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Effects of Circulating and Local Uteroplacental Angiotensin II in Rat Pregnancy

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Abstract—The renin-angiotensin (Ang) system is important during placental development. Dysregulation of the renin-Ang system is important in preeclampsia (PE). Female rats transgenic for the human angiotensinogen gene crossed with males transgenic for the human renin gene develop the PE syndrome, whereas those of the opposite cross do not. We used this model to study the role of Ang II in trophoblast invasion, which is shallow in human PE but deeper in this model. We investigated the following groups: PE rats, opposite-cross rats, Ang II–infused rats (1000 ng/kg per day), and control rats. Ang II infusion increased only circulating Ang II levels (267.82 pg/mL), opposite cross influenced only uteroplacental Ang II (13.52 fmol/mg of protein), and PE increased both circulating (251.09 pg/mL) and uteroplacental (19.24 fmol/mg of protein) Ang II. Blood pressure and albuminuria occurred in the models with high circulating Ang II but not in the other models. Trophoblast invasion increased in PE and opposite-cross rats but not in Ang II–infused rats. Correspondingly, uterine artery resistance index increased in Ang II–infused rats but decreased in PE rats. We then studied human trophoblasts and villous explants from first-trimester pregnancies with time-lapse microscopy. Local Ang II dose-dependently increased migration by 75%, invasion by 58%, and motility by 282%. The data suggest that local tissue Ang II stimulates trophoblast invasion in vivo in the rat and in vitro in human cells, a hitherto fore unrecognized function. Conceivably, upregulation of tissue Ang II in the maternal part of the placenta represents an important growth factor for trophoblast invasion and migration. (*Hypertension*. 2010;56:311-318.)

Key Words: preeclampsia ■ angiotensin II ■ transgenic rats ■ trophoblasts ■ decidua ■ placenta ■ vascular remodeling

Various studies have implicated the renin-angiotensin (Ang) system in preeclampsia (PE).^{1–3} Female rats harboring the human angiotensinogen gene develop hypertension and proteinuria late in pregnancy when mated with male rats harboring the human renin gene; the result is a PE-like syndrome.⁴ However, instead of showing shallow trophoblast invasion associated with PE in humans, trophoblast invasion and spiral artery remodeling are actually increased in this animal model.^{4,5} Trophoblast invasion is highly relevant in pregnancy. These fetal cells invade the maternal decidua and remodel the spiral arteries, a process that includes replacing the smooth muscle cell layer with fibrinoid material. The remodeling of the uterine spiral arteries causes the vessels to increase in diameter, allowing for an increase in blood flow to the intervillous space of the placenta.⁶ The role of Ang II in this process is unclear.

The hallmark of PE is a shallow trophoblast invasion accompanied by a reduced percentage of remodeled spiral arteries in the decidua and no remodeling in the inner myometrium.⁶ Preliminary observations in our transgenic rat model indicated that the opposite cross (OC), namely dams harboring a human renin gene mated with males harboring human angiotensinogen, did not develop hypertension or a PE-like syndrome.⁴ In addition to the circulating renin-Ang system, several authors have described a local, uteroplacental renin-Ang system in pregnancy.^{7–10} However, the function of the uteroplacental renin-Ang system is unknown. Consistent with the observations in rat pregnancy, we showed earlier that components of the tissue renin-Ang system are substantially higher in the maternal part of the human placenta (the decidua), than in the fetal part (placenta), suggesting a role for local Ang II in the trophoblast-decidua interaction.¹¹

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We investigated the effects of uteroplacental Ang II and circulating Ang II in rat pregnancy in more detail. We studied our conventional PE-syndrome cross, the OC, and chronic Ang II-infused pregnant rats and appropriate control rats. We also extended our observations to in vitro Ang II-related effects on cells derived from human trophoblasts and villous explants from first-trimester pregnancies. Our data show that uteroplacental Ang II has opposite in vivo effects on trophoblast invasion, migration, uteroplacental vascular remodeling, and maternal endothelial dysfunction compared with circulating Ang II. Local Ang II could serve as a potent regulator promoting trophoblast migration and invasion early in pregnancy.

Methods

Animal Studies

Details on the TGR models (PE and OC) have been published previously.^{4,12} On day 9 of gestation, Sprague-Dawley (SD) dams were randomly assigned to 1 of 2 experimental groups: SD plus vehicle, which served as the control group (n=9) or SD plus Ang II (n=8), at a dose of 1000 ng/kg per min (Ang II). The animals were followed to term (day 21), anesthetized, and then euthanized. Doppler studies were performed as described earlier.¹³ Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured from 3 cardiac cycles. The resistance index (RI=[PSV-EDV]/PSV) was calculated when EDV was >0 to quantify the pulsatile arterial blood velocity waveforms.

Tissue sections were immediately snap frozen in liquid nitrogen and homogenized as described previously.^{4,11,13} Tissue Ang II and circulating Ang II were measured by radioimmunoassay.⁷ Tissue preparations and immunostaining protocols were performed as described earlier.^{5,14} For the quantitative analysis of depth of invasion and spiral artery remodeling, the KS-400 image analysis system (Carl Zeiss) was used, and the length of each traced line was measured and calculated in relation to spiral artery lumen contour. More detailed information is given in the online Data Supplement (please see <http://hyper.ahajournals.org>).

Cell Culture

SGHPL-4 cells (derived from primary human first-trimester extravillous trophoblasts transfected with the early region of SV40, known previously as MC418) were cultured as described earlier.¹⁵ The effect of 48-hour Ang II stimulation in matrigel on the invasive trophoblast outgrowth was investigated as described earlier.¹⁶ Human placental villous tissue explants from first-trimester pregnancies were established and characterized as described previously.¹⁷ Migration experiments were conducted with collagen-coated chambers after 72-hour Ang II exposure. More detailed information is given in the online Data Supplement.

Statistics

Data are presented as mean \pm SEM. We tested Kolmogorov-Smirnov for distribution. For group differences we tested 1-way ANOVA with Bonferroni post hoc test, Dunnett T3, or Kruskal-Wallis test and Mann-Whitney *U* test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Circulating and Local Uteroplacental Ang II

Circulating Ang II was increased in PE rats and in rats after Ang II infusion (Figure 1A). OC rats had similar plasma Ang II levels as SD. In contrast to the circulating plasma, local uteroplacental tissue concentration of Ang II was increased in both PE and OC rats (Figure 1B). Ang II infusion did not lead to an increase in uteroplacental Ang II. Thus, PE is a rat

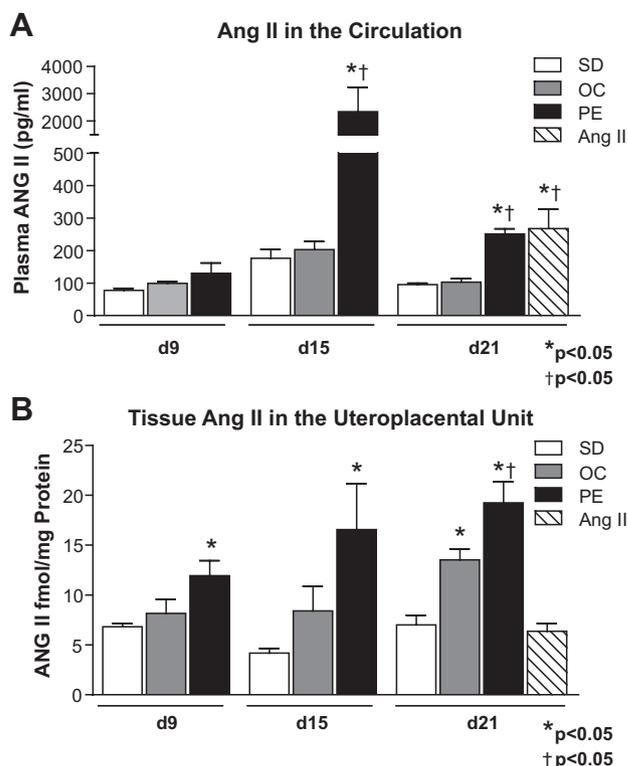


Figure 1. A, Circulating Ang II levels on days 9, 15, and 21 of pregnancy. These values were highest in PE rats. Ang II-infused rats had correspondingly elevated values. OC rats had values no different from SD rats. B, Ang II levels in the uteroplacental unit on days 9, 15, and 21 of pregnancy. PE and OC placentas had increased values on day 21. Ang II infusion placentas had lower values, similar to SD. Values are mean \pm SEM. * $P < 0.05$ vs SD; † $P < 0.05$ vs OC.

model with high uteroplacental and circulating Ang II levels. OC results in solely high uteroplacental Ang II values at term and normal circulating values. Human renin and angiotensinogen (hAogen) mRNA expressions were significantly elevated in the OC and PE uteroplacental units and were undetectable in SD and Ang II (Figure S1, available in the online Data Supplement). hAogen expression was significantly higher in PE compared with OC, especially in the mesometrial triangle. Ang II infusion raised the circulating Ang II values but not the uteroplacental Ang II concentrations.

Blood Pressure, Albuminuria, and Intrauterine Growth Retardation

Telemetric blood pressure measurements showed that mean arterial blood pressure significantly increased PE and Ang II and decreased in OC in the last third of pregnancy. Blood pressure was significantly increased in PE and Ang II compared with SD. Mean arterial blood pressure was significantly decreased in OC versus control (Figure 2A). Albuminuria was present in PE and Ang II-infused rats, but not in OC or SD rats, in the last third of pregnancy (Figure 2B). Placental weight was lower in PE and Ang II-infused rats. OC rats had placental weights no different from SD rats (Figure 2C, left). Fetal body weight was reduced in PE, OC, and Ang II-infused rats (Figure 2C, middle). To further

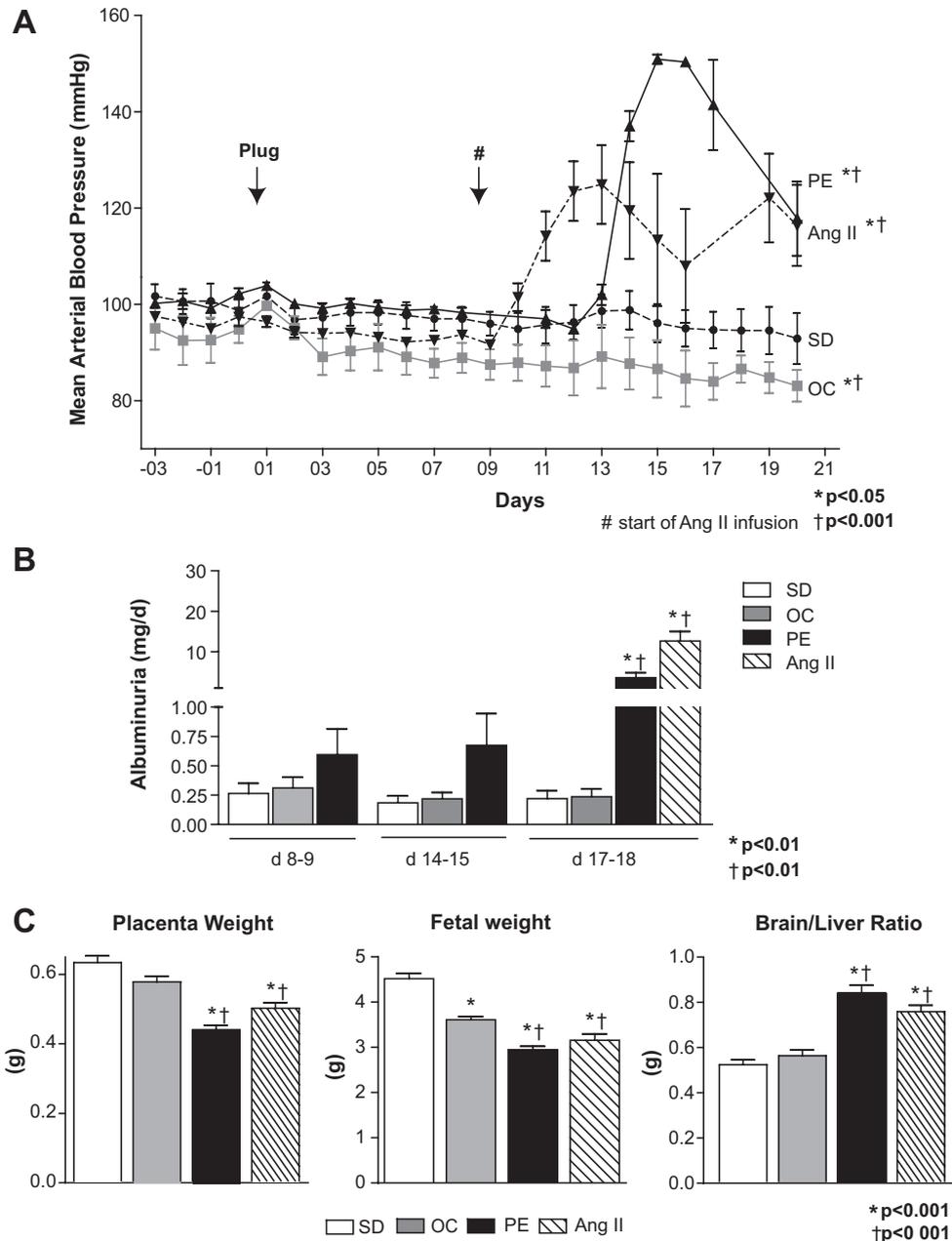


Figure 2. A, Telemetrically measured mean arterial blood pressures. PE rats and Ang II-infused rats showed a significantly increased blood pressure over time, whereas mean arterial pressure within OC rats significantly decreased toward the end of pregnancy (**P*<0.05). Analyzing the different groups among each other, mean arterial blood pressure was significant for PE, Ang II, and OC vs SD controls (†*P*<0.05). The arrow with plug indicates the observation of plug in the vagina, indicating day 1 of pregnancy. B, Albuminuria, which occurred in PE and Ang II-infused rats (**P*<0.01 vs SD; †*P*<0.01 vs OC). C, Placenta weight (left), fetal weight (middle), and brain:liver weight ratio (right). Placental weight was reduced in PE and Ang II rats. Fetal weight was decreased in PE, Ang II, and OC rats. Brain:liver ratio showed the difference in PE and Ang II rats, indicating IUGR in these 2 groups but not in OC rats. Values are mean±SEM (**P*<0.001 vs SD; †*P*<0.001 vs OC).

characterize intrauterine growth retardation (IUGR), we investigated the brain:liver ratio in the fetuses. In PE and Ang II-infused rats, we found a reduced liver and preserved brain weight, leading to an increased brain:liver ratio consistent with IUGR (Figure 2C, right). The OC rats had a normal brain:liver weight ratio and a normal placenta weight, so that the criteria for IUGR were not fulfilled, although these rats had pups small for gestational age compared with SD rats. These data indicate that PE-like features and IUGR were only present in the groups with high circulating Ang II levels.

However, slow fetal growth without signs of IUGR took place when uteroplacental Ang II was elevated, even with normal circulating Ang II levels.

Trophoblast Invasion and Spiral Artery Vascular Remodeling

Endovascular trophoblast invasion was evaluated in the mesometrial triangle in 3 zones according to the immunohistograph picture, which was stained with cytokeratin 7 to identify trophoblasts (Figure 3A, left) and as described

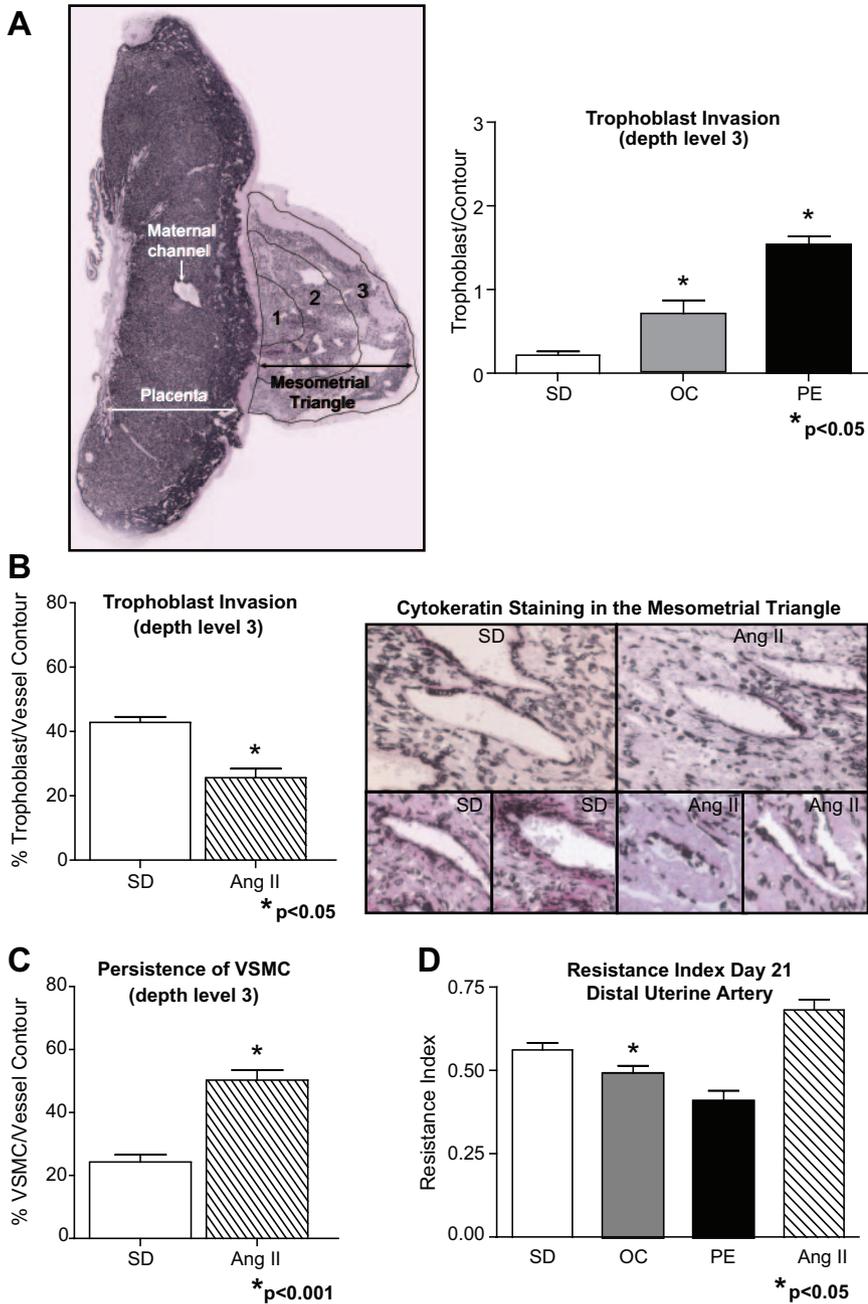


Figure 3. A, A rat placenta with associated mesometrial triangle stained with cytokeratin 7, identifying trophoblasts. Trophoblast invasion in the mesometrial triangle was evaluated in 3 depth levels from the central spiral artery to the periphery (left). Invasion was determined in terms of percentage trophoblast/vessel contour.¹⁸ As described earlier, trophoblast invasion was more intense and deeper in PE rat placentas, as shown by the trophoblast/vessel contour (reproduced with permission from Geusens et al,¹⁴ copyright Elsevier 2010). OC rat placentas showed a significantly higher percentage of endovascular trophoblast/vessel contour indicating more deep trophoblast invasion than SD (right). B, Reduced trophoblast invasion in Ang II-infused rats compared with controls (left). The invasion actually decreased. Right is shown a representative immunohistochemistry picture for trophoblast staining verifying reduced trophoblast invasion in Ang II-infused rats. Cell number of trophoblasts is lower in Ang II-infused compared with SD placenta, confirming this result. C, Increased persistence of VSMCs in the spiral arteries in Ang II-infused rats, indicating reduced vascular remodeling, confirming B. D, Doppler ultrasound of the uterine arteries. Resistance index was increased in Ang II-infused rats on day 21 of gestation compared with controls. OC and PE rats show reduced resistance index. Values are mean±SEM (*P<0.05 vs SD).

earlier.^{5,13,18} We showed earlier that deep endovascular trophoblast invasion was significantly increased in PE and OC rats, resulting in altered vascular remodeling of spiral arteries (Figure 3A, right).^{5,13} In contrast, Ang II infusion resulted in a reduced deep-trophoblast invasion in the mesometrial triangle compared with SD controls (Figure 3B, left). Examples of reduced trophoblast invasion by Ang II infusion compared with SD are shown (Figure 3B, right). In accord with alterations in trophoblast invasion, persistence of vascular smooth muscle cells (VSMC) was increased in Ang II-infused compared with SD controls (Figure 3C). We measured the functional consequences of the altered trophoblast and VSMC expression by B-mode Doppler ultrasound (Figure 3D). The measurements showed increased resistance index with Ang II infusion compared with SD controls,

indicating that placental blood flow had decreased. We found a decrease in the resistance index in PE rats and OC rats, consistent with our earlier report.¹³

Invasion, Migration, and Motility of Trophoblasts

We next investigated human trophoblasts from first-trimester pregnancies to determine the effect of uteroplacental Ang II. We stimulated SGHPL-4 cells, which were derived from primary human first-trimester extravillous trophoblasts. The cells were treated with 2 concentrations of Ang II and were studied with time-lapse microscopy (Figure 4A). Ang II dose-dependently increased the various aspects of invasion (showing more and deeper invasion) and motility. The effects of Ang II were blocked completely with losartan. To determine whether Ang II has an effect on the migration, villous

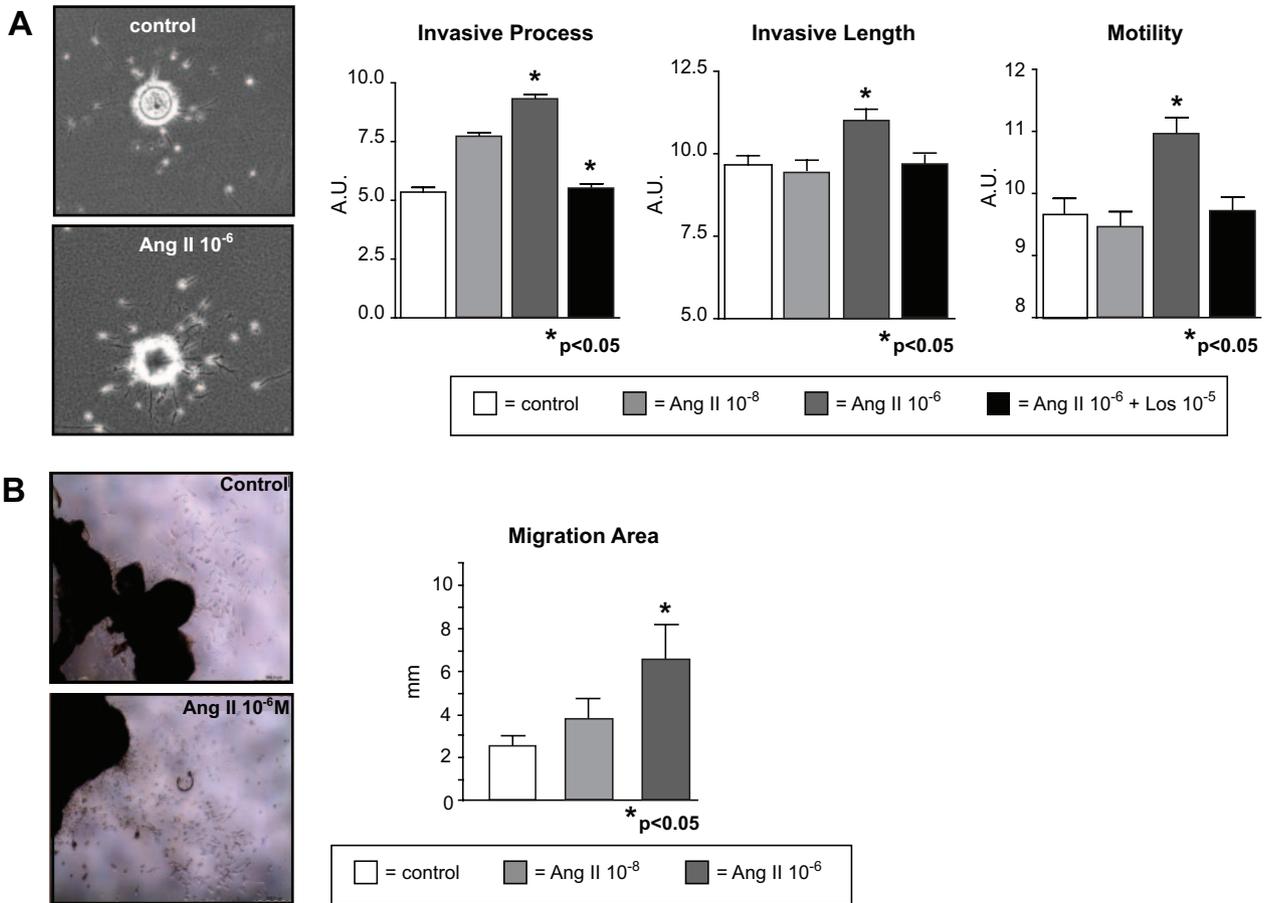


Figure 4. SGHPL-4 cells, which were derived from primary human first-trimester extravillous trophoblasts, were exposed to 2 different Ang II concentrations, and time-lapse microscopy was performed. A, A representative result from the time-lapse microscopy under control (left) and Ang II stimulation (right), and average results is showing that Ang II affects the invasive process, invasion length, and motility. All of the effects were blocked by losartan. B, Villous explants from first-trimester pregnancies in migration experiments. Above is shown a representative experiment in collagen-coated chambers. Ang II increases the migration area in a dose-dependent manner. Values are mean ± SEM (*P < 0.05 vs control).

explants from first-trimester normal pregnancies were used for in vitro invasion assay experiments in collagen-coated chambers (Figure 4B). Ang II for 72 hours resulted in a strong increase in the migration of extravillous trophoblasts cells compared with control cells. These results were reproducible in 3 separate experiments.

Discussion

The novel finding in our study was that circulating Ang II reduced trophoblast invasion and vascular remodeling in the uteroplacental unit. We found opposite effects of increased circulating and uteroplacental Ang II in pregnant rats. Ang II-infused rats exhibited an increased blood pressure in the third trimester, albuminuria, and IUGR. The OC model, in which only uteroplacental tissue Ang II is increased, showed increased trophoblast remodeling. Blood pressure was lower than in controls, no albuminuria was present, and pup weights were reduced. In the PE cross, in which circulating and tissue Ang II are increased, we observed hypertension in the last trimester, albuminuria, IUGR, increased trophoblast invasion, and uteroplacental vascular remodeling. Finally, by applying Ang II directly to human first-trimester trophoblasts and villous explants, we found that invasion, migration, and

motility were increased in response to local Ang II. We believe that our results show a different and unique function for uteroplacental Ang II in pregnancy. Uteroplacental Ang II but not circulating Ang II increased trophoblast invasion and vascular remodeling.

Several studies have shown the presence of a local tissue-specific renin-Ang system in the uteroplacental unit of normal pregnancies.^{2,7-10} Estrogen increases both tissue and circulating levels of angiotensinogen and renin.⁷ Plasma Ang II, together with angiotensinogen and plasma renin activity, is increased during normal pregnancy.¹⁹ However, the physiological function of a stimulated uteroplacental renin-Ang system during pregnancy is unknown. Our results of an increased trophoblast invasion by tissue Ang II are in line with the human results that we reported earlier in that increased renin, angiotensinogen, Ang-converting enzyme (ACE), and Ang II are substantially increased in the maternal part of the uteroplacental unit, the deciduas, compared with the fetal part of the placenta.¹¹ We now suggest that the maternal-fetal Ang II gradient contributes to trophoblast invasion and migration. Rosenfeld and Gant²⁰ reported that Ang II infusion increased blood pressure in sheep but reduced uteroplacental

perfusion, which was measured invasively with chronically implanted flow probes around both major uterine arteries. Their experiments suggested an autoregulation of uterine blood flow. Earlier, Ferris et al²¹ showed that an increase of uterine renin production resulted in an increase in uterine perfusion in rabbits. Uterine perfusion was determined with radioactive microspheres lodged in the uterus and placenta after injection in the left ventricle in that study. Li et al²² were the first to show that endocrine-active decidual cells express both renin and angiotensinogen in the human uteroplacental unit. They suggested that decidual cells regulate a tissue renin-Ang system expression in the endometrium. Anton and Brosnihan⁷ were the first to suggest a different regulation for circulating and uteroplacental Ang II in PE. They observed contrasting changes for Ang II levels in the circulation and chorionic villi in human preeclamptic pregnancy. Although circulating Ang II was significantly decreased, the expression of local tissue Ang II was augmented in the placenta of the preeclamptic women. Uteroplacental Ang II in the OC and PE groups was elevated because the expression of both human renin gene and hAogen mRNA in the uteroplacental unit was increased. hAogen was significantly higher in PE, indicating that hAogen concentrations were responsible for the different Ang II levels in OC and PE rats.²³ Morgan et al²⁴ were able to show increased expression of an angiotensinogen variant in human decidual spiral arteries that are involved in the generation of atherotic (a histopathologic term applied to PE-related vascular changes resembling atherosclerosis) lesions overrepresented in PE.

Trophoblast cells of the human placenta proliferate, migrate, and invade the pregnant uterus and remodel its vasculature. Trophoblast-associated spiral artery remodeling is an important uterine adaptation mechanism of human pregnancy, allowing an adequate maternal blood supply to the placenta. Few factors have been shown to promote trophoblast invasion and survival of these fetus-derived cells in the maternal surroundings, including corticotropin-releasing hormone, insulin-like growth factor binding protein 1, and various cytokines, such as interleukin 1.²⁵ The role of many growth factors and proto-oncogenes has not yet been thoroughly studied in the decidua.

We found that fetal weight was decreased in the Ang II-infused group compared with controls. Similar to patients with PE, we observed a shallow trophoblast invasion, as well as persistence of VSMCs and reduced spiral artery remodeling in the group with chronic Ang II infusion. The increased resistance indicates a disturbed placental function with reduced transformation of the arterioles into a highly dilated vessel. In accordance with the human pathology in PE, the increased vascular resistance led to IUGR and decreased birth weight in this group. The pathophysiological processes underlying IUGR are highly complex and incompletely understood. The finding that the fetal weight was decreased in the OC group appears surprising, because trophoblast invasion is increased and vascular resistance decreased. The pups exhibited lower birth weight, but not IUGR, suggesting a different mechanism compared with the Ang II-infused group that did

exhibit IUGR. In earlier studies, we showed that atherosclerosis is present in OC, which also might contribute to impaired fetal growth.^{4,5} We noted focal necrosis in the arterial wall in 25% of spiral arteries in that study. The atherosclerosis in these models is similar to the acute atherosclerosis associated with placental infarcts observed in uteroplacental spiral arteries of preeclamptic women.²⁶ The impact of this vasculopathy on fetal growth is unclear.^{6,27} Shibata et al²⁸ showed that Ang II suppresses Na⁺-K⁺-ATPase in human placental villi, consistent with possible adverse effects of enhanced placental Ang II on fetal growth. These findings are consistent with those of our OC.

We showed that local Ang II caused human trophoblasts to migrate and invade, similar to the trophoblast behavior in the PE and OC models, where uteroplacental Ang II is elevated. These data could suggest similar effects and functional roles of local uteroplacental Ang II in our rat model and in the decidua of pregnant women. Furthermore, our findings could suggest that Ang II plays a central role in signaling trophoblast invasion and, ultimately, spiral artery remodeling. Ang II induces migration of various cells, including dendritic cells, VSMCs, and glomerular mesangial cells.²⁹ The results in trophoblasts are controversial. Xia et al³⁰ found that Ang II inhibits migration of the trophoblast cell line, HTR-8, via plasminogen activator inhibitor 1 activation. Ishimatsu et al³¹ found the opposite. They showed that direct-application Ang II induces migration in human cytotrophoblast-like choriocarcinoma cell lines. Local Ang II also influences endothelial cadherin, platelet-endothelial adhesion molecule 1, vascular endothelial adhesion molecule 1, and $\alpha 4$ integrins. These factors are important in facilitating the adhesion-phenotype switch in trophoblasts, which is required for successful endovascular invasion and normal placentation.³² Taken together, these data suggest that local Ang II is an important uteroplacental factor regulating the temporal and spatial regulation of trophoblast invasion in a paracrine way.

Circulating Ang II levels in the PE cross increased between day 9 and day 15, exactly when we started the Ang II infusion in the Ang II-infused group. However, a shorter time of exposure to Ang II in the Ang II-infused group compared with the transgenic group cannot be excluded as a variable contributing to the observed phenotypes. We also excluded the possibility that proteases are dysregulated in the Ang II-infused group. Aminopeptidase A, Prolyl-carboxypeptidase, Neprilysin, ACE-1, and ACE-2 were not induced in the Ang II-infused group compared with controls (data not shown). Aminopeptidase A, ACE-1, and ACE-2 were upregulated in OC and PE compared with control and Ang II (data not shown).

Ours and other laboratories have described activating autoantibodies directed at the Ang II type 1 receptor in women with human PE.^{3,33} We made similar observations in PE rats.^{4,34} The role of these antibodies is still unclear. However, upregulation of the decidual Ang II type 1 receptor is also present in PE.^{11,35} Both events could represent a possible physiological adaptation, suggesting the necessity of increased Ang II-like activity in promoting trophoblast inva-

sion for spiral artery remodeling. The antibodies could represent malfunction of this mechanism resulting in PE in some women.

The rodent transgenic renin/angiotensinogen model was originally described in mice by Takimoto et al³⁶ Hypertension, proteinuria, and IUGR described in that study were very similar to the rat PE model that we report. However, placental abnormalities were different in the 2 species. Takimoto et al³⁶ detected necrosis in spongiotrophoblasts and decidual cells, edematous enlargement, and congestion in chorion cells. In a follow-up study, the group showed that the interaction between fetal vasculature and maternal blood canal in the labyrinth was distorted and the expression patterns of key molecules in neovascularization were dysregulated.³⁷ The maternal plasma level of sFlt-1 was significantly increased after midterm in that model.³⁷ In contrast to the mouse, the rat develops deep endovascular and interstitial trophoblast invasion beyond the decidua into the mesometrial triangle, showing a deep endovascular trophoblast-associated spiral artery remodeling. Thus, we can investigate the labyrinth placenta and the mesometrial triangle separately. sFlt-1 is not dysregulated in the rat placenta or in the maternal serum in PE rats.¹³ In spite of the species differences, both models underscore the notion that cell-specific expression of renin and angiotensinogen in the fetomaternal interface strongly influences IUGR and placenta development.

A limitation in our study is that an ACE inhibitor and/or Ang II type 1 receptor blocker was not tested in our models. Saito et al showed that genetic deletion of the maternal Ang II type 1a receptor, and cross-breeding with the transgenic mouse model significantly ameliorated the phenotype in their mice.³⁸ The role of Ang II inhibition on trophoblast function, vascular remodeling in the uteroplacental unit, and IUGR requires substantial further study in animal models, particularly because human studies along these lines cannot be performed.

Perspectives

Uteroplacental Ang II promoted trophoblast migration and invasion in rats and humans. This effect could mediate a cross-talk between invasive trophoblasts and the maternal endometrium. Such a paracrine Ang II function in normal pregnancy could also be further activated in pregnancies with abnormal placentation that exhibit shallow trophoblast invasion, as in PE. The concept of a fetal-maternal Ang II gradient warrants further investigations, such as the determination of Ang II in the amniotic fluid.

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Disclosures

None.

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Online supplement

Effects of circulating and local uteroplacental angiotensin II in rat pregnancy

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Methods

Animal studies

Local authorities approved the studies along American Physiological Society guidelines. Mean blood pressure, heart rate, and ambulatory activity were continuously recorded by radiotelemetry (Data Sciences International). Sprague-Dawley females were mated with Sprague-Dawley males as controls, namely pregnant Sprague-Dawley (SD).

For Doppler studies, the animals were anesthetized with 1.5% isoflurane via an oxygen mask. The pregnant rats were imaged at embryonic days 9.0, 15.0, 18.0, and 21.0 with an ultrasound bio-microscope and a 30-MHz or 40-MHz transducer at 30 frames per second (Model Vevo 660, VisualSonics). The Doppler waveforms were obtained from the proximal uterine artery, arcuate artery, and distal of the main branch of the uterine artery between 2 embryonic implantation sites.

Blood for hormone analysis was drawn by aortic puncture into pre-chilled tubes containing EDTA (6.25 mmol/L) and phenanthroline (26 mmol/L) as anticoagulant and inhibitor of Ang II breakdown in vitro, respectively. The direct renin inhibitor, aliskiren (10^{-6} M), was added to plasma samples for Ang II measurement to prevent Ang II formation in vitro.

For tissue immunostaining protocols all implantation sites (placenta with associated mesometrial triangle) were removed, fixed in JB-Fix fixative, and embedded into Paraplast Plus (Sherwood Medical Co) from each rat. Parallel sections were cut step-serially from each implantation site parallel to the mesometrial-fetal axis. A set containing a central maternal arterial channel was selected for further analysis. Parallel, serial cross-sections were obtained and stained for anti- α -actin (clone 1A4, Dako, diluted 1/200), anti cyokeratin (clone MNF116, Dako), and anti CD31 (clone TLD-3A12, BDPharmingen). For the quantitative analysis of depth of invasion and spiral artery remodeling, the KS-400 image analysis system (Carl Zeiss) connected to a Zeiss microscope (Axioskop 50) fitted with a color camera (Axiocam MRc5) was used. Briefly, the whole mesometrial triangle was photographed and the images were shade-corrected. The border of the triangle was manually delineated on the α -actin immunostained section, and the triangle was divided into three concentric depth levels of equal thickness for the evaluation of the depth of invasion. The lumen of each spiral artery (SA) cross-section was manually delineated and stretches of endovascular trophoblast, fibrinoid, vascular smooth muscle and CD31 positive endothelium were traced separately over the lumen contour tracing. For every cross-section, the length of each traced line was measured and percentages of all lengths were calculated in relation to the total length of the spiral artery lumen contour.

Cell culture

SGHPL-4 cells were derived from primary human first trimester extravillous trophoblasts (EVT) and transfected with the early region of SV40. The cells were cultured in Ham's F10 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μ g/ml). These cells show similar invasive capabilities to primary EVT and retain features of normal extravillous trophoblast, including expression of HLA-G, cyokeratin-7, BC-1, CD9 and human placental lactogen. Ang II stimulation (10^{-6} M, 10^{-8} M, Calbiochem) and 10^{-6} M losartan (Calbiochem) were applied for 48h in 0.5% serum-reduced medium in matrigel. The effect on the invasive trophoblast outgrowth (number of invasive processes, length of invasion) was assessed by phase contrast microscopy (Olympus) and Image Pro Plus Software (Media Cybernetics). For this purpose, the cells were grown on beads. Motility of SGHPL-4 cells was monitored by time-lapse microscopy (TLM) using an Olympus IX70 inverted fluorescence microscope with a motorized stage and cooled CCD camera (Hamamatsu C4742-95) enclosed in a humidified chamber at 37°C with 5% CO₂ in

air at 15 min intervals for 6 hours as described previously. Forty cells were scored in each field of view.

Human placental villous tissue explants from first trimester pregnancies were established and characterized as previously described.²⁰ Four placentas were obtained from healthy women after legal termination of pregnancy (5-10 weeks of gestation). Informed, written consent was obtained after due approval from the local institutional ethical review board. Briefly, the placentas were washed in sterile phosphate-buffered saline (PBS) until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and were grown on collagen-coated (Sigma) plates in DMEM/Hams F12 supplemented with 1% P/S/Amphotericin B and 10% human serum. Migration experiments were conducted with collagen coated chambers (24-well plates; BD Biosciences) according to the manufacturer's instructions. The effects of Ang II images were taken after 72 h and the area of trophoblast outgrowth was analyzed with ImageJ software.

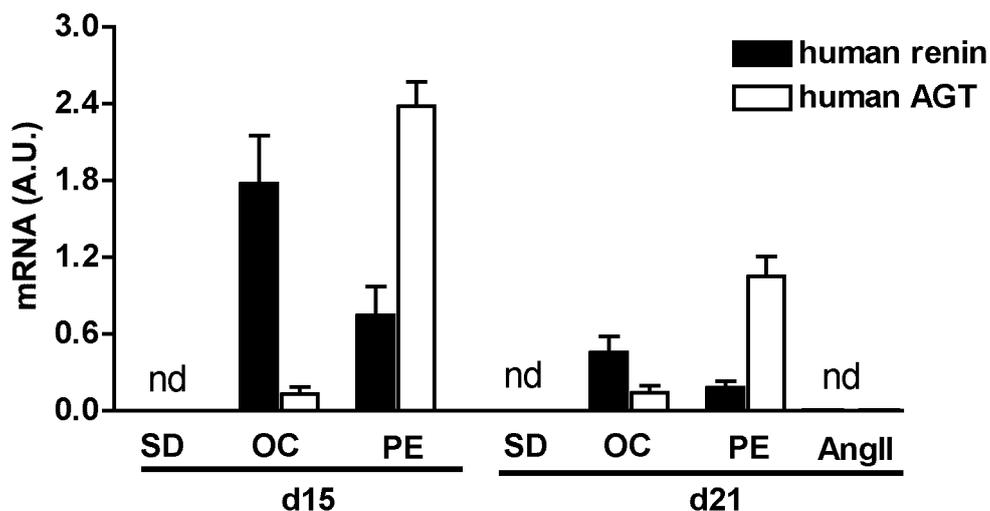


Figure S1. Expression levels of the transgenes human renin and human angiotensinogen (AGT) in the uteroplacental unit of the SD-, OC-, PE- and AngII-group at days 15 and 21 of pregnancy. (nd = not detectable)