 of pregnancy (Fig. 3A); while the abundance of ACE protein was decreased by1.7-fold in LP group at Day 19 of day, and not changed by diet at Day 22 of pregnancy (Fig. 3B). Both *Ace2* mRNAs and protein were not detectable in rat uterine artery by q-PCR and western blotting, respectively (Data not shown).

Expression of Agtr1a, Agtr1b, Agtr2, Esr1, Esr2 *in rat uterine artery*
The mRNA levels of *Agtr1a* in uterine artery were increased by 3.5-fold ($P < 0.001$) and 5.0-fold ($P < 0.001$) in LP at Days 19 and 22 of pregnancy, respectively (Fig. 4A). The expression of *Agtr1b* in uterine artery were also increased in LPD group by 6.4-fold ($P < 0.001$) and 3.5-fold ($P < 0.001$) at Days 19 and 22 of pregnancy, respectively (Fig. 4B). The abundance of AGTR1 protein in rat uterine artery was increased by1.7-fold in LP group compared to control at Day 19 of pregnancy, but not changed by diet at Day 22 of pregnancy (Fig. 4C).

The mRNA levels of *Agtr2* were not changed by diet at both Days 19 and 22 of pregnancy (Fig. 5A). In contrast, the abundance of AGTR2 in rat uterine artery was increased by 1.5–fold in LP group compared to control at Day 19 of pregnancy and not changed at Day 22 of pregnancy (Fig. 5B).

Expression of Esr1*and* Esr2 *in rat uterine artery*
The expression of *Esr1* was increased in LP group by 2.6-fold ($P < 0.001$) and 2.1-fold ($P < 0.05$) at Days 19 and 22 of pregnancy, respectively (Fig. 6A). The expression of *Esr2* was marginally detectable by q-PCR, and Ct value was in a range of 35.2-38.4 (data not shown).

Expression of Ace, Agtr1a, Agtr1b *and* Agt2 *in rat uterine artery was regulated by female sex hormone*
The expression of *Ace* was not changed by either E2 or P4 (Fig. 7A). Both E2 and P4 reduced the expression of *Agtr1a* by 1.4- and 1.9-fold, respectively ($P < 0.05$, $P < 0.001$) (Fig.7B); E2 increased the expression of *Agtr1b* by 2.5-fold ($P < 0.001$) (Fig. 7C); E2 increased the expression of *Agtr2* by 2.9-fold ($P < 0.001$) (Fig. 7D).

DISCUSSION
This study, to our knowledge, reports for the first time that gestational protein restriction increases plasma levels of angiotensin II in pregnant dams and alters gene expression of RAS in uterine artery. Numerous studies examined the effects of maternal protein restriction on RAS of the offspring as reviewed by Moritz [10], however, whether gestational protein restriction affects the expression of RAS components and their functions in the maternal side has been largely ignored. This study shows that gestational protein restriction does affect RAS in pregnant dams and also provides frame work for the investigation of RAS function in response to gestational protein restriction. We suggest that these changes in RAS in response to gestational protein restriction may contribute to the reduced vascular relaxation and thus, reduce blood flow to the uteroplacental unit, resulting in fetal growth restriction (Fig. 1).

Reductions in uterine arterial blood flow can occur through elevated angiotensin II levels in the plasma and/or elevated AGTR1 receptors in the uterine artery. Previous studies in late pregnant ewes have shown a reduction in uterine artery blood flow when plasma angiotensin II levels are elevated [43]. This study demonstrates elevated angiotensin II levels in the plasma for protein restricted pregnant rats compared to

controls. In control group, the plasma levels of angiotensin II were increased from Day 19 to Day 22 of pregnancy (Fig. 2). This is similar to the changes in angiotensin II in human pregnancy [44]. LP did not alter this temporal pattern, but increased the plasma levels of angiotensin II compared to control group at both Days 19 and 22 of pregnancy (Fig. 2). The elevated angiotensin II levels may exacerbate the arterial constriction by these following mechanisms. 1) The net bioavailability of angiotensin II for the uterine artery is increased in LP dams, when the elevated levels of circulating angiotensin II overcome the reduction in the local production of angiotensin II in uterine artery due to the decreased abundance of ACE protein by LP (Fig. 3B). Angiotensin II binds to AGTR1 and finally causes vasoconstriction; 2) angiotensin II up-regulates the expression of *Agtr1* and down-regulates the expression of *Agtr2* [45]; 3) the non-expression of *Ace2* in uterine artery (data not shown) makes it impossible to convert angiotensin II to angiotensin (1-7) and reduce the angiotensin II-induced vasoconstriction and 4) in long term, angiotensin II promotes vascular smooth muscle proliferation via AGTR1 in arteries [3;46;47]. As a result, gestational protein restriction may stimulate uterine artery smooth muscle cell proliferation and differentiation.

In the vascular tissue including uterine artery, AGTR1 and AGTR2 mediate the physiological function of angiotensin II, vasoconstriction or vasodilatation, respectively. Recently, it has been reported that AGTR2 may reduce AGTR1 mediated contractile response to angiotensin II in mouse uterine artery [18], possibly by stimulating nitric oxide production [48]. During the mid and late pregnancy, the vasoconstricting response to angiotensin II, primarily mediated by AGTR1[49], is increased in mouse uterine artery, although the AGTR2 mediated vasodilatation is increased as well [48]. Although it is known that the affinity of AGTR1 and AGTR2 to angiotensin II in rat heart is similar [50], in the uterine artery, angiotensin II is suggested to interact primarily with AGTR1 instead of AGTR2 [51], as the inhibitory effect mediated by AGTR2 on vasoconstriction by AGTR1 is reduced in the late pregnancy [52]. In ovine uterine artery, the ratio of AGTR1/AGTR2 is increased during pregnancy [53]. These may indicate that AGTR1 may be predominant over AGTR2 in regulating vascular reactivity in the uterine artery. In this study, at Day 19 of pregnancy the abundance of both AGTR1 and AGTR2 proteins are increased by LP (Fig. 4B, 5B), with the unaltered ratio of AGTR1 and AGTR2 in LP. Notably, AGTR1 proteins detected by Western Blotting in this study contain the products of two genes, *Agtr1a* and *Agtr1b*, because the antibody can not differentiate the two proteins due to their high identity (95%) [54]. *Agtr1a* and *Agtr1b* are different genes, but they show high identity in both nucleotide and amino acid sequences [54] and their proteins mediate similar vasoconstriction in response to angiotensin II [55;56]. It has been reported that protein restriction impairs vascular relaxation of mesenteric arteries in pregnant rats [17] and global dietary restriction reduces the uteroplacental blood flow [16]. Thus, it is reasonable to predict that the overall vasoconstriction in the LP uterine artery may be enhanced and therefore, the uterine blood flow is impaired in the LP group. This will be investigated in our future studies.

The expressions of *Ace* and *Agtr2* in uterine artery may be regulated at the posttranscriptional level. The direct evidence in this study is that the protein and mRNA levels of these genes do not match well (Figs. 3, 5). This phenomenon also occurs in these gene expressions in rat placental labyrinth zone [57]. Recently, several studies have demonstrated that gene expressions of RAS components such as *Ace* and *Agtr2* are post-transcriptionally regulated by microRNAs [12;13]. Up-regulation of miRNAs, mmu-MIR27A and 27B, by maternal protein restriction reduces the ACE proteins in the brains of offspring [13]. However, to date, the existence of miRNAs regulating the expression of *Ace* in uterine artery has never been reported. As for the functional significance, the reduction of ACE protein at late pregnancy in response to gestational protein restriction may be an attempt to reduce the local angiotensin II levels and increased AGTR2 proteins may promote vasodilatation. These may represent positive adaptation to the maternal protein restriction when fetuses grow rapidly in late pregnancy. The underlying mechanisms warrant further investigation.

The expressions of *Agtr1* and *Agtr2* are regulated by female sex hormones. Pregnancy demonstrates remarkable changes in hormones, metabolism and cardiovascular functions. It has been reported that

gestational protein restriction increases the maternal plasma levels of estradiol [26], while reducing the plasma levels of progesterone [27;28]. Accumulating evidence indicate that estrogen regulates gene expression of RAS components in a tissue-specific manner [29-33]. Estrogen stimulates the renal *Agtr1a* and *Agtr2* expression in rodents [29;30]. In contrast, estrogen down-regulates expression of *Agtr1a* in hypothalamus [32]. Similarly in this study, we found that the expression of *Agtr1a* in uterine artery was reduced by both E2 and P4 (Fig. 7B). It has been reported that gestational protein restriction decreased the plasma levels of P4 in rat [27;28], therefore, the elevated expression of *Agtr1a* in response to gestational protein restriction may be caused partly by the reduced levels of P4 [27;28], as angiotensin II also stimulates the expression of *Agtr1* [45]. This study also demonstrates that estrogen increases the expression of *Agtr1b* and *Agtr2* in rat uterine artery (Figs. 7C, 7D). Further, the expression of *Esr1* in the uterine artery is elevated in protein restricted pregnant rats (Fig. 6). Thus, elevated expression of *Esr1* and plasma levels of estrogen in protein restricted mothers could have contributed to the increased expressions of *Agtr1b* in uterine artery. As a result, although estrogen is a potent vasodilator and critical for the cardiovascular adaption to pregnancy, estrogen induced RAS changes in the uterine artery in response to gestational protein restriction failed to restore the normal vasoactivity, shown by the impaired vascular relaxation [17].

In summary, this study reveals that gestational protein restriction increases the maternal plasma levels of angiotensin II and *Agtr1* expression in uterine artery and, thus, may exacerbate the vasoconstriction in uterine artery and that female sex hormones also play a role in this process. In future studies, the following relevant questions will be addressed: 1) Is vasoconstricting response of uterine artery to angiotensin II elevated in the rats with gestational protein restriction, 2) which type of angiotensin II receptors play predominant roles in vasoconstriction of uterine artery in vivo or ex vivo and 3) Can the altered expression of RAS by gestational protein restriction be reversed by supplementation or blockage of sex hormones? These will provide valuable information for the role of altered RAS in the fetal programming on adulthood hypertension in response to nutritional disruptions.

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FIGURE LEGENDS

Figure 1. Fetal weights in rat with (LP) or without (CT) gestational protein restriction at Day 19 and 22 of pregnancy. The error bar represents the mean ± SEM. *, $P < 0.05$.

Figure 2. Plasma levels of angiotensin II in pregnant rats with protein restriction (LP) and controls (CT). The error bar represents the mean ± SEM. (*, $P < 0.05$).

Figure 3. Expressions of *Ace* in rat uterine artery with (LP) or without (CT) gestational protein restriction at Day 19 and 22 of pregnancy. A) q-PCR analysis of mRNA levels of *Ace.* The error bar represents the least mean ± SEM expressed as relative units of mRNA standardized against *Rn18s* (n = 6); B) Western blot analysis of ACE protein. The error bar represents the mean ± SEM expressed as the ratio of density of the ACE band to that of ACTB (n = 3-4) (*, $P < 0.05$).

Figure 4. Expressions of *Agtr1* in rat uterine artery with (LP) or without (CT) gestational protein restriction at Day 19 and 22 of pregnancy. A) q-PCR analysis of mRNA levels of *Agtr1a;* B) q-PCR analysis of mRNA levels of *Agtr1b.* The error bar represents the least mean ± SEM expressed as relative units of mRNA standardized against *Rn18s* (n = 6); C) Western blot analysis of AGTR1 protein. The error bar represents the mean ± SEM expressed as the ratio of density of the AGTR1 band to that of ACTB (n = 3-4) (*, $P < 0.05$; ***, $P < 0.001$).

Figure 5. Expressions of *Agtr2* in rat uterine artery with (LP) or without (CT) gestational protein restriction at Day 19 and 22 of pregnancy. A) q-PCR analysis of mRNA levels of *Agtr2.* The error bar represents the least mean ± SEM expressed as relative units of mRNA standardized against *Rn18s* (n = 6); B) Western blot analysis of AGTR2 protein. The error bar represents the mean ± SEM expressed as the ratio of density of the ACE band to that of ACTB (n = 3-4) (**, $P < 0.01$).

Figure 6. Expressions of *Esr1* in rat uterine artery with (LP) or without (CT) gestational protein restriction at Day 19 and 22 of pregnancy by q-PCR analysis. The error bar represents the least mean ± SEM expressed as relative units of mRNA standardized against *Rn18s*(n = 6) (*, $P < 0.05$; **, $P < 0.01$).

Figure 7. Hormonal regulation on the expressions of *Ace* (A), *Agtr1a* (B), *Agtr1b* (C) and *Agtr2* (D) in rat uterine artery. The error bar represents the least mean ± SEM expressed as relative units of mRNA standardized against *Rn18s* (n = 5) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

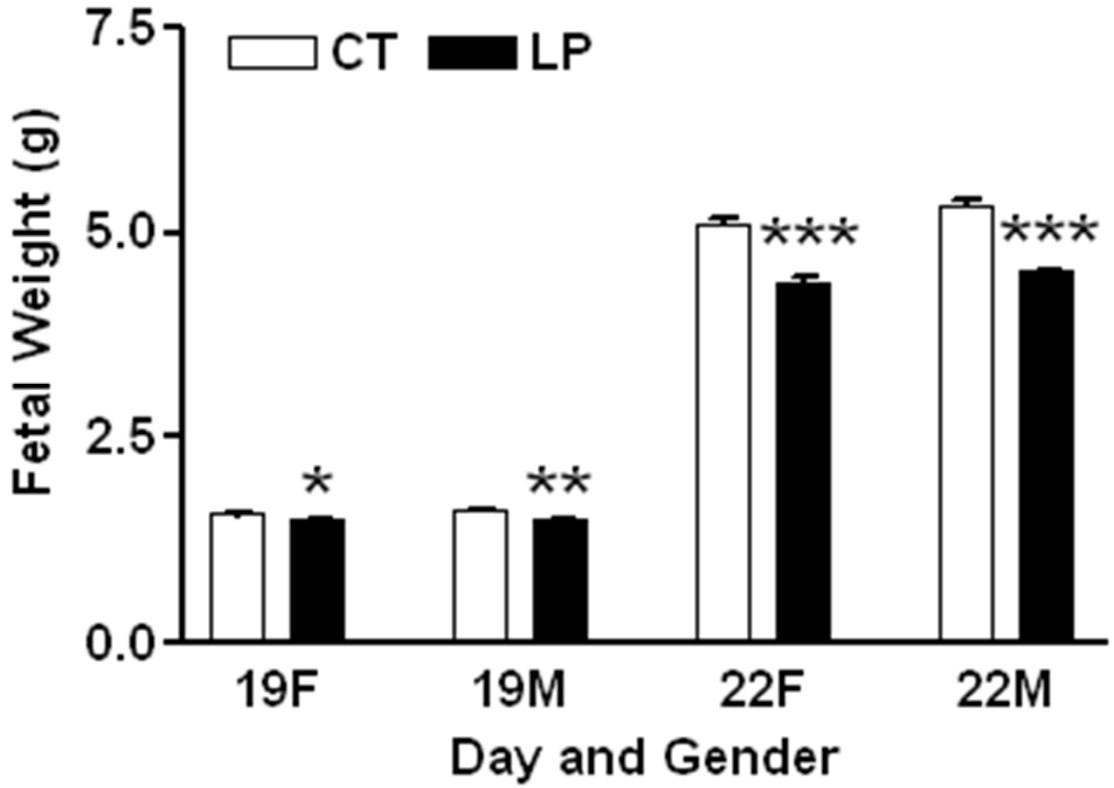


Figure 1

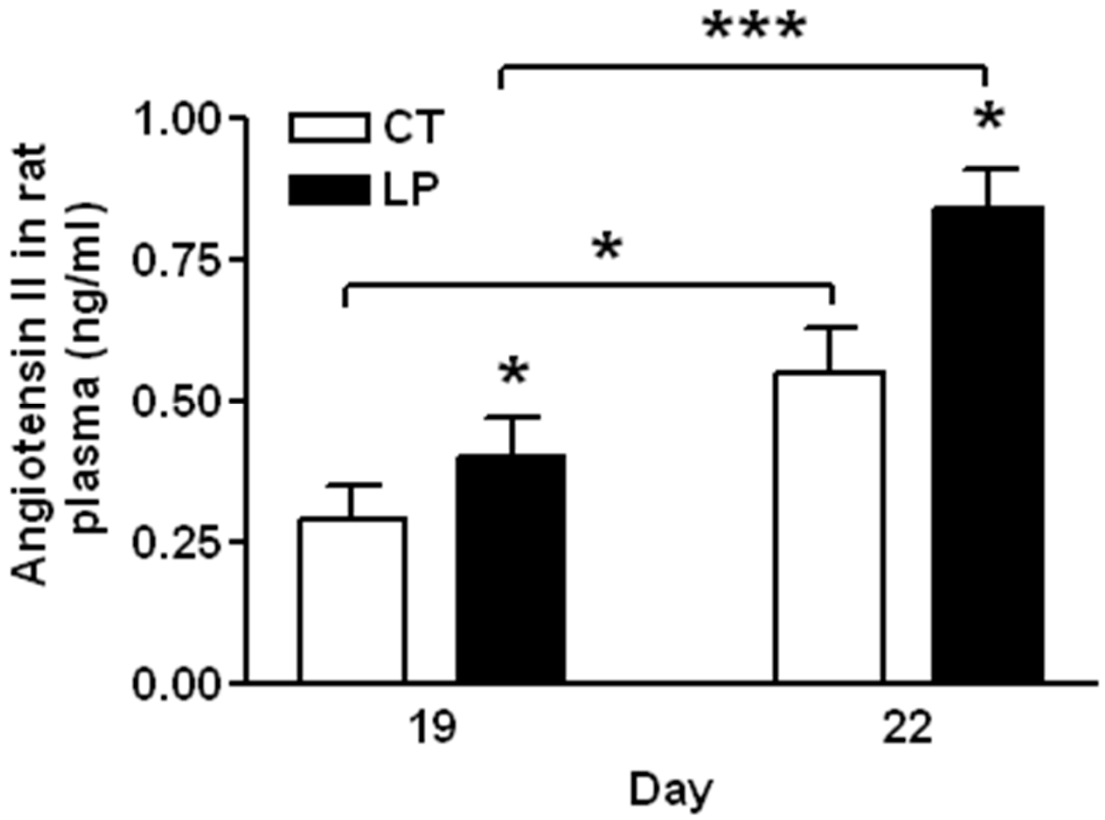
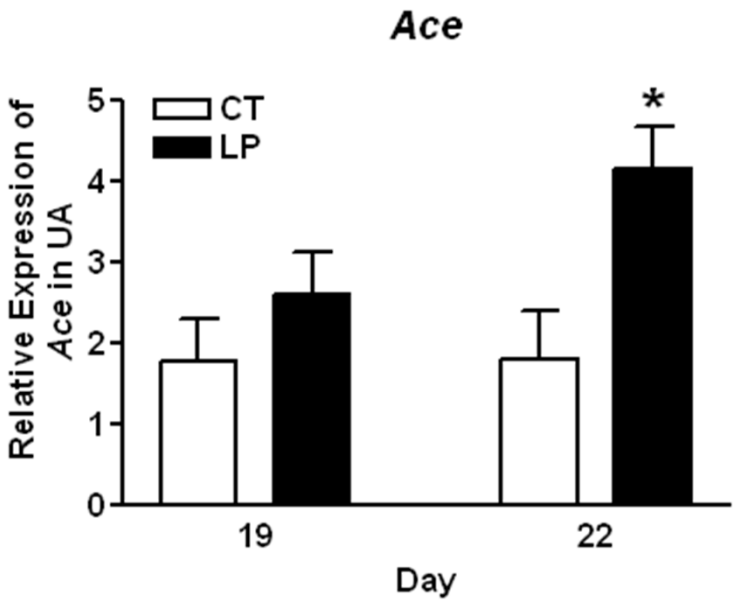

***
*
*
*
CT
LP
1.00
0.75
0.50
0.25
0.00
Angiotensin II in rat plasma (ng/ml)
19
22
Day

Figure 2

A)


Ace
Relative Expression of Ace in UA
CT
LP
*
5
4
3
2
1
0
19
22
Day

B)

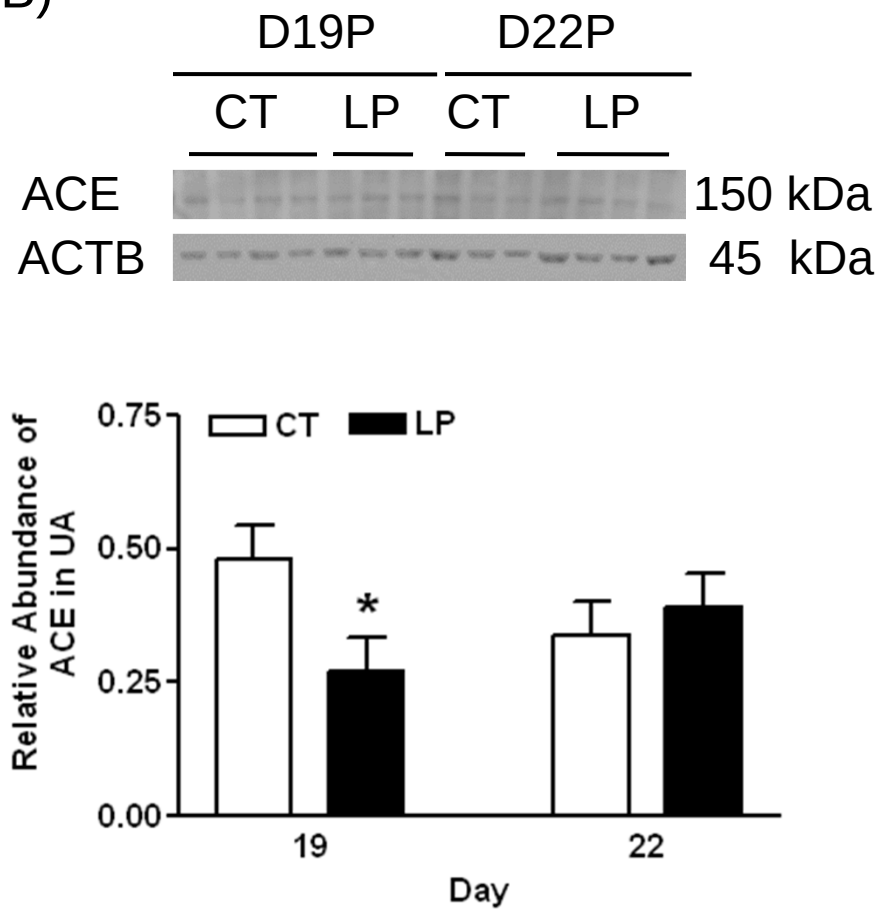
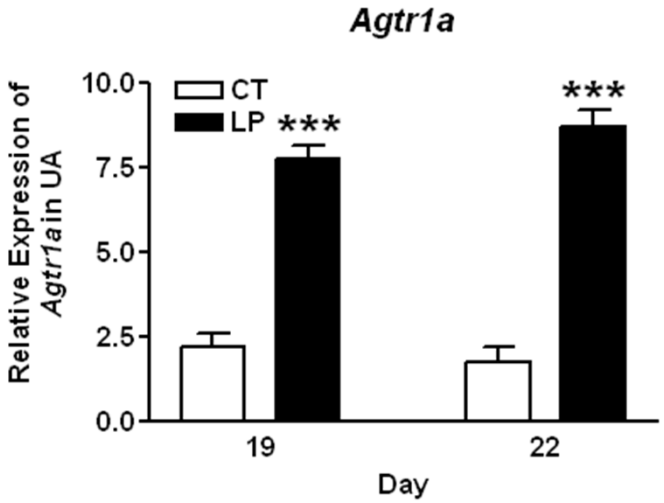
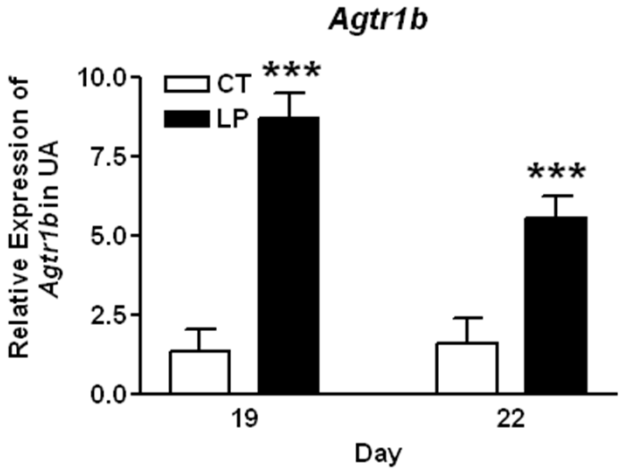

D19P
D22P
CT
LP
CT
LP
ACE
150 kDa
ACTB
45 kDa
Relative Abundance of ACE in UA
0.75
0.50
0.25
0.00
CT
LP
*
19
22
Day

Figure 3

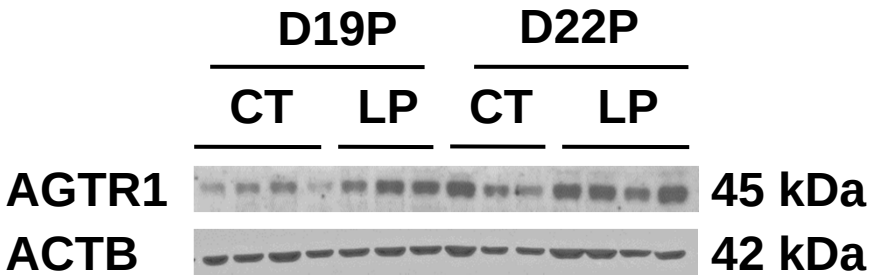
A)


Agtr1a
Relative Expression of Agtr1a in UA
10.0
7.5
5.0
2.5
0.0
CT
LP
***
***
19
22
Day

B)


Agtr1b
Relative Expression of Agtr1b in UA
10.0
7.5
5.0
2.5
0.0
CT
LP
***
***
19
22
Day

C)


D19P
D22P
CT
LP
CT
LP
AGTR1
45 kDa
ACTB
42 kDa

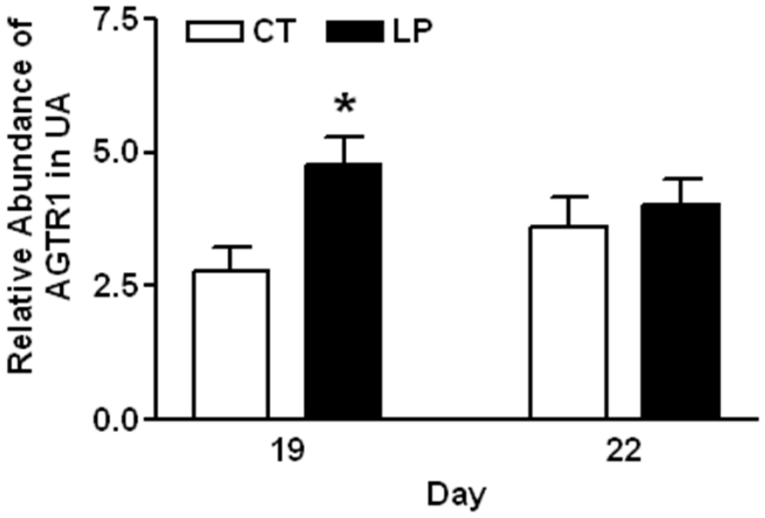
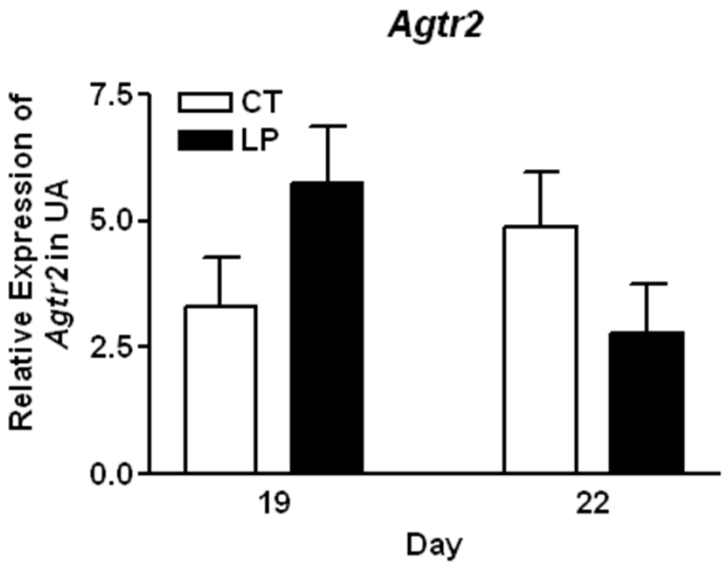

Relative Abundance of AGTR1 in UA
7.5
5.0
2.5
0.0
CT
LP
*
19
22
Day

Figure 4

A)


Agtr2
Relative Expression of Agtr2 in UA
7.5
5.0
2.5
0.0
CT
LP
19
22
Day

B)

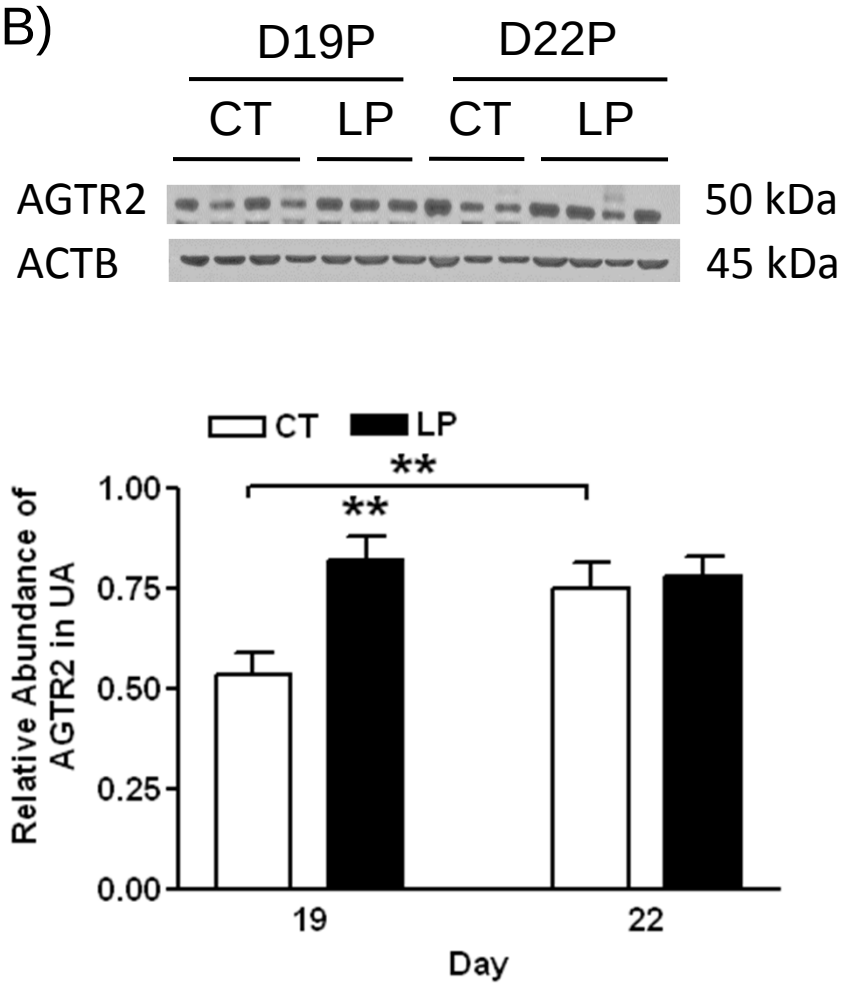

D19P
D22P
CT
LP
CT
LP
AGTR2
50 kDa
ACTB
45 kDa
CT
LP
**
**
Relative Abundance of AGTR2 in UA
1.00
0.75
0.50
0.25
0.00
19
22
Day

Figure 5

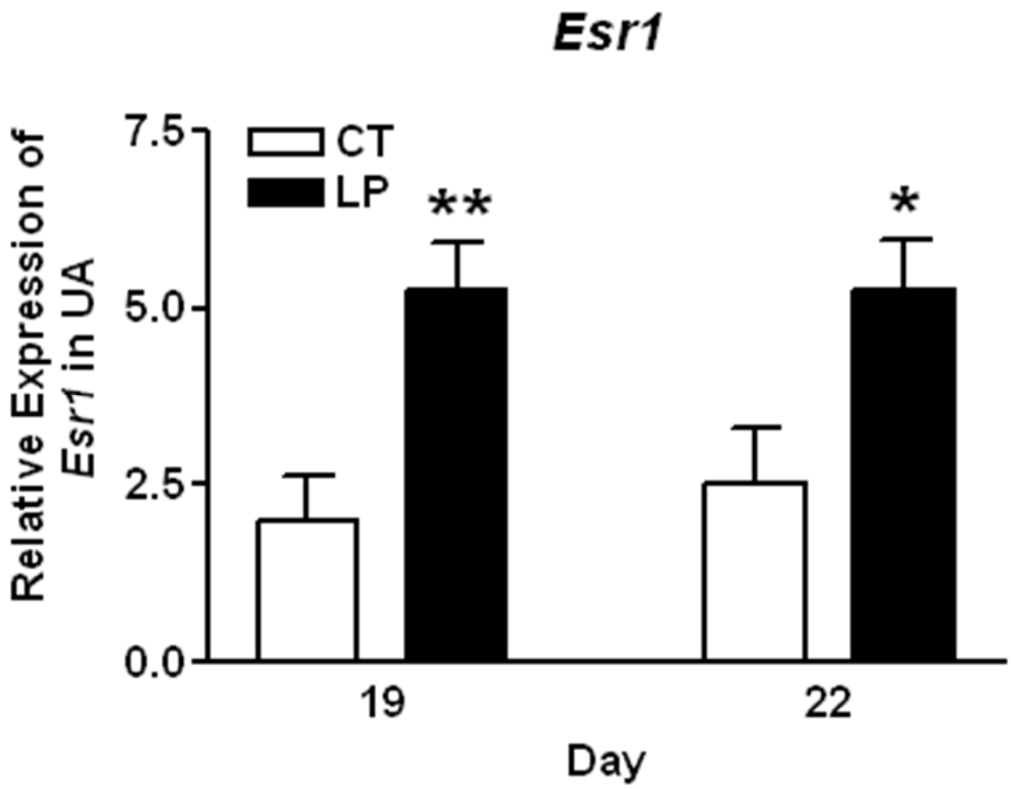

Esr1
Relative Expression of Esr1 in UA
7.5
5.0
2.5
0.0
CT
LP
**
*
19
22
Day

Figure 6

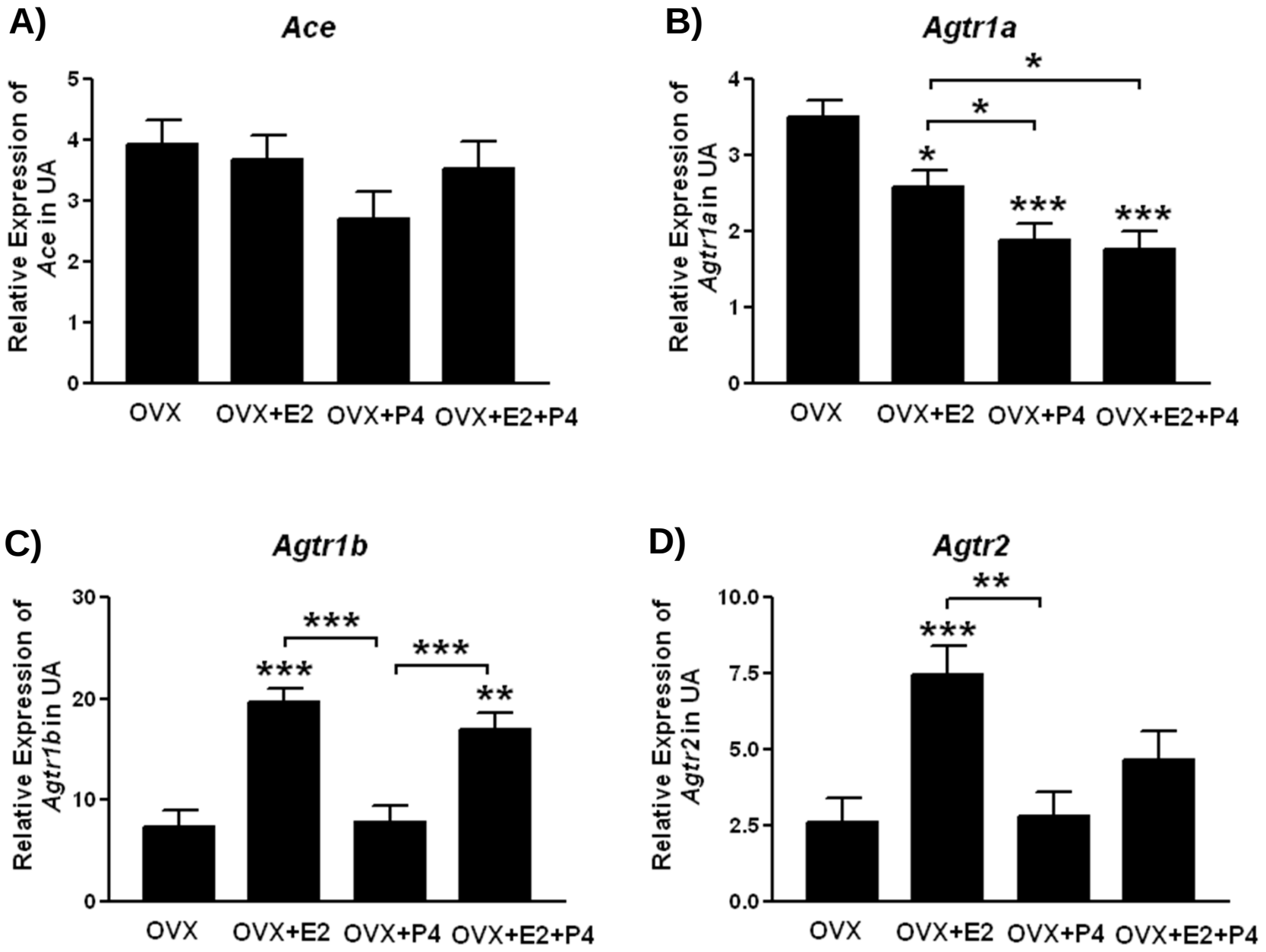

A)
Ace
Relative Expression of Ace in UA
0
1
2
3
4
5
OVX
OVX+E2
OVX+P4
OVX+E2+P4
B)
Agtr1a
Relative Expression of Agtr1a in UA
0
1
2
3
4
*
*
*
***
***
OVX
OVX+E2
OVX+P4
OVX+E2+P4
C)
Agtr1b
Relative Expression of Agtr1b in UA
0
10
20
30
***
***
***
**
OVX
OVX+E2
OVX+P4
OVX+E2+P4
D)
Agtr2
Relative Expression of Agtr2 in UA
0.0
2.5
5.0
7.5
10.0
**
***
OVX
OVX+E2
OVX+P4
OVX+E2+P4

Figure 7