Angiotensin II Type 1 Receptor Antibodies and Increased Angiotensin II Sensitivity in Pregnant Rats

Katrin Wenzel, Augustine Rajakumar, Hannelore Haase, Nele Geusens, Norbert Hubner, Herbert Schulz, Justin Brewer, Lyndsay Roberts, Carl A. Hubel, Florian Herse, Lydia Hering, Fatimunnessa Qadri, Carsten Lindschau, Gerd Wallukat, Robert Pijnenborg, Harald Heidecke, Gabriela Riemekasten, Friedrich C. Luft, Dominik N. Muller, Babetta Lamarca, Ralf Dechend

Abstract—Pregnant women who subsequently develop preeclampsia are highly sensitive to infused angiotensin (Ang) II; the sensitivity persists postpartum. Activating autoantibodies against the Ang II type 1 (AT1) receptor are present in preeclampsia. In vitro and in vivo data suggest that they could be involved in the disease process. We generated and purified activating antibodies against the AT1 receptor (AT1-AB) by immunizing rabbits against the AFHYESQ epitope of the second extracellular loop, which is the binding epitope of endogenous activating autoantibodies against AT1, from patients with preeclampsia. We then purified AT1-AB using affinity chromatography with the AFHYESQ peptide. We were able to detect AT1-AB both by ELISA and a functional bioassay. We then passively transferred AT1-AB into pregnant rats, alone or combined with Ang II. AT1-AB activated protein kinase C-α and extracellular-related kinase 1/2. Passive transfer of AT1-AB alone or Ang II (435 ng/kg per minute) infused alone did not induce a preeclampsia-like syndrome in pregnant rats. However, the combination (AT1-AB plus Ang II) induced hypertension, proteinuria, intrauterine growth retardation, and arteriolosclerosis in the uteroplacental unit. We next performed gene-array profiling of the uteroplacental unit and found that hypoxia-inducible factor 1α was upregulated by Ang II plus AT1-AB, which we then confirmed by Western blotting in villous explants. Furthermore, endothelin 1 was upregulated in endothelial cells by Ang II plus AT1-AB. We show that AT1-AB induces Ang II sensitivity. Our mechanistic study supports the existence of an “autoimmune-activating receptor” that could contribute to Ang II sensitivity and possible to preeclampsia.

Key Words: preeclampsia • angiotensin II • immunology • autoimmune disease
immunization should be able to elicit such antibodies and cause a similar syndrome.16 Jahns et al17 demonstrated that generation of antibodies against the β1-adrenergic receptor induced dilatative cardiomyopathy. Similar long-term active immunization experiments have also been performed for other G protein–coupled receptors.18,19 However, such experiments have not been done in pregnant rats. We generated and isolated AT1 antibodies (AT1-AB) in rabbits using the peptide sequence AFHYESEQ of the second extracellular loop detected as a binding epitope of AT1-AAs from preeclamptic patients. We then characterized the AT1-ABs and investigated their effects in pregnant rats alone and in combination with infused Ang II.

Materials and Methods

AT1-AB Generation, Purification, and Functional Testing

We immunized rabbits with the peptide sequence AFHYESEQ (Bio-syntan GmbH, Berlin, Germany) to generate AT1-ABs. To purify the AT1-ABs from sera, the corresponding peptides were covalently bound to ε-aminocapryl agarose (Sigma-Aldrich, Munich, Germany) to yield epitope-specific affinity beads. The preparation of antibodies and the cardiomyocyte contraction assay were carried out as earlier described.20 AT1-ABs were detected by an AT1-AB ELISA. ELISA for α1-adrenergic and β1-adrenergic receptor autoantibodies were used as negative controls (CellTrend, Luckenwalde, Germany). Because AT1-ABs were raised in rabbits, we detected them with a peroxidase-labeled antirabbit IgG antibody (Johnson & Johnson).

Chinese hamster ovary (CHO) cells stably transfected with human AT1-receptor (CHO/AT1R) were cultured in F12 HAM medium supplemented with glutamine, 10% FCS, and 1% penicillin/streptomycin. Protein kinase C-α activity in CHO/AT1R cells was detected with AT1-ABs (2.5 and 25.0 μg/mL of medium) using an MRC 1024 confocal imaging system (Bio-Rad, Munich, Germany) with an argon/krypton laser.20 As positive control, the AT1-receptor agonist Ang II (100 nmol/L to 1 μmol/L) was used, and for inhibition experiments, irbesartan (1 μmol/L; Sanofi-Aventis, Paris, France) was used. For extracellular-regulated kinase 1/2 phosphorylation, CHO/AT1R, cells were maintained in serum-free medium for 4 hours, respectively, and treated with AT1-ABs (25 and 50 μg/mL of medium) for 15 minutes, respectively. For inhibition experiments, 1 μmol/L of irbesartan was added. Western blot experiments were carried out as described earlier.20

Rats

Sprague-Dawley rats (R. Janvier Breeding Center, Le Genest St Isle, France) were outfitted with radiotelemetry pressure transducers (TA11PA-C20).18 Animals were then mated and pregnancy monitored. For Ang II infusion, osmotic pumps (ALZET, Cupertino, CA) were implanted 9 days later. The animals received 435 ng of Ang II per kilogram of body weight per minute. AT1-AB or rabbit control IgG were IV injected on days 9 and 11 of pregnancy (1 μg per gram of body weight, 6 animals per group). Rat serum was sampled on days 11 and 13 for rabbit IgG detection by Western blotting using a peroxidase-coupled antirabbit IgG antibody (Thermo Fisher Scientific, Bonn, Germany). We maintained the animals in metabolic cages on days 17 and 18 for 24-hour albumin excretion by ELISA (CellTrend GmbH). Animals were euthanized on day 20 of pregnancy. The quantitative analysis of depth of trophoblast invasion and staining for atherosis-like lesions has been described earlier.21,22 Briefly, all of the implantation sites (placenta with associated mesometrial triangle) were removed, fixed in JB-Fix fixative, and embedded. The whole mesometrial triangle was photographed, and the images were shade corrected. The lumen of each spiral artery cross-section was manually delineated, and stretches of endovascular trophoblast were traced separately over the lumen contour tracing. Local authorities (Berlin, Germany) approved the animal protocol that complied with criteria outlined by the American Physiological Society.

Gene Expression, Cell Culture, ELISA, and Data Analysis

We extracted total RNA from the mesometrial triangle and placenta using the RNeasy Purification kit (Qiagen). RNA was treated by deoxyribonuclease 1 (Qiagen). We compared the gene expression profile in the mesometrial triangle in nonpooled microarray experiments (RatRef-12 Expression BeadChip kit; 22 523 rat targets, Illumina). After quantile normalization without background correction (Illumina BeadStudio 1.0.2, 20706 Platform), an offset addition16 and log-2 transformation were performed. The general expression profile over the 6 experimental conditions with respect to the 3 to 6 replicates was characterized using ANOVA statistic followed by false discovery rate multiple-testing correction. Consistently low-expressed transcripts were removed (BeadStudio detection \(P > 0.05\) before using test statistic. We found that 16 041 of 22 523 transcripts remained after filtering. A total of 998 transcripts were significantly differentially expressed at 5% false discovery rate level.

Human umbilical vein endothelial cells were pretreated with serum-free medium, Ang II (10−7 mol/L), AT1-AB, or Ang II + AT1-AB for 6 hours. Endothelin 1 (ET-1) was measured using ELISA and normalized to total cellular protein per flask. For detection of soluble fms-like tyrosine kinase 1 (sFLT-1) and placenta-like growth factor (PLGF) protein concentration, soluble vascular endothelial growth factor R1 Immunoblot (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) and placenta-like growth factor Immunoboyssay (R&D Systems GmbH) were used, respectively. Villous explant cultures, treatment, and Western blot analysis were performed as described earlier.23 We relied on the Mann-Whitney test at \(P < 0.05\) and expressed the data as mean ± SD.

Results

AT1-AB Assays and Characteristics

AT1-ABs increased the beating rate of neonatal rat cardiomyocytes, similar to isolated autoantibodies from preeclamptic patients (AT1-AA). The positive chronotropic effect was blocked by the AT1 receptor antagonist irbesartan and by the AFHYESEQ peptide, respectively (Figure 1A). Using the purified AT1-AB as capture antibody, we recovered a positive signal in a cell-based ELISA after CHO cells were transfected with AT1 receptor (CHO/AT1R), but not when CHO cells remained untransfected or were transfected with α1- or β1-adrenergic receptor (Figure 1B). We next examined the effects of AT1-AB on signal transduction in stably AT1 receptor–transfected cells (CHO/AT1R). We concentrated on protein kinase C-α and extracellular-regulated kinase 1/2, because both are important in AT1 receptor signaling. We found that AT1-AB exposure to CHO/AT1R resulted in protein kinase C-α activation, as did the positive Ang II control. Irbesartan blocked these effects (Figure 1C). Incubation of CHO/AT1R cells with AT1-ABs resulted in transient extracellular-regulated kinase 1/2 phosphorylation. The specificity of phosphorylation was shown by inhibition with irbesartan (Figure 1D).

AT1-AB and Ang II in Pregnant Rats

We performed passive transfer experiments in pregnant rats. We verified the presence of the rabbit AT1-AB in the rat sera on days 11 and 13 by rabbit IgG detection in an immunoblot (Figure 2A). On day 20, AT1-ABs were detectable by ELISA (Figure 2B). AT1-AB or control rabbit IgG injection in pregnant rats did not influence
blood pressure. Ang II at a high dose (435 ng/kg per minute) also did not increase blood pressure. However, AT1-AB plus Ang II resulted in a significant increase in mean arterial blood pressure in our pregnant rats (Figure 2C). We next performed acute Ang II infusions. Ang II (100 ng/mg per minute) was administered via the jugular vein to controls and chronic AT1-AB–infused pregnant rats on day 19 of gestation as a bolus. Blood pressure increased to 141 \text{mm Hg} in normal pregnant rats but markedly increased to 168 \text{mm Hg} in AT1-AB pretreated pregnant rats (Figure 2D). AT1-AB transfer did not induce albuminuria (317.3 \text{g/mL per 24 hours}), and Ang II alone induced modest albuminuria in the rats compared with no treatment (2014.5 \text{g/mL per 24 hours}). However, Ang II plus AT1-AB produced albuminuria (4388.2 \text{µg/mL/24 hours} \text{g/mL per 24 hours}).

We determined the body, kidney, and placental and mesometrial triangle weights. There were no significant differences between the groups (data not shown). Ang II, IgG, or AT1-AB treatment did not influence the fetal weights, but fetal weights were significantly decreased in the Ang II plus AT1-AB–treated group. Fetal weights of the Ang II plus IgG-treated group were also lower compared with control (P=0.05; Figure 3A). We also looked for intrauterine growth restriction. An increased brain:liver ratio is an important indicator for fetal stress resulting in intrauterine growth restriction. The brain:liver ratio was significantly increased only in the Ang II plus AT1-AB group. All of the other groups showed no increased brain:liver ratio (Figure 3B). In an earlier study involving an Ang II–dependent transgenic rat model for preeclampsia, we identified focal necrosis in the walls of spiral arteries. We termed these changes “atherioendotheliopathy” because they resembled the “atherosclerosis” of human preeclampsia.21 This atherioendotheliopathy of the spiral arteries was located in the mesometrial triangle, which
represents the maternal part of the uteroplacental unit and has strong homologies to the human placenta.24 Signs for such arteriolosclerotic lesions were significantly higher in Ang II/H11001 AT1-AB–treated rats compared with the other groups (Figure 3C). We then investigated the capacity to further remodel the spiral arteries in the uteroplacental unit. As in our earlier study, we calculated the amount of trophoblast cells per vessel contour.24 In this study, the complete mesometrial triangle trophoblast invasion into spiral arteries was reduced in the Ang II plus AT1-AB group compared with the AT1-AB plus IgG group. Surprisingly, Ang II alone had no effect, but AT1-AB alone also reduced trophoblast-induced spiral artery remodeling (Figure 3D).

Molecular Mechanisms
We measured the serum concentration of sFLT-1 and PLGF by ELISA. There were no differences in serum concentration of sFLT-1 or PLGF comparing all of the treated groups with the untreated controls. We then tested the hypothesis that binding of the AT1-AB to the AT-1 receptor increases endothelial cell ET-1 production. Basal ET-1 production increased modestly in response to AT1-AB and to Ang II. In sharp contrast, ET-1 production increased markedly in response to Ang II in the presence of AT1-AB. These data support the hypothesis that AT1-ABs increase endothelial cell sensitivity to Ang II (Figure 4A). To find novel pathways induced by AT1-AB and Ang II, we analyzed the transcriptome of mesometrial triangle by gene expression profiling using Illumina Arrays. We found that the transcription factor hypoxia-inducible factor (HIF) 1α was robustly induced by AT1-AB plus Ang II (Figure 4B). The results were confirmed in villous explants. Ang II and AT1-AB both induced HIF-1α protein expression in villous explants compared with controls, however, to a lesser degree than the combined treatment (Figure 4C). We encountered other less prominently differentially expressed genes in the mesometrial triangle (Table S1).

Discussion
Our novel finding is that AT1-ABs increase Ang II sensitivity, possibly explaining the increased Ang II sensitivity observed in preeclamptic patients. We report that AT1-ABs can be generated by immunization and that purified AT1-ABs induced a chronotropic effect in the cardiomyocyte contraction assay, initiated extracellular-regulated kinase 1/2 phosphorylation, and stimulated protein kinase C-α activation. We were able to detect AT1-ABs with ELISA. Passive transfer of AT1-ABs alone into pregnant rats did not induce a preeclamptic syndrome. However, the combination of AT1-ABs and
Ang II produced hypertension, proteinuria, abnormal placental vascular remodeling, and intrauterine growth restriction. We also found that AT1-AB plus Ang II induced HIF-1/β, which was associated with arteriolosclerotic lesions in the spiral arteries and altered trophoblast invasion. Finally, AT1-AB plus Ang II induced ET-1 expression.

Gant et al infused Ang II into pregnant patients from week 10 of pregnancy onward and showed that those who later developed preeclampsia required diminishing amounts of Ang II to obtain a similar pressor response. Baker et al confirmed these findings. They observed increased Ang II–induced calcium signaling in platelets of pregnant women who subsequently developed preeclampsia. Siddiqui et al showed that AT1-AAs occur in 95% of preeclamptic women. They also found AT1-AAs in women with gestational hypertension. AbdAlla et al postulated that AT1-bradykinin 2 receptor heterodimerization mediates an increased Ang II response in preeclampsia. Systemic alterations in AT1 receptor expression did not correlate with increased Ang II sensitivity.

We do not know how AT1-AAs activate the AT1 receptor. They could alter the receptor conformation so that the receptor’s ability to bind circulating Ang II is enhanced.11 The activation may involve a cross-linking between receptors, thereby holding receptors in their activated conformation and preventing tachyphylaxis. Cruz et al reported an increase in fetoplacental vascular responsiveness to Ang II in isolatedperfused placenta from preterm pregnancies with absent tachyphylaxis, whereas marked tachyphylaxis was present in all of the preparations from term pregnancies. Saxena et al showed recently that increased sensitivity to Ang II persists postpartum in preeclamptic women. In line with these data, we found that AT1-AAs persisted in 20% of former preeclamptic patients 1 year postpartum. Although blood pressure returns to normal after delivery, women with preeclampsia have an increased risk of cardiovascular disease later in life. Several reports postulate that the abnormalities in vascular function persist, which predisposes these women to the increased risk. AT1-AAs could conceivably exercise long-term effects.

Vasodilatation of the maternal systemic circulation is an important physiological adaptation of mammalian pregnancy. We observed that a substantial Ang II infusion (435 ng/kg per minute) did not increase blood pressure in pregnant rats. This dose regularly increases blood pressure in nonpregnant rats. Yu and Khraibi showed that an IV Ang II infusion
at 200 ng/kg per minute is required to increase blood pressure in anesthetized midpregnancy rats in a short-term experiment. Zhou et al.\textsuperscript{13} reported that passive transfer of isolated IgG and purified AT1-AAs in pregnant mice on day 13 induced hypertension. Similarly, LaMarca et al.\textsuperscript{15} reported that AT1-AAs infused IP into pregnant rats from day 12 to 19 gestation via miniosmotic pumps induced a hypertensive response. However, these studies and our work have important differences. Our aim was to produce activating antibodies in a biological system distinct from the patients, as a “proof of principle.” Zhou et al.\textsuperscript{13} isolated AT1-AAs from patients directed against the second extracellular loop. We used an epitope-specific isolation procedure. LaMarca et al.\textsuperscript{15} administered their AT1-AAs differently than in our study. Liu and colleagues\textsuperscript{19,32} found differences between AT1-AAs from preeclamptic women and AT1-ABs generated by long-term active immunization in male rats. They found that their AT1-ABs alone did not induce hypertension.\textsuperscript{19,32}

Naturally occurring autoantibodies and antibodies generated by immunization behave differently, although they are directed against the same epitope.\textsuperscript{33} Posttranslational modifications, such as acetylation, lipidation, citrullination, glycosylation, and so forth, could also influence the antibodies.\textsuperscript{34} Although the generated AT1-ABs showed effects similar to AT1-AAs in in vitro assays, we cannot exclude the possibility that various autoantibodies have additional properties that are necessary to induce the preeclamptic phenotype in vivo. In addition, different affinity and avidity of the antibodies may also influence the effects as shown for autoantibodies to monosialotetrahexosylganglioside 1, a major ganglioside important to Guillain-Barré syndrome.\textsuperscript{35} Thus, antibodies directed to the same epitope may exhibit different receptor responses.

Iizuka et al.\textsuperscript{36} showed that mouse autoantibodies differ, even in mice with the same genetic background and major histocompatibility complex class, when the antigen is either a “foreign” or a “self” antigen. In mice deficient in the M3 muscarinic acetylcholine receptor, functional antibodies were induced by immunization with the M3 receptor. However, these antibodies were not present when studied from mice having tolerance to this autoantigen, namely, littermates that were also immunized with M3 receptor. Furthermore, major histocompatibility complex class I molecules are different among different species and determine the expression of
peptides on T cells, positive and negative selection of the immune response, and antibody specificity in vivo.34 By studying the autoimmune response against certain autoantigens in different lupus models, Monneaux et al17 reported that major histocompatibility complex and nonmajor histocompatibility complex genes are responsible for the autoantibody response and the fine specificity of the autoantibodies. These responses could be completely different in an antibody generated by immunization. We do not believe that our results are specifically related to low antibody titers.17,38 We also dosed the pregnant rats twice. We detected the transferred AT1-ABs for ≥11 days after injection. Siddiqui et al10 concluded that, in healthy pregnant women, AT1-AA concentrations were not high enough or the AT1-As were not present for sufficient duration. Furthermore, Ang II levels are increased 2.2-fold in normal human pregnancy.59 The levels that we obtained in the rat did not reach the physiological human pregnancy levels. Although preeclampsia, scleroderma, and non-human leukocyte antigen (HLA)–triggered acute kidney transplant rejection share vasculopathy as a common feature,11,40,41 which permissive factors are responsible for the preference of the different vascular beds is unclear.

We identified HIF-1α by means of gene expression analysis. We then supported this finding with Western blotting. Placental ischemia-hypoxia is the major inducer for HIF-α and protein stabilization in preeclampsia.42 Whether Ang II plus AT1-ABs induced HIF-1α directly or whether the response was secondary to hypoxia cannot be discerned from our study. However, Rajakumar et al33 suggested earlier that HIF-1α contributes to preeclampsia. The arteriolosclerotic lesions in the spiral arteries occurring in our experiments could have contributed to uteroplacental hypoxia. Furthermore, Burton et al43 proposed recently that turbulent blood flow could damage villous architecture. Reactive oxygen and the redox-sensitive part of the oxygen-sensing pathway are also important inducers of HIF-1α.44 Under situations of increased oxidative stress, changes in the redox state within a cell may contribute to an efficient and fast-responding O2-sensing system.45

We also found that ET-1 might be involved in mediating the blood pressure increases that we observed. In the reduced uterine perfusion pressure preeclampsia-like model, a selective endothelin (endothelin A) receptor antagonist attenuated the blood pressure response.46 Furthermore, LaMarca et al15 showed that the hypertensive effects elicited by rat AT1-As were blocked by an endothelin A receptor antagonist. These results are in line with in vitro observations from Yang et al,32 who found that AT1-As from preeclamptic patients caused vascular constriction in conduit and small resistance arteries from male rats and mechanistically implicated ET-1.

Perspectives
We provide further evidence to support the “immune” theory of preeclampsia and suggest that interventions along the lines of renin-Ang-system attenuation could be therapeutically useful. We recognize the fact that our findings are still not definitive and that we will have to conduct further studies.


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Supplement

**AT1 receptor antibodies and increased angiotensin II sensitivity in pregnant rats**

Running title: Autoantibodies, Ang II, pregnancy

Katrin Wenzel¹,², Augustine Rajakumar³, Hannelore Haase², Nele Geusens⁴, Norbert Hubner², Herbert Schulz², Justin Brewer², Lyndsay Roberts⁵, Carl Hubel⁶, Florian Herse¹,², Lydia Hering², Fatimunnisa Qadri¹,², Carsten Lindschau⁷, Gerd Wallukat², Robert Pijnenborg⁴, Harald Heidecke⁸, Gabriela Riemekasten¹, Friedrich C. Luft¹,², Dominik N. Muller², Babette Lamarca⁵ and Ralf Dechend²,⁹
Supplement figure S1. Twenty four-hour albumin excretion on day 17-18 of pregnancy. Albumin was significantly increased in the Ang II/AT1-AB group compared to the Ang II/IgG group (P = 0.0157). The mRNA of neutrophil gelatinase-associated lipocalin (N-Gal) suggested as early marker of acute kidney injury was only significantly upregulated in the Ang II/AT1-AB group.
Supplement figure S2. No increased fibrosis was detectable in kidneys stained by Masson-Trichrome.
Supplement figure S3. Hematoxylin-Eosin staining of kidneys.
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Supplement table S1. Significantly different expressed genes in Ang II+AT1-AB treated group (FC ≥ 1.5)