Hypertension in Response to Placental Ischemia During Pregnancy
Role of B Lymphocytes

Babbette LaMarca, Kedra Wallace, Florian Herse, Gerd Wallukat, James N. Martin, Jr, Abram Weimer, Ralf Dechend

Abstract—Preeclampsia is associated with innate inflammatory response resulting in elevated tumor necrosis factor-α, agonistic autoantibodies to the angiotensin II type I receptor, and activation of endothelin 1 (ET-1). This study was designed to determine the role of B-cell depletion, resulting in agonistic autoantibodies to the angiotensin II type I receptor suppression to mediate hypertension via activation of ET-1 in the placental ischemic reduced uterine perfusion pressure (RUPP) rat model of preeclampsia. To achieve this goal we examined the effect of RUPP on mean arterial pressure and ET-1 in the presence and absence of chronically infused rituximab (R; 250 mg/kg), a B-lymphocyte–suppressive agent used clinically to treat autoimmune diseases. Mean arterial pressure was 103±1 mm Hg in normal pregnant (NP) rats; 103±3 mm Hg in NP+R versus 133±2 mm Hg in RUPP rats, and 118±2 mm Hg in RUPP+R rats (P<0.001 vs RUPP controls). B lymphocytes decreased from 6.0±0.5% gated cells in RUPP to 3.7±0.8% gated cells in RUPP+R rats. Importantly, agonistic autoantibodies to the angiotensin II type I receptor decreased from 18±1 bpm in RUPP rats to 10±1 bpm in RUPP+R rats. ET-1 decreased 1.5-fold in kidneys and 4-fold in the placenta (P<0.01) of RUPP+R versus RUPP rats. Media ET-1 excretion from endothelial cells exposed to serum from NP, RUPP, NP+R, or RUPP+R rats was determined. ET-1 from endothelial cells treated with NP serum was 53±13 pg/mg and increased to 75±10 pg/mg with RUPP serum. In contrast, ET-1 secretion decreased in response to B-cell–depleted RUPP serum to 50±8 pg/mg and was unchanged in response to NP+R sera (46±12 pg/mg). These data demonstrate the important roles that B-lymphocyte activation and agonistic autoantibodies to the angiotensin II type I receptors play in the pathophysiology of hypertension in response to placental ischemia. (Hypertension. 2011;57:865-871.)

Key Words: hypertension ■ inflammation ■ renin-angiotensin system ■ pregnancy ■ endothelin

Preeclampsia has long been considered an immunologic disorder during pregnancy.1-4 Recent developments in preeclampsic research confirm these initial speculations. In recent years we have learned that preeclamptic women display characteristics similar to various chronic inflammatory diseases, such as elevated inflammatory cytokines, activated circulating immune cells, autoantibodies, and, most recently, autoimmune associated T cells and cytokines (T-helper 17 and interleukin 17, respectively).3-13 In recent years much research has been performed to determine the role of the agonistic autoantibody to the angiotensin II type I receptor (AT1-AA) to mediate much of the pathophysiology associated with preeclampsia.12-21 For B-cell maturation and IgG production, several costimulatory signals must be occur between the antibody-producing B lymphocyte and CD4+T-helper cells.22,23 One of these includes stimulation of the CD20 receptor on the surface of the B cell.24,25 This recognition stimulates the B cell to enter the circulation and mass produce specific immunoglobulin. To treat various autoimmune diseases, many therapeutic agents inhibiting specific interactions between immune molecules and cells have been developed. In recent years ours and other laboratories have examined a potential use of specific anti-inflammatory therapeutic agents to suppress the pathophysiology associated with autoimmune diseases in various animal models of the disease.26-33 In this study we used a new chemotherapeutic agent that has shown efficacy among autoimmune patients by blocking the CD20 costimulatory molecule, thereby inhibiting B lymphocytes from entering the circulation and secreting antibody, a process coined as “B-cell depletion.”22-28,33

Our laboratory reported recently that infusion of the rat form of AT1-AA into normal pregnant (NP) rats during the later stages of gestation increases blood pressure, the antian- giogenic factor sFlt-1, and sEndoglin, and increases local transcription of the potent vasoconstrictor peptide, endothelin-1 (ET-1).34,35 Although these studies demonstrate

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a role for the AT_1-AA to mediate hypertension during pregnancy and to activate several mechanisms contributing to the phenotype of preeclampsia, these studies fail to evaluate the importance of endogenously generated AT_1-AA in mediating hypertension in response to placental ischemia. Therefore, we tested the hypothesis that B-cell depletion, as a mechanism of suppressing endogenously generated AT_1-AA, blunts increases in blood pressure and ET-1 activation in response to placental ischemia in pregnant reduced uterine perfusion pressure (RUPP) rats. To test this hypothesis we chronically administered rituximab (CD20 blockade) to induce B-cell depletion in NP and RUPP rats and evaluated hypertension and ET-1 activation in response to placental ischemia.

Methods

All of the studies were performed in timed pregnant Sprague-Dawley rats purchased from Harlan Sprague-Dawley, Inc (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 hour light/dark cycle. All of the experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals, and the University of Mississippi Medical Center Institutional Animal Care and Use Committee approved all of the protocols.

B-Cell Depletion and AT_1-AA Suppression Decrease Mean Arterial Pressure in Response to Chronic Reductions in Uterine Perfusion Pressure

Experiments were performed in the following groups of rats: pregnant control (n=23); NP treated with rituximab (250 mg/kg; Genentech, Inc) from day 14 to day 19 of gestation (n=12); RUPP pregnant rats (n=28); and RUPP pregnant rats treated with rituximab (250 mg/kg) from day 14 to day 20 of gestation (n=28). Rituximab is a chimeric monoclonal anti-CD20 antibody that is used to induce B-cell depletion in vivo.23-27 The dose used was based on the maximal doses identified in the medicinal pamphlet provided by the manufacturer. All of the pregnant rats undergoing surgical procedures were anesthetized with 2% isoflurane (WA Butler Co) delivered by an anesthesia apparatus (Vaporizer for Forane Anesthetic, Ohio Medical Products). Pregnant rats entering the RUPP group underwent the clipping procedure at day 14 of gestation, as described previously.29,32,36-39 Groups of rats treated with rituximab underwent intraperitoneal osmotic minipump insertion performed on day 14 of gestation.

Measurement of Arterial Pressure in Chronically Instrumented Conscious Rats

Pregnant rats were instrumented with arterial catheters on day 18 of gestation under anesthesia, and arterial pressure was determined in all of the groups of rats at day 19 of gestation, as described previously.29,32,34-40

Determination of Circulating AT_1-AA

On day 18 of gestation, blood was collected and immunoglobulin was isolated from 1 mL of serum by specific anti-rat-IgG column purification. AT_1-AA was purified from the rat IgG by epitope binding to the amino acid sequence corresponding with the second extracellular loop of the AT_1 receptor covalently linked to Sepharose 4B cyanogen bromide–activated gel. Unbound IgG was washed away, and bound IgG was eluted with 3 mol/L of potassium thiocyanate. AT_1-AA activity was measured using a bioassay that evaluates the beats per minute of neonatal cardiomyocytes in culture.13-18,34,35,41,42 AT_1-AAs were assessed in NP controls, RUPP controls, and rituximab-treated RUPP rats. Because AT_1-AA is not elevated in NP rats, the effect of rituximab on B cells or AT_1-AA in NP rats was not determined in this study.

Determination of Circulating Tumor Necrosis Factor-α and Interleukin 6

On day 18 of gestation isolated serum was collected and used for determination of circulating cytokines measured via ELISA from R&D Systems. The minimal detectable dose for the rat quantikine interleukin (IL) 6 ELISA was 21 pg/mL with an intra-assay/interassay precision of 8.8 and 10%, respectively. The minimal detectable dose for the tumor necrosis factor (TNF)-α quantikine ELISA was <5 pg/mL with an intra-assay/interassay precision of 5 and 9.7%, respectively.

Determination of Renal and Placental Preproendothelin mRNA Levels

The placenta, cortex, and medulla of the kidneys were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the Quiagen kit after the tissue was crushed in liquid nitrogen with a mortar and pestle. Isolation procedure was then performed as outlined in the instructions provided by the manufacturer. cDNA was synthesized from 1 μg of RNA with BioRad Iscript cDNA reverse transcriptase, and real-time PCR was performed using the BioRad Syber Green supermix and iCycler, as described previously.32,34,40 Levels of mRNA expression were calculated using the mathematical formula for mean normalized cycle threshold recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997).

Endothelial Cell ET-1 Production

Cell Culture

Human umbilical vein endothelial cells (HUVECs), passage 2, were cultured in 50:50 DMEM/M199 (Gibco BRL) with 10% FBS (Hyclone) and 1% antimycotic antibiotic (Gibco BRL) in a humidified atmosphere of 5%CO_2-20%O_2-75%N_2 at 37°C. Seventy-percent confluent monolayers were incubated for 48 hours in serum-free medium before exposure to experimental conditions. The experimental protocol to determine whether endogenous B-cell depletion and the resulting decrease in AT_1-AA and hypertension in response to placental ischemia had profound effects to decrease vascular (HUVEC) secretion of ET-1 was performed as shown previously by LaMarca et al42 and Roberts et al43 Aliquots of sample were taken after 6 and 18 hours of cultivation, after exposure to experimental media to determine whether ET-1 secretion increased with time. Cells were trypsinized and total protein collected to normalize ET-1 secretion.32,43

Determination of Endothelin Concentration

Endothelin concentration was determined using 100 μL of media collected and measured using the ET-1 Quantikine ELISA kit from R&D Systems. The assay displayed a sensitivity of 0.023 to 0.102 pg/mL, interassay variability of 8.9%, and intra-assay variability of 3.4%.32,43

Statistical Analysis

Data are reported as mean±SEM with P values <0.05 considered statistically significant. Differences between control and experimen-
Arterial Pressure Response to B-Lymphocyte Depletion and Rituximab in NP and RUPP Rats

Administration of rituximab (250 mg/kg), an agent used for B-lymphocyte depletion, to NP rats from day 14 to day 19 of gestation had no effect on mean arterial pressure (MAP) (Figure 1). In sharp contrast, administration of rituximab decreased MAP significantly; however, it did not completely attenuate hypertension in placental ischemic RUPP rats.

**Results**

Arterial Pressure Response to B-Lymphocyte Depletion and Rituximab in NP and RUPP Rats

Administration of rituximab (250 mg/kg), an agent used for B-lymphocyte depletion, to NP rats from day 14 to day 19 of gestation had no effect on mean arterial pressure (MAP) (Figure 1). In sharp contrast, administration of rituximab decreased MAP significantly; however, it did not completely attenuate hypertension in placental ischemic RUPP rats.
B-cell depletion decreased the placental ET-1 mRNA transcript in response to placental ischemia. Top panel shows a ET-1 transcript quantitated by real-time PCR in the renal cortex and placenta (bottom) from all of the experimental groups. Although rituximab had no significant effect on ET-1 in NP rats, it did decrease ET-1 in the renal cortices and placenta of RUPP rats compared with control RUPP rats (P<0.05).

**Figure 3.** B-cell depletion decreased the placental ET-1 mRNA transcript in response to placental ischemia. Top panel shows a ET-1 transcript quantitated by real-time PCR in the renal cortex and placenta (bottom) from all of the experimental groups. Although rituximab had no significant effect on ET-1 in NP rats, it did decrease ET-1 in the renal cortices and placenta of RUPP rats compared with control RUPP rats (P<0.05).

### Pup and Placental Weights Decrease in Response to RUPP in Pregnant Rats and Were Unchanged by Administration of Rituximab

Pup and placental weights are smaller in response to RUPP in pregnant rats compared with control NP rats. Administration of rituximab had no effect on pup or placental weights in either RUPP or control pregnant rats. Only viable pups are counted and weighed, and the average from each group is reported. The average weight for pups from NP rats was 2.39±0.11 g and was 2.17±0.07 g in NP+R rats. The average weight of pups from RUPP rats was 1.9±0.08 g and was 1.96±0.10 g in RUPP+R rats (P<0.05 versus NP). Placental weights in NP rats averaged 0.55±0.04 g and were 0.55±0.02 g in NP rats treated with rituximab. Placental weights in RUPP rats averaged 0.50±0.04 g and were 0.51±0.03 g in RUPPs treated with rituximab.

### Rituximab Blunts Local Production of Tissue Endothelin

Real-time PCR was used to measure preproendothelin in the renal cortex and the placenta of NP or RUPP rats. Preproendothelin was increased 2.5-fold in the cortex from RUPP rats compared with NP rats (P<0.02) and decreased 1.5-fold in RUPP+R compared with control RUPP rats; however, this difference was not significant (Figure 3).

Likewise, preproendothelin significantly increased in the placenta of RUPP rats compared with NP rats. Administration of rituximab did reduce preproendothelin expression in NP+R placentas. However, treatment of RUPP rats with rituximab significantly decreased placental levels of preproendothelin compared with control RUPP rats (Figure 3). These data indicate that B-lymphocyte activation and elevations in AT₁-AA in response to placental ischemia contribute to increased transcription of ET-1 in both the kidney and placentas of pregnant RUPP rats. However, additional factors, such as TNF-α, which also influence ET-1 expression in RUPP rats, or other secondary factors resulting from B-lymphocyte activation may play an equally important role as AT₁-AAAs to stimulate ET-1 transcription. These other factors will be examined in future studies characterizing the effects of B-cell depletion to suppress hypertension in response to placental ischemia.

### Rituximab Decreased Circulating TNF-α, But Not IL-6, in RUPP Rats

As we have shown previously, we see a 2-fold increase in circulating TNF-α and significant elevations in circulating IL-6 in RUPP rats versus NP controls. Therefore, we examined the effect of B-cell depletion on circulating levels of these 2 proinflammatory cytokines. We have shown previously that IL-6 is significantly increased in response to placental ischemia. In this study we demonstrate that IL-6 increased from 503±53 to 753±75 pg/mL from NP to placental ischemic RUPP rats. Because of significant revitalization of the R&D quantikine ELISA, these IL-6 levels are significantly different from those published from our laboratory in 2006. Importantly, the trend in response to placental ischemia is the same as that shown previously. Chronic administration of rituximab had no significant difference in circulating IL-6 levels in NP or RUPP rats. IL-6 in NP+R rats was 601±100 pg/mL and was 688±75 pg/mL in RUPP+R rats. As shown previously, TNF-α increased from 24±7 pg/mL in NP rats to 50±10 pg/mL in RUPP rats. In NP+R rats, TNF-α levels were 26.5±8.0 pg/mL. Interestingly, TNF-α decreased in RUPP+R rats to 13.2±2.0 pg/mL (P<0.05 versus RUPP controls).

### B-Cell Depletion Decreased Vascular ET-1 Secretion in Response to Placental Ischemia

We previously demonstrated a 2-fold increase in ET-1 secretion, a marker for endothelial cell activation, from endothelial cells in response to 6 hours of exposure to serum from RUPP rats compared with serum from NP rats. After an 18-hour incubation, the ET-1 response increased 3-fold in response to RUPP-induced placental ischemia compared with NP rats. To examine the role of B-cell depletion in mediating endothelial activation and dysfunction in response to placental ischemia, the response of HUVECs to serum collected from control RUPP rats and serum from RUPP rats treated with rituximab. Samples were taken at 6 and 18 hours of cultivation after exposure to rat serum. At both 6 and 18 hours, the ET-1 response of HUVECs to serum from rats with placental ischemia (n=9) is increased compared with the ET-1 response of HUVECs to serum from NP rats (n=8; Figure 4). In contrast, ET-1 secretion decreased in response to treated RUPP serum (n=9) and was unchanged in response to NP+R sera (n=7; Figure 4). These results indicate that B-cell depletion in pregnant rats with placental ischemia may provide protection, at the cellular level, against the actions of AT₁-AA and/or TNF-α to induce endothelial cell activation and dysfunction.
Discussion

One mechanism whereby AT1-AAs increase arterial pressure in pregnant rats is activation of the ET-1.34 We have reported recently that infusion of purified rat AT1-AA into NP rats increased serum AT1-AA, blood pressure, and tissue levels of preproendothelin.34 AT1-AA–induced hypertension in pregnant rats was attenuated by either oral administration of the angiotensin II type 1 receptor antagonist losartan or an endothelin type A receptor antagonist. In addition, the increase in ET-1 transcript in response to AT1-AA–induced hypertension was abolished by administration of an angiotensin II type 1 receptor antagonist. In this study we used the technique of B-cell depletion to suppress lymphocyte entry into the circulation and, as a direct result, suppression of AT1-AA production in response to placental ischemia. RUPP rats treated with rituximab and having suppressed AT1-AA exhibited less blood pressure increase in response to induced placental ischemia. Furthermore, B-cell–depleted RUPP rats had lower tissue ET-1 transcript in renal cortices and placentas compared with RUPP control rats. In addition, we have shown previously that circulating factors in serum from placental ischemic rats cause endothelial cell activation as measured by ET-1 secretion. This response is attenuated by angiotensin II type 1 receptor blockade, thus suggesting a role for the AT1-AA in response to placental ischemia to induce endothelial cell activation.43 In this study we examined this hypothesis by exposing endothelial cells to serum from B-cell–depleted, AT1-AA–suppressed placental ischemic rats. In support of this theory we found that ET-1 secretion from cultured endothelial cells in response to RUPP in pregnant rats was completely attenuated in response to serum from B-cell–depleted placental ischemic rats.

Local synthesis of ET-1 has been assessed in preeclamptic women, and investigators have found preproendothelin mRNA to be elevated, thus indicating that endothelin may play an important role in mediating pathophysiological changes that occur during preeclampsia.2,4,9,34–50 Previous studies in the RUPP model have indicated that chronic reductions in placental perfusion are associated with enhanced-production ET-1 and that chronic administration of a selective endothelin A receptor antagonist markedly attenuated the hypertension in RUPP pregnant rats.51–54 In recent studies we have shown that TNF-α directly simulates endothelial cell ET-1 secretion and stimulates hypertension and tissue ET-1 in pregnant rats.32,40,43 In addition, TNF-α–induced hypertension is attenuated with an endothelin A receptor antagonist.40 Interestingly, RUPP rats treated with rituximab not only had suppressed AT1-AA but also exhibited significantly lower circulating TNF-α than did control RUPP rats. We had previously examined the role of endogenous TNF-α to mediate hypertension via ET-1 activation.32 By administering a single injection of etanercept to RUPP rats, both hypertension and tissue ET-1 were suppressed. Furthermore we examined endothelial cell activation in the presence of serum from TNF-α–suppressed RUPP rats and serum from RUPP rats with etanercept exogenously added to the experimental media. In both in vitro studies HUVEC-secreted ET-1 was decreased to levels significantly less than ET-1 secreted in response to control RUPP serum.32 Although these studies demonstrate a role for TNF-mediated increases in ET-1 to mediate hypertension in response to RUPP, they did not address the role of TNF-mediated AT1-AA to cause hypertension in response to placental ischemia. Importantly, we have demonstrated that TNF-α excess is also a stimulus of AT1-AA in pregnant rats but not in virgin rats. This hypertension also was blocked by an angiotensin II type 1 receptor antagonist.42 Conversely, Irani et al55 demonstrated that injection of human AT1-AA stimulated preeclampsia features including elevated TNF-α in pregnant but not virgin mice. Furthermore, coinjection of AT1-AA with a TNF-α neutralizing antibody decreased the bioavailability of the circulating cytokine and attenuated much of the preeclampsia features associated with AT1-AA–induced hypertension in pregnant mice. The authors concluded that AT1-AA is a novel stimulus for the elevation in circulating TNF-α during preeclampsia. Our data presented in this study strongly support these novel findings from the Xia laboratory,55 illustrating that, with B-cell depletion and AT1-AA suppression, TNF-α production is attenuated in response to placental ischemia. Moreover, via B-cell depletion, suppression of AT1-AA and TNF-α completely attenuated ET-1 stimulated from vascular endothelial cells and placental ET-1 transcript in response to placental ischemia. Because anti-CD20 is specific for destruction of B cells, we hypothesize that the mechanism for decreased TNF-α in response to placental ischemia must be secondary to B-cell depletion and suppression of AT1-AA production. Likewise, the converse remains in question. It remains unknown whether AT1-AA production was inhibited in RUPP rats chronically treated with etanercept. However,
these studies were not designed to address these specific questions, but they could be an area of future investigations.

Nevertheless, our data from this study indicate that administration of rituximab not only resulted in B-cell depletion, but also significantly suppressed AT₁-AA levels in RUPP rats (Figure 2). Likewise, arterial pressure and local and cellular ET-1 production in response to RUPP were significantly decreased (Figures 1, 3, and 4). Although these data indicate the importance of endogenous AT₁-AA in mediating hypertension in response to placental ischemia, they do not address the role of mechanisms stimulated in response to placental ischemia to mediate the residual blood pressure effect observed in response to placental ischemia during pregnancy. We have shown previously that hypertension in response to RUPP is associated with increased reactive oxygen species, inflammatory cytokines, antiangiogenic factors sFlt-1 and soluble endoglin, and decreased NO.⁴–⁷,10,29,32,34–40,42–44,51–54 Furthermore, as stated previously, IL-6 is stimulated in response to placental ischemia but remained unaffected by administration of rituximab. In addition, we have shown that AT₁-AA–induced hypertension is a stimulus not only for ET-1 but also oxidative stress, sFlt-1, and soluble endoglin. Future studies using this model of B-cell depletion and AT₁-AA suppression can be used to determine the role of these other factors to mediate the phenotype observed in response to placental ischemia. Moreover, it is unknown whether direct inhibition of the AT₁-AA with the 7 amino acid peptide reduces blood pressure, ET, and reactive oxygen species in RUPP rats. Although a recent Nature Medicine study demonstrated that an antibody neutralizing 7-amino acid epitope peptide was effective in abolishing the blood pressure response to chronic infusion of AT₁-AA in mice, it is unknown whether the antibody neutralizing 7-amino acid epitope peptide attenuates the blood pressure response to placental ischemia. Furthermore, it is unknown whether administration of an antibody neutralizing 7-amino acid epitope peptide would be effective in abolishing the rise in TNF-α in response to placental ischemia. Therefore, additional studies examining the importance of endogenous AT₁-AA to increase blood pressure and activate the ET-1 system, reactive oxygen species, TNF-α, or antiangiogenic factors in RUPP rats are necessary to further our understanding of the pathophysiological ramifications of AT₁-AA production during pregnancy.

Perspectives

In this study we use the technique of B-cell depletion to examine the role of endogenously produced AT₁-AA to mediate hypertension during pregnancy. Administration of rituximab inhibits the release of B cells into circulation and subsequent secretion of antibody and, therefore, allows one to partially examine mechanisms of AT₁-AA production in response to placental ischemia. In this study we learned that, via B-cell depletion and AT₁-AA suppression, the proinflammatory cytokine, TNF-α, was attenuated in the serum of placental ischemic rats. Furthermore, we learned that B-cell–depleted RUPP rats had lower ET-1 and blood pressure response to placental ischemia. The findings could have profound implications on our understanding and future therapies developed for treating women with preeclampsia. Although well-controlled human trials of pregnant women treated with rituximab have yet to be performed, nontherapeutic effects were observed in pregnant monkeys treated with rituximab (manufacturer pamphlet provided with purchased rituximab). Immune functions studies were performed in infants of dams treated with rituximab, and the immediate decrease in B cells and immune function noted in these offspring compared with NP dams returned to normal levels and function within 6 months postbirth. Furthermore, in our study we noted no difference in pup weights of experimental groups compared with controls. Because of innate developmental variations between animals and humans, this and any other therapeutic agent should only be administered during pregnancy if the potential benefit outweighs the potential risk to the mother and infant experiencing a devastating disease.

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Disclosures

None.

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