Hypertension in Response to Autoantibodies to the Angiotensin II Type I Receptor (AT1-AA) in Pregnant Rats
Role of Endothelin-1

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Abstract—Agonistic autoantibodies to the angiotensin II type I receptor (AT1-AA) and endothelin-1 (ET-1) are suggested to be important links between placental ischemia and hypertension during preeclampsia. Activation of the angiotensin II type I receptor (AT1R) increases endothelial cell production of ET-1; however, the importance of ET-1 in response to AT1-AA-mediated AT1 R activation during preeclampsia is unknown. Furthermore, the role of AT1-AA-mediated increases in blood pressure during pregnancy remains unclear. The objective of this study was to test the hypothesis that AT1-AA, increased to levels observed in preeclamptic women and placental ischemic rats, increases mean arterial pressure (MAP) by activation of the ET-1 system. Chronic infusion of purified rat AT1-AA into normal pregnant (NP) rats for 7 days increased AT1-AA from 0.68 ± 0.5 to 10.88 ± 1.1 chronotropic units (P < 0.001). The increased AT1-AA increased MAP from 99 ± 1 to 119 ± 2 mm Hg (P < 0.001). The hypertension was associated with significant increases in renal cortices (11-fold) and placental (4-fold) ET-1. To determine whether ET-1 mediates AT1-AA-induced hypertension, pregnant rats infused with AT1-AA and NP rats were treated with an ET\textsubscript{A} receptor antagonist. MAP was 100 ± 1 mm Hg in AT1-AA + ET\textsubscript{A} antagonist-treated rats versus 98 ± 2 mm Hg in ET\textsubscript{A} antagonist-treated rats. Collectively, these data support the hypothesis that one potential pathway whereby AT1-AAs increase blood pressure during pregnancy is by an ET-1-dependent mechanism. (Hypertension. 2009;54:905-909.)

Key Words: preeclampsia ■ hypertension ■ kidney ■ placenta ■ inflammation

The initiating event in early onset preeclampsia is postulated to involve inadequate vascularization of the subplacental decidua with reduced placental perfusion that leads to hypertension during pregnancy by mechanisms not yet elucidated.\(^1,2\) Recent studies have suggested that the production of agonistic autoantibodies to the angiotensin II (Ang II) type I receptor (AT1-\textsubscript{AA}) may be an important link between placental ischemia and hypertension in preeclamptic women.\(^3,8\) The AT1-AA induces signaling in vascular cells that are blocked by an AT1 receptor antagonist including activating protein-1, calcineurin, and nuclear factor kappa B activation.\(^3,4,7\) Recent studies by Zhou et al demonstrate that immunoglobulin isolated from preeclamptic women increases systolic blood pressure 4 days after retro-orbital injection into pregnant mice.\(^7,8\) This hypertensive response was attenuated by administration of an AT1 receptor antagonist. Although these findings suggest that AT1-AAs from preeclamptic women increases blood pressure in pregnant mice, possibly by activation of the AT1 receptor, it remains unclear by what mechanism purified AT1-AA mediates hypertension during pregnancy.

We recently reported that hypertension in response to reductions in uterine perfusion pressure in pregnant rats (RUPP) is associated with increased circulating levels of the AT1-\textsubscript{AA}.\(^9,10\) Moreover, we found that the increased blood pressure response in RUPP pregnant rats decreased markedly by antagonism of the AT1 receptor. In addition we previously reported that the hypertension in RUPP rats is associated with significant increases in renal expression of preproendothelin, and this blood pressure response is attenuated by administration of a selective ET\textsubscript{A} receptor antagonist. In contrast, ET\textsubscript{A} receptor blockade had no significant effect on blood pressure in the normal pregnant animal suggesting a role for ET-1 in mediating the hypertension in response to placental ischemia.\(^11\)

Although our recent data implicate that AT1-\textsubscript{AA} and ET-1 are produced in response to placental ischemia and activation of the AT1R and ET\textsubscript{A} receptors contribute to hypertension in...
RUPP rats, it is unclear whether AT1-AAs have a direct effect to enhance ET-1 production or whether chronic AT1-AA increases arterial pressure during pregnancy via an ET-dependent mechanism. Therefore, the overall goal of this study was to test the hypothesis that the AT1-AA stimulate ET-1 production and increases mean arterial pressure in pregnant rats by an ET-1–dependent mechanism. To achieve this goal we examined the effects of isolated column-purified active rat AT1-AA, on blood pressure and ET-1 production in pregnant rats. In addition, to determine a role for ET-1 activation in response to the purified rat AT1-AA, we compared the blood pressure effects of AT1-AA in pregnant rats in the presence of an ET\textsubscript{A} receptor antagonist.

**Methods**

**Isolation and Purification of Rat AT1-AA**

The female h\text{Aogen} x male h\text{Ren} (MDC, Berlin) transgenic rats (MDC, Berlin) were used as the source of rat AT1-AA. This model develops hypertension associated with the AT1-AA. On day 18 of gestation blood was collected and immunoglobulin was isolated from 1 ml of serum by specific antiratIgG column purification. AT1-AA was purified from rat IgG by epitope binding to the amino acid sequence corresponding to the second extracellular loop of the AT1 receptor covalently linked to Sepharose 4B CNBr-activated gel. Unbound IgG was washed away and bound IgG was eluted with 3 mol/L potassium thiocyanate. AT1-AA activity was measured using a bioassay that evaluates the beats per minute (bpm) of neonatal cardiomyocytes in culture.

**Protocol 1a: Effect of Rat AT1-AA on MAP in Pregnant Rats**

All studies were performed in timed pregnant Sprague Dawley rats purchased from Harlan Inc (Indianapolis, Ind). Animals were housed in a temperature controlled room (23°C) with a 12:12 light: dark cycle. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center. Twelve microliters/d of (1:50) purified rat AT1-AA fraction (collected as described above) diluted in saline was infused into pregnant rats for seven days. This protocol was adapted from a study by Dragun et al that used similar fraction of isolated AT1-AA to demonstrate renal transplant rejection in rats. Purified rat AT1-AA was infused intraperitoneally from day 12 to 19 of gestation via mini-osmotic pumps (model 2002, Alzet Scientific Corporation) into normal pregnant rats (n = 17) and pregnant rats treated chronically with AT1-AA (n = 16). A second group of normal pregnant rats were chronically treated with diluted control IgG (n = 4) to compare the effects of the AT1-AA with an IgG molecule serving as a control to mediate hypertension during pregnancy. On day 18 of gestation, all rats were surgically instrumented with a carotid catheter for subsequent arterial pressure measurement. At day 19 of gestation arterial pressure was measured. Subsequently, a blood sample was collected, kidneys and placentas were harvested, and litter size and pup weights were recorded while dams were under anesthesia using isoflurane (Webster) delivered by an anesthesia apparatus (Vaporizer for Forane Anesthetic, Ohio Medical Products). A catheter of V-3 tubing (SCI) was inserted into the carotid artery for blood pressure monitoring. The catheter was tunneled to the back of the neck and exteriorized after implantation. On day 19 of gestation, pregnant rats were placed in individual restraining cages for arterial pressure measurements. Arterial pressure was monitored with a pressure transducer (Cobe III Transducer CDX Sema) and was recorded continuously for a 2-hour period after a 1-hour stabilization.

**Determination of Kidney and Placental Preproendothelin mRNA Levels**

The cortex and medulla of kidneys were separated immediately after harvesting and quickly frozen in liquid nitrogen and stored at −80°C. Placentas were weighed and quickly frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the Totally RNA kit supplied by Ambion after the tissues were crushed in liquid nitrogen with a mortar and pestle. The isolation procedure was then performed as outlined in the instructions provided by the manufacturer. Real-time PCR was used, as previously described, to determine tissue ppET-1. Statistical Analysis

**Statistical Analysis**

Differences between control and AT1-AA infused rats were analyzed using an unpaired t test for blood pressure analysis and bioassay results. ANOVA with Tukey-Kramer multiple comparison test was used when comparing blood pressure analysis between control and experimental groups (groups treated with either losartan or ET\textsubscript{A} receptor antagonist). Data were considered statistically different at probability values <0.05. Statistical analysis of real time PCR results was calculated using the mean normalized cycle threshold (delta/ delta CT) values and compared using ANOVA and Tukey-Kramer multiple comparison tests.

**Results**

**Serum AT1-AA Levels and Mean Arterial Pressures in Control and Chronic AT1-AA–Treated Pregnant Rats**

AT1-AA activity, determined via rat cardiomyocyte bioassay, increased from 0.68±0.5 bpm to 10.88±1.1 bpm (P <0.001) with chronic AT1-AA (rat AT1-AA [1:50]) infusion into
normal pregnant rats (Figure 1). Chronic infusion of purified rat AT1-AA into pregnant rats resulted in significant increases in arterial pressure, from 99±1 mm Hg in NP controls to 119±2 mm Hg (P<0.001) in AT1-AA–treated pregnant rats (Figure 2). In contrast, chronic infusion of a control IgG into pregnant rats had no effect on MAP compared to normal pregnant rats (102±2 versus 102±2 mm Hg).

Hypertension in Response to AT1-AA Is Blocked by an AT1-Receptor Antagonist

MAP of pregnant AT1-AA–treated rats that were administered AT1 receptor antagonist was not different than in pregnant rats chronically treated with AT1 receptor antagonist alone (96±2 mm Hg versus 103±3 mm Hg; Figure 2).

Preproendothelin mRNA Levels in Placenta and Kidneys in Control and AT1-AA–Treated Pregnant Rats

Real-time PCR was used to measure preproendothelin in the placenta and the renal cortex and medulla. Preproendothelin mRNA levels in the renal cortices and placenta of the AT1-AA–treated rats were significantly increased compared to normal pregnant rats (Figure 3). Conversely, medullary preproendothelin mRNA levels were not different in response to chronic AT1-AA excess compared to normal pregnant rats.

In addition, the increase in preproendothelin in response to AT1-AA–induced hypertension was attenuated with administration of an AT1 receptor antagonist.

Effect of ETα Receptor Antagonist on AT1-AA–Induced Hypertension

The selective ETα receptor antagonist (ABT-627) was administered for 5 days to normal pregnant control rats and pregnant rats chronically treated with AT1-AA. Normal pregnant rats treated with ETα receptor antagonist alone served as controls. Administration of an ETα receptor antagonist attenuated AT1-AA–induced hypertension (100±1 mm Hg in AT1-AA+ETα pregnant rats versus 98±2 mm Hg in ETα rats; Figure 2).

Placental and Pup Weight in Response to Chronic AT1-AA

There was no difference in pup weight (2.3±0.04 g versus 2.2±0.03 g versus 2.3±0.03 g versus 2.2±0.03 g) or litter size (14 versus 13 versus 14 versus 14) between normal pregnant, AT1-AA, ETα receptor antagonist treated, or AT1-AA plus ETα receptor antagonist pregnant rats, respectively. Furthermore, placental weights were statistically unchanged (0.55±0.2 versus 0.54±0.2 versus 0.51±0.1 versus 0.48±2 g) between normal pregnant, AT1-AA, ETα receptor...
antagonist–treated, or AT1-AA plus ET\textsubscript{A} receptor antagonist pregnant rats, respectively.

**Discussion**

In this study we demonstrated that increasing levels of AT1-AA to levels observed in preeclamptic women and in placental ischemic rats increases mean arterial pressure (MAP) in pregnant rats by activation of the endothelin system. We report that infusion of purified rat AT1-AA, isolated from serum collected from a pregnant transgenic rat cross overproducing components of the renin angiotensin system,\textsuperscript{12,13} into pregnant rats from day 12 to day 19 of gestation, increased serum AT1-AA, blood pressure, and tissue levels of preproendothelin. Finally, we report that AT1-AA–induced hypertension in pregnant rats was attenuated by either oral administration of the AT1 receptor antagonist losartan or an ET type A receptor antagonist. In addition, the increase in endothelin transcript in response to AT1-AA–induced hypertension was abolished by administration of an AT1 receptor antagonist.

Although a role for the AT1-AA to mediate hypertension during pregnancy has been suggested by other laboratories, potential mechanisms whereby the autoantibody increases pressure has remained undefined. In this study we demonstrate that administration of the AT1-AA to pregnant rats significantly increased ET-1 levels in renal cortices and placenta of pregnant rats (Figure 3). To confirm a role for the increase in ET-1 as a potential mechanism of AT1-AA–induced hypertension, pregnant rats and chronically AT1-AA–treated pregnant rats were administered a selective ET\textsubscript{A} receptor antagonist in their drinking water. The hypertensive response to the AT1-AA in pregnant rats was attenuated with ET\textsubscript{A} blockade (Figure 2). These data indicate that the increased local production of ET-1 in response to chronic AT1-AA plays an important role in hypertension during pregnancy.

Although AT1-AA are reportedly elevated in preeclamptic women,\textsuperscript{5,6,10–20} the importance of immune activation in mediating the cardiovascular and endothelial dysfunction in response to placental ischemia during pregnancy remains unclear. Although the AT1-AA has been implicated in endothelial dysfunction associated with preeclampsia, the exact mechanism linking enhanced production of ET-1 to placental ischemia in pregnant rats or in preeclamptic women is unknown.\textsuperscript{10,11,16–21} A recent study from our laboratory demonstrated that sera from pregnant rats exposed to chronic reduced uterine perfusion pressure (RUPP) increased ET-1 production by cultured endothelial cells and this response was attenuated by AT1 receptor antagonism.\textsuperscript{21} This study suggested that the AT1-AA was a circulating component stimulated in response to placental ischemia. Further in vitro evidence included studies demonstrating that AT1-As induce signaling in vascular cells through activating protein-1, calcineurin and nuclear factor kappa B which were all blocked by AT1 receptor antagonism.\textsuperscript{3,4,7,8} The signaling resulted in increased reactive oxygen species and sFlt-1 production, both of which have been implicated in preeclampsia.\textsuperscript{3,4} Finally, a recent study by Yang and colleagues demonstrated that human AT1-AA caused vascular constrictive hypertension in isolated rat thoracic aortic rings, middle cerebral artery, and coronary artery segments in a concentration-dependent fashion.\textsuperscript{22} The vasoconstrictive effect of AT1-AA was completely blocked by an AT1-receptor antagonist.

Recent studies from Zhou et al demonstrated that immunoglobulin isolated from preeclamptic women increases systolic pressure in pregnant mice. This increase in systolic pressure was associated with a reduction in placental size and fetal weight. This phenotype was ameliorated with coinjection of an AT1 receptor antagonist. These findings suggest a role for the AT1-AA component of the human IgG to mediate hypertension and intrauterine growth restriction in pregnant mice.\textsuperscript{7,8} These well designed and executed studies, however, did not determine the pathophysiological mechanisms associated with the hypertension. In this study we infused purified species specific AT1-AA into normal pregnant rats to test the role of the AT1-AA to induce hypertension during pregnancy. In addition, we confirm the presence of active rat AT1-AA 7 days postinfusion by a previously established and validated bioassay used to determine increased chronotropic effects of the AT1-AA to activate AT1 receptors on cardiomyocytes in vitro.\textsuperscript{3–6,12} Finally, the findings reported in the present study are the first to demonstrate that one potential mechanism of the AT1-AA to elicit hypertensive effects is by ET-1 activation during pregnancy.

Data suggest that reduced placental perfusion in both humans and in animal models of preeclampsia is an important stimulus for AT1-AA production.\textsuperscript{5–10,21} Using the cardiomyocyte contraction assay, Walther et al found that the AT1-AA was detectable between 18 to 22 weeks gestation in women with abnormal uterine perfusion 23. These women, when followed to term, fell into 3 groups: those who developed preeclampsia, those characterized by fetuses with intrauterine growth retardation, and those with a normal pregnancy outcome. AT1-AA was not observed in second trimester women with normal Doppler. At term, the AT1-AA was present in preeclamptic women, those exhibiting intrauterine growth retardation, and in healthy pregnant women with a history of abnormal uterine perfusion in the second trimester.\textsuperscript{23} The authors concluded that AT1-AA were present in patients with pathological uterine artery Doppler findings independent of preeclampsia, suggesting that AT1-AA may not be the primary cause of preeclampsia. We recently reported that chronic reductions in uterine perfusion pressure in the pregnant rat increases arterial pressure, impairs endothelial function, and is associated with intrauterine growth restriction and production of the AT1-AA.\textsuperscript{9} Although reductions in placental perfusion are associated with AT1-AA production, we did not see intrauterine growth restriction or decreased pup viability with purified AT1-AA administration into pregnant rats, suggesting that this phenotype could result from a synergism between AT1-AA and other placental derived factors to elicit deleterious effects on the fetus.

**Perspectives**

Although the data presented in this study demonstrate that hypertension in response to AT1-AA during pregnancy is mediated via an ET-1–dependent mechanism, there are a number of unanswered question in this field of investigation.
Although AT1-AA causes significant hypertension, the role of the AT1-AA in mediating impaired renal hemodynamics or proteinuria during pregnancy is unclear. Experiments inhibiting the generation of the AT1-AA in rats with placental ischemia will contribute to further defining the pathophysiological role of the autoantibody to mediate hypertension and renal dysfunction during pregnancy.

Furthermore, studies from our laboratories have shown that the AT1-AA is not produced in normal virgin rats indicating that the antigen to which the autoantibody is produced is predominantly expressed during pregnancy. The exact antigen to which the AT1-AA is produced remains to be determined. Thus, the immunologic mechanisms that leads to AT1-AA production in response to reductions in uterine perfusion remains to be an important area of investigation.

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Disclosures
None.

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