Dysregulation of the Circulating and Tissue-Based Renin–Angiotensin System in Preeclampsia

Florian Herse, Ralf Dechend, Nina K. Harsem, Gerd Wallukat, Jürgen Janke, Fatimunnisa Qadri, Lydia Hering, Dominik N. Muller, Friedrich C. Luft, Anne C. Staff

Abstract—The renin–angiotensin system (RAS) participates in preeclampsia; however, the relative contributions from the circulating RAS and the tissue-based, uteroplacental RAS are unknown. We hypothesized that the tissue-based uteroplacental RAS is dysregulated in preeclampsia. We performed microarray and gene expression studies and confirmed the findings on the protein level by immunohistochemistry in uteroplacental units from 10 preeclamptic women and 10 women with uneventful pregnancies. All of the women were delivered by cesarean section. We also analyzed plasma renin activity and circulating agonistic angiotensin II type 1 (AT1) receptor autoantibodies. In preeclampsia, we found that the angiotensin II AT1 receptor gene was 5-fold upregulated in decidua (maternal origin). We also found AT1 autoantibodies in preeclamptic women and in their offspring by neonatal cardiomyocyte bioassay compared with women with normal pregnancies and their infants (mother: 17.5±2.2 versus 0.05±0.4; fetus: 14.5±1.8 versus 0.5±0.5 Δbpm). Gene expressions for renin (35.0-fold), angiotensin-converting enzyme (2.9-fold), and angiotensinogen (8.9-fold) were higher in decidua than placenta (fetal origin) in both control and preeclamptic women, whereas the AT1 receptor was expressed 10-fold higher in placenta than in decidua in both groups. Our findings elucidate the uteroplacental unit RAS in preeclamptic and normal pregnancies. We found that, in preeclampsia, the AT1 receptor expression is particularly high in decidua, combined with pregnancy-specific tissue RAS involving decidual angiotensin II production and AT1 autoantibodies. We also showed that AT1 autoantibodies cross the uteroplacental barrier. These components could participate in the pathophysiology of preeclampsia. (Hypertension. 2007;49[part 2]:604-611.)

Key words: preeclampsia • renin • angiotensin • AT1 receptor • gene expression • proteomics

Preeclampsia is devastating; however, the mechanisms are imperfectly defined. Abnormal placentation associated with shallow trophoblast invasion (fetal cells from outer cell layer of the blastocyst) into endometrium (decidua) and improper spiral artery remodeling in the decidua are initial pathological steps. This state of affairs leads to inefficient uteroplacental perfusion and a relatively hypoxic placenta in preeclampsia. Oxidative stress is involved in the release of placenta products, namely, trophoblast debris, angiogenic-associated factors, or other placenta-derived factors. Release of placenta-derived factors into the maternal circulation leads to a generalized maternal endothelial dysfunction, excessive vascular inflammation, and preeclampsia-associated hypertension and proteinuria. Placental gene expression studies have identified 2 upregulated genes, namely, a vascular endothelial growth factor-receptor splice variant (sFlt-1) and soluble endoglin, a coreceptor for transforming growth factor-β. No gene array data for decidua have been published because of technical difficulty in obtaining the tissue. We have established a safe decidual suction method yielding sufficient decidual tissue for morphological and functional studies of extravillous trophoblast function and spiral artery remodeling.

The renin–angiotensin system (RAS) participates in preeclampsia. We have described circulating agonistic autoantibodies directed at the angiotensin (Ang) II type 1 (AT1) receptor in women with preeclampsia (AT1-AA). The AT1 receptor may heterodimerize with itself or with other G protein–coupled receptors. A tissue-based RAS has been described in the uteroplacental unit in the gravid state. For this study, we selected only women undergoing cesarean section so that the tissues would not be perturbed. We separately investigated the 2 types of tissues from the uteroplacental unit (decidua and placenta) in the same pregnant women with or without preeclampsia. This enabled us to explore the role of RAS in the 2 types of placenta with different cellular origin; the “fetal placenta” tissue (hereafter named “placenta”) compared with the “maternal placenta” tissue, named the “decidua,” corresponding with the maternal endometrium in pregnancy. Our hypothesis was that, in
addition to changes in the maternal and fetal circulating RAS, the RAS in the uteroplacental unit is distinctly abnormal and contributes to the disease.

Methods

The pregnant women included in this study were nonsmokers, white, and previously healthy with uncomplicated pregnancies. None had chronic hypertension, renal disease, or diabetes. Women in both preeclampsia (n = 10) and uneventful pregnancy group (n = 10) were delivered by cesarean section. None were in active labor nor had any infection at the time of cesarean delivery. Control women were normotensive and were undergoing cesarean delivery because of breech presentation or other medical indications. Preeclampsia was defined as the rise in blood pressure after 20 weeks of gestation to >140/90 mm Hg on ≥2 occasions 6 hours apart in a previously normotensive woman, combined with proteinuria.1 Proteinuria was defined as a protein dipstick reading of ≥1+ or a 24-hour urinary excretion of ≥0.3 g protein in ≥2 urine samples, in the absence of urinary tract infection. Korotkoff phases I and V were used to determine systolic and diastolic blood pressure, respectively. Informed written consent was obtained from all of the participants, and the Regional Committee of Medical Research Ethics in Eastern Norway approved the study.

Samples

Abdominal adipose, muscle, and placenta biopsies were obtained at cesarean section. Decidual tissue was collected through vacuum suctioning of the placental bed, as described previously.8,9 Histopathologic confirmation of the decidual nature of the tissue was made on a random portion of each sample (by N.K.H.). All of the tissues were immediately frozen in liquid nitrogen and stored at −80°C. Maternal blood was collected from an antecubital vein. Immediately after delivery of the placenta, blood from umbilical vein was collected. All of the blood samples were prepared and stored as described previously.10,13

mRNA Isolation and Gene Array

Total mRNA was isolated with the Qiagen RNeasy mini kit (including the RNase-Free DNase set) according to the manufacturer’s protocol from decidual, placenta, adipose, and muscle tissue. RNA quantity and quality were confirmed by RNA 6000 Chip in the 2100 Bioanalyzer (Agilent Technologies) and NanaDrop UV/VIS-Spectrometer (PeqLab). Equal amounts of total RNA were pooled from control and preeclamptic groups, and in each group, 20 μg of pooled total RNA were reverse transcribed into cDNA and in vitro transcribed to complementary RNA as described previously.14,15 GeneChip experiments were performed with chip U133 plus 2 according to the Affymetrix protocols, corresponding with the Minimum Information About a Microarray Experiment (MIAME) criteria.13 Raw data analysis was performed using Affymetrix software and can be accessed at http://www.ncbi.nlm.nih.gov/geo with the GEO accession No. GSE6573.16,17 Genes were defined as differentially expressed if the fold change was ≥4-fold.

RT-PCR

Two micrograms of total RNA were reverse transcribed and analyzed in triplicate with the ABI 5700 sequence detection system (PE Biosystems) as described previously.14 Acidic ribosomal protein 36B4 (decidual, placental, and adipose tissue) and TATA-box binding protein (muscle tissue) were chosen as endogenous control genes. Primer and probes were designed with PrimerExpress 2.0 (Applied Biosystems), and sequences are shown in Table I in a data supplement, available online at http://hyper.ahajournals.org.

Immunofluorescence

Frozen decidual and placental tissues were cryosectioned at 6-μm thickness and prepared as described previously.14 Staining was performed with primary antibodies anti-human-renin (friendly gift from Hoffman-La Roche), anti-AT1-Receptor (N-10; Santa Cruz), anti-cytokeratin 7 (Santa Cruz), and the Cy3-labeled secondary anti-rabbit antibody (Jackson Immunoresearch Laboratories). Preparations were examined under a Zeiss Axioscop-2 microscope. Fifteen different areas of each sample were analyzed. Samples were examined without knowledge of the disease state.

Contraction and Plasma Renin Activity Assays

IGF fractions from 12 preeclamptic patients (7 fetal samples) and 10 control patients (6 fetal samples) were prepared as outlined in detail elsewhere.10 Briefly, single cells were dissociated from the minced venules of Wistar rats and were cultured as monolayers. The AT1-AAAs were measured by counting the beating rates of focal contractile neonatal rat cardiomyocytes before and after treatment with the purified IGF fraction. Immunoglobulin fractions, agonist and antagonist drugs, and peptides were added singly or cumulatively as indicated. Plasma renin activity (PRA) was measured at pH 7.4. The samples were examined without knowledge of the disease identity.

Statistical Analysis

The mRNA expression results are expressed as means from triplicates. All of the data were tested with SPSS 12.0 for normal distribution with the Kolmogorov–Smirnov test. Normally distributed data were analyzed with a t test. Otherwise, the Mann–Whitney test was applied. P < 0.05 was considered statistically significant.

Results

The clinical characteristics are shown in Table 1. The control group and preeclamptic women differed in terms of blood pressure, proteinuria, pregnancy duration at delivery, and newborn weight. Neonatal weight percentiles did differ between these groups, but only 1 baby in the preeclampsia

<table>
<thead>
<tr>
<th>TABLE 1. Patient Demographic Data (Median and Range)</th>
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<td>Diastolic BP at delivery, mm Hg</td>
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BMI indicates body mass index; BP, blood pressure.
group was small for gestational age (below the 10th weight percentile). We found various genes significantly upregulated (≥4 times) in the preeclamptic decidua compared with the decidua from control women, as shown in Table II and Table 2 (most relevant genes). We found a 128-fold upregulation of a cDNA termed FLJ13785 and a 32-fold upregulation of pregnancy-specific β-1 glycoprotein, as well as several expressed sequence tags. We were particularly interested in RAS components and observed a 4-fold increase in the AT1 receptor expression in the decidua of preeclamptic women compared with control women. We then confirmed increased decidual AT1 receptor mRNA by semiquantitative PCR (Figure 1A) and found also increased protein expression by immunohistochemistry in preeclamptic decidua (Figure 1B). The immunopositive cells are morphologically trophoblasts exhibiting staining with cytokeratin 7, a trophoblast marker (data not shown).

We also performed microarray analysis from placenta (Table 3, most relevant genes, and Table III), but no RAS-related genes differed significantly (4-fold) between the patient groups. Two genes represented here are of particular interest. The gene encoding sFlt was 4-fold upregulated, as was the gene encoding the pentraxin-related gene responsible for the C-reactive protein (CRP). The gene encoding endoglin, a recently reported preeclampsia-associated gene, was found (data not shown). A pathogenic role for sFlt and endoglin.

PRA reflecting Ang I generation per unit time was reduced in preeclamptic patients compared with control subjects (Figure 2A). The cardiomyocyte contraction assay was used to identify activating autoantibodies against the AT1 receptor (AT1-AA). The IgG fractions from the preeclamptic women caused a 17.5 ± 2.2-bpm increase in contraction rates (Figure 2B). IgG from control patients had no effect. IgG from 8 of 10 preeclamptic individuals and from none of the control subjects showed significant biological activity. Stimulation was inhibited by losartan and the peptide sequence AF-HYESQ, as reported earlier (data not shown), demonstrating active antibodies. We then determined whether or not AT1-AAs were also detectable in fetal serum, which proved to be the case (Figure 2C), and only in preeclampsia. No AT1-AA–negative mother had an AT1-AA–positive fetus.

We next investigated whether or not decidua and placenta showed different profiles in terms of mRNA expression for renin, angiotensinogen, Ang-converting enzyme (ACE), and the AT1 receptor. Renin, angiotensinogen, and the ACE were more expressed in decidua than in placenta, in both preeclamptic women and control subjects (Figure 3A). The AT1 receptor gene expression was higher in placenta than in decidua for both groups. However, as reported above (Figure 1A), the decidua of preeclamptic women had a significantly higher (5-fold) AT1 receptor gene expression than control subjects (ordinate is a log scale). Immunohistochemistry was then performed, also showing higher renin expression in the decidua, compared with the placenta, at the protein level (Figure 3C).

**Discussion**

We found that the gene encoding the AT1 receptor was upregulated in the decidua of preeclamptic women, but not in normal women, as verified by microarray, RT-PCR, and the protein level. The upregulation was confined to decidua and was not present in placenta. Thus, RAS may exert its pathogenic effects primarily in the preeclamptic decidua. The upregulated AT1 receptor in the preeclamptic decidua could have 2 potential partners, Ang II or AT1-AA. Renin, ACE, and angiotensinogen were more expressed in decidua than in placenta, irrespective of the preeclamptic state. On the other hand, AT1 receptor expression was weaker in decidua than in placenta, underscoring the importance of AT1 receptor upregulation in preeclamptic decidual tissue. We also found AT1-AAs in maternal serum in preeclampsia. We documented AT1-AA in the fetus of preeclamptic pregnancy, suggesting cross-placental transfer. Our findings also support a pathogenic role for sFlt and endoglin.

Hodari et al and Symonds et al were the first to convincingly report that renin is produced outside of the kidney; they verified renin production in placenta, chorion, and uterus, irrespective of preeclampsia status. Verification
of extrarenal sources, that renin is present in anephric persons,\textsuperscript{20} in the gastrointestinal tract,\textsuperscript{21} and in the brain,\textsuperscript{22} were described later. The notable findings linking preeclampsia to the renin Ang system are supported by several important observations. First, PRA is increased in normal pregnancy\textsuperscript{22} because of increased renin substrate,\textsuperscript{23} but PRA is actually decreased in preeclampsia.\textsuperscript{24} Concurrently, we observed 3-fold higher PRA values in nonpreeclamptic women compared with preeclamptic women in our study. Second, Ang II in the circulation is also decreased in preeclampsia.\textsuperscript{25} Third, despite a substantial increase in total body sodium and total body water, circulating aldosterone concentrations are decreased in preeclamptic, compared with nonpreeclamptic, women.\textsuperscript{26} Nevertheless, for a given PRA value, aldosterone concentrations in preeclamptic women are relatively increased compared with nonpreeclamptic women.\textsuperscript{27} Seminal observations showed that the response to Ang II is markedly increased in preeclamptic women.\textsuperscript{28} Upregulation of the AT\textsubscript{1} receptor, as found on platelets in preeclampsia,\textsuperscript{29} could enhance this finding. Finally, a host of publications suggest that genetic variants in RAS-related genes contribute to preeclampsia.\textsuperscript{30} These observations, coupled with our own findings concerning the existence of AT1-AA, prompted this investigation.\textsuperscript{10}

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\caption{A, RT-PCR verification of AT\textsubscript{1} receptor results (n=10 each) from decidua of preeclamptic women. B, Immunofluorescent verification of AT\textsubscript{1} receptor upregulation in preeclamptic decidua vs control decidua; *P<0.001.}
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Gant et al.33 had a major influence on the concept of RAS involvement in preeclampsia. Ang II up to 8 ng/kg per minute was infused in pregnant women, sufficient to raise diastolic pressure by 20 mm Hg. In the group of women that remained normotensive during pregnancy, the necessary dose was significantly higher than in the women who developed pregnancy-induced hypertension. These data receive support with nonpreeclamptic women. We were able to confirm their work and found an ∼3-fold upregulation of renin gene expression in decidua, although not statistically significant, but the decidua sample collection methods differed. The decidua vera was dissected by Shah et al.33 from the delivered placenta, whereas our decidual biopsies were obtained by suction from the uterine wall after placental removal. Our samples were obtained at cesarean section before labor had commenced, avoiding unpredictable oxidative stress resulting from various lengths and intensities of labor and thereby a possible effect on the test results. In decidua, renin expression was >10-fold higher than in placenta, irrespective of preeclampsia. Similar findings were observed for ACE mRNA and angiotensinogen mRNA. These observations are in accord with those of Shah et al.,35 who found that human decidua is a major source of renin.

Our most striking finding was upregulation of the AT1 receptor in the decidua of preeclamptic women, compared with nonpreeclamptic women, a finding verified on mRNA and protein levels. Interestingly, AT1 receptor expression was substantially higher in placenta, compared with decidua, irrespective of preeclampsia. These findings suggest a physiological upregulated local Ang II production in the decidua with low expression of the AT1 receptor. Thus, the increased decidual AT1 expression in preeclampsia may be the initial step for a profound RAS activation. Placental and decidual tissues are distinct in terms of cellular origin. The placenta is derived from the fetus; the villous cytotrophoblasts and syncytiotrophoblasts appear to exhibit a tissue RAS that may be distinct from the maternal decidual tissue.36 Decidual tissue consists of the uterine endometrium invaded by fetal extravillous trophoblasts, and includes a plethora of maternal cells (including decidual cells and maternally derived immune cells), as well as fetally derived invading extravillous cytotrophoblasts. How these pregnancy-specific tissues interact in terms of Ang II generation cannot be answered from our study. Conceivably, the decidua is responsible for Ang II generation, whereas the placental tissue is the target organ. Upregulation of the AT1 receptor in the decidua during pregnancy-induced hypertension. These data receive support with nonpreeclamptic women.

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<th>TABLE 4. Quantitative Real-Time RT-PCR Results</th>
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<td>MR</td>
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n.s. indicates not significant; n.d., not detectable; AGT, angiotensinogen; R, receptor; EGFR, epidermal growth factor receptor; MR, mineralocorticoid receptor.
preeclampsia could possibly compromise the maternal–placental unit.

We confirmed our previous finding regarding the presence of AT1-AAs in preeclamptic patients. The AT1-AAs were active, because their effect in the bioassay could be blocked by losartan in both mother and fetus. We have not explored any pathophysiological significance of these fetal findings in the present study. Previously, we showed that AT1-AAs are able to activate cells via the AT1 receptor and initiate signaling events that could very well contribute to development of preeclampsia.37,38 Other investigators have reported similar findings and showed that AT1-AAs can induce

Figure 2. A, PRA was reduced in preeclamptic women vs control subjects (n=10 each). B, Agonistic AT1 receptor antibodies were present in preeclamptic maternal sera (n=12) vs control sera (n=10; bioassay results). The effect could be blocked with losartan. C, Agonistic AT1 receptor antibodies were also present in sera from the infants of preeclamptic mothers (control: n=6; preeclampsia: n=7; *P<0.001; **P=0.015).

Figure 3. A, Renin gene expression in placenta and decidua (open symbols are control subjects; closed symbols are preeclamptic patients). Renin gene expression was higher in decidua than in placenta. ACE gene expression was similar in decidua and placenta, as was angiotensinogen gene expression. No differences in expression of these genes, between preeclamptic and normal subjects, were observed. B, AT1 receptor gene expression. AT1 receptor gene expression in decidua, but not placenta, was augmented in preeclamptic patients vs control subjects. C, Immunofluorescence of increased renin protein expression in decidua vs placenta in 3 different patients (2 controls and 1 preeclamptic; *P<0.001).
calcium signaling via the AT₁ receptor. The maternal and fetal circulation could possibly represent a molecular link between the epidemiologically shown augmented cardiovascular risk later in life for both women with preeclampsia and their offsprings. Interestingly, the RAS, in addition to oxidative stress, has been proposed as 1 of several possible mechanisms of in utero programming of adult disease. Finally, we supply a list of upregulated genes in placenta and decidua that we have not yet been further evaluated. We noted that pentraxin is among these genes. CRP is a recognized marker for preeclampsia during pregnancy. We showed earlier that Ang II increases CRP in a model of target-organ damage. Shagarsuren et al showed recently that human CRP contributes to cardiovascular disease and that the effect can be abrogated by a small molecule inhibitor of CRP. Conceivably, such a molecule could be useful to clinically explore in preeclamptic patients. Our microarray findings also support a pathogenic role of placenta in preeclampsia in excess production of sFlt and endoglin. The leptin gene expression was also upregulated in preeclamptic placentas as compared with controls. In an earlier study we also found increased plasma and placental leptin expression in preeclampsia. Our study was necessarily limited in size. Moreover, we could not correct for gestational length. Although we did not find any significant correlation between gestational age and any of the study tests, differences related to gestational age cannot be ruled out completely.

Perspectives
Attractive preeclampsia mediators include sFlt1, soluble endoglin, and RAS components, notably, AT1-AAs. We relied on serum and uteroplacental unit tissue samples to further advance these questions. ACE inhibition and AT1 receptor blockade treatment during pregnancy are unacceptable because of teratogenicity. However, a decoy sequence to neutralize circulating AT1-AAs could be useful, and similar constructs to neutralize sFlt and soluble endoglin are in development. Hopefully these efforts will result in improved perspectives for preeclamptic patients and their offsprings.

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Disclosures
None.

References
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