Enhanced Endothelin Synthesis by Endothelial Cells Exposed to Sera From Pregnant Rats With Decreased Uterine Perfusion

Lyndsay Roberts, B. Babbette D. LaMarca, Lillian Fournier, Jennifer Bain, Kathy Cockrell, Joey P. Granger

Abstract—The initiating event in preeclampsia is thought to be reduced uteroplacental perfusion. Although we have reported previously that chronic reductions in uterine perfusion pressure (RUPP) in pregnant rats results in hypertension and enhanced endothelin production, the factors linking placental ischemia and endothelial cell activation remain unclear. The purpose of this study was to determine the role of angiotensin II type-1 (AT1) receptor activation on endothelin production induced by serum from pregnant rats exposed to reductions in uterine perfusion. To achieve this goal, human umbilical vein endothelial cells were exposed to sera collected from RUPP rats or normal pregnant rats. Arterial pressure was significantly higher in RUPP rats (135±2 mm Hg) than in normal pregnant rats (106±1 mm Hg). Six hours after exposure to RUPP serum (n=17), cell media endothelin concentration was 18.4±2.7 pg/mL as compared with 9.22±1.3 pg/mL from cells exposed to serum from normal pregnant rats (n=9). Eighteen hours after exposure to RUPP serum (n=7), endothelin concentration was 30.5±3.8 pg/mL as compared with 12.8±5.3 pg/mL from cells exposed to normal pregnant rat serum (n=6). In contrast, serum from RUPP rats did not increase endothelin production in human umbilical vein endothelial cells pretreated with an AT1 receptor antagonist, losartan (15 μmol/L). Eighteen hours after exposure to RUPP serum and losartan (n=14), endothelin concentration was 21.3±2.2 pg/mL as compared with 16.4±3.3 pg/mL from cells exposed to normal pregnant rat serum and losartan (n=10). These data indicate that serum from pregnant rats exposed to reductions in uterine perfusion enhances endothelin production by endothelial cells via by AT1 receptor activation. (Hypertension. 2006;47[part 2]:615-618.)

Key Words: pregnancy, hypertension ■ angiotensin II ■ endothelin

The initiating event in preeclampsia is thought to be reduced uteroplacental perfusion, which leads to widespread dysfunction of the maternal vascular endothelium.1–4 The fact that serum from preeclamptic women causes endothelial activation/dysfunction suggests that a circulating factor may be involved.5–8 Circulating factors, such as inflammatory cytokines, vascular endothelial growth factor receptor antagonists (sflt1), and agonistic autoantibodies to the angiotensin II (Ang II) type-1 (AT1) receptor are elevated in preeclamptic women and are proposed to be important links between placental ischemia and endothelial dysfunction.1,9–11 Although we have reported previously that chronic reductions in uterine perfusion pressure (RUPP) in pregnant rats results in hypertension and enhanced endothelin production, the factors linking placental ischemia and enhanced endothelin production in this rat model of preeclampsia remain unclear.12–15 Indeed, it is not known whether serum from pregnant rats with placental ischemia activates endothelial cells. Thus, the first goal of this study was to determine whether serum isolated from pregnant rats with chronic reductions in uterine perfusion enhances endothelin production by endothelial cells.

Agonistic autoantibodies to the AT1 receptor are elevated in preeclamptic women and are proposed to be an important link between placental ischemia and endothelial dysfunction.9 During normal pregnancy, plasma renin activity and Ang II increases. In contrast, plasma renin activity, angiotensinogen, and Ang II concentrations are suppressed in women with preeclampsia.16,17 However, the vascular sensitivity to Ang II is markedly enhanced in preeclamptic women.16,17 Chronic RUPP in pregnant rats result in hypertension that is also associated with enhanced sensitivity to Ang II.18 Moreover, the increase in arterial pressure in RUPP rats is significantly blunted in RUPP rats pretreated with an Ang II receptor antagonist.18 Collectively, these data suggest that AT1 receptor activation may play an important role in mediating the
endothelial dysfunction and hypertension in response to reductions in uterine perfusion in pregnant rats. Although we and others have reported previously that AT₁ receptor activation by Ang II is a potent stimulus for endothelin production, the role of AT₁ receptor activation in mediating the enhanced endothelin production induced by serum from pregnant rats exposed to chronic reductions in uterine perfusion is unknown.¹⁹,²⁰ Thus, the second aim of this study was to determine the role of AT₁ receptor activation on endothelin production induced by serum from pregnant rats exposed to chronic reductions in uterine perfusion.

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 Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all protocols.

Effect of Chronic RUPP on Arterial Pressure
Experiments were performed in the following groups of rats: pregnant controls (n=15) and RUPP pregnant rats (n=24). All of the pregnant rats undergoing surgical procedures were anesthetized with 2% isoflurane (W.A. 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 in detail.³ After a midline incision, the lower abdominal aorta was isolated, and a silver clip (0.230 mm ID) was placed around the aorta above the iliac bifurcation. Branches of both the right and left ovarian arteries were clipped using a silver clip (0.100 mm ID) as described previously.¹⁴–¹⁵ Rats were also surgically instrumented with a carotid catheter for subsequent arterial pressure measurement on day 18. At day 19 of gestation, arterial pressure were recorded, and blood samples were collected.

Effect of Sera From RUPP Rats or Normal Pregnant Rats on Endothelin Production
Cell Culture
Human umbilical venous endothelial cells (HUVEC), 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% CO₂–20% O₂–75% N₂, at 37°C. Seventy percent confluent monolayers were incubated for 48 hours in serum-free medium before exposure to experimental conditions.

Experimental Protocol
Culture medium was removed, and experimental medium containing 1 mL of experimental rat serum and 1 mL of serum-free medium was laid onto the cells for 24 hours. The experimental medium was removed, fresh serum-free medium was added, and cells were cultured for an additional 18 hours. Aliquots of sample were taken after 6 hours and 18 hours. Cells were trypsinized and total protein collected. Tumor necrosis factor (TNF) α (10 ng/mL) was used to determine specificity of Losartan pretreatment to block sera-induced production of endothelin.²¹,²²

Assay Methods
Measurement of Endothelin Concentration
Endothelin was determined using 100 μL of medium 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 intraassay variability of 3.4%.

Isolation of Total Protein
Total protein was isolated and used to standardize immunoassay results. After trypsinization, cells were collected by centrifugation (5 minutes at 2 rpm), washed with 200 μL of Dulbecco’s PBS, and centrifuged. Two hundred microliters of protein lysis buffer were added, and cells were disrupted by vortexing. The lysate was placed on ice for 5 minutes, and cell debris was collected by centrifugation at full speed for 2 minutes. The protein lysate was extracted and placed in a clean tube. Total protein was quantitated using the BCA protein quantitation kit from Pierce.

Statistical Analysis
All of the data are expressed as mean±SEM. Comparisons of control with experimental groups were analyzed by ANOVA. A value of P<0.05 was considered statistically significant.

Results
Mean Arterial Pressure in Response to RUPP in Pregnant Rats
Figure 1 shows that mean arterial pressure was significantly higher (P<0.05) in RUPP rats (135±2 mm Hg) than in pregnant rats (106±1 mm Hg).

Effect of Sera From RUPP Rats or Normal Pregnant Rats on Endothelin Production
Figure 2 illustrates that 6 hours after exposure to RUPP serum (n=17), cell medium endothelin concentration was 18.4±2.7 pg/mL as compared with 9.22±1.3 pg/mL from cells exposed to serum from normal pregnant rats (n=9). Eighteen hours after exposure to RUPP serum (n=7), cell medium endothelin concentration was 30.5±3.8 pg/mL as compared with 12.8±5.3 pg/mL from cells exposed to normal pregnant rat serum (n=6).

Effect of Sera From RUPP Rats or Normal Pregnant Rats on Endothelin Production: Role of AT₁ Receptor Activation
Pretreatment of HUVECs with an AT₁ receptor antagonist, losartan (15 μmol/L), markedly attenuated the increase in
endothelin production induced by serum from RUPP rats (Figure 3). Eighteen hours after exposure to RUPP serum (n = 14), cell medium endothelin concentration was 21.3 ± 2.2 pg/mL as compared with 16.4 ± 3.3 pg/mL from cells exposed to normal pregnant rat serum (n = 10).

TNF-α was used to determine the specificity of losartan pretreatment to block sera-induced production of endothelin. In control cells exposed to 10 ng TNF-α, endothelin cell medium concentration increased by 268%. Losartan pretreatment had no effect on TNF-induced stimulation of endothelin production.

Discussion

Roberts and others1,6,8 were among the first to propose that alterations in endothelial cell function by circulating agent(s) produced by the placenta initiates the clinical syndrome of preeclampsia. Although a number of studies have confirmed that sera from preeclamptic women cause endothelial cell activation, it was unclear whether the presence of these circulating agent(s) in preeclamptic patients was directly related to reductions in uterine perfusion.5–8 Thus, an important finding of the present study is that sera isolated from pregnant rats with chronic RUPP in pregnant rats enhance endothelin production by endothelial cells. Moreover, we report that the enhanced endothelin production induced by sera from pregnant rats exposed to chronic reductions in uterine perfusion is, in part, mediated by AT₁ receptor activation.

Compelling evidence indicates that the endothelin system plays an important role in the pathogenesis of preeclampsia.3,4 Endothelin production is increased in women with preeclampsia.3,4 We have also reported that the hypertension in response to chronic reductions in uteroplacental perfusion pressure in the pregnant rat is associated with significant increases in endothelin production.14 Furthermore, selective blockade of the endothelin type A (ET₁) receptor virtually abolished the hypertension in response to chronic reductions in uteroplacental perfusion pressure in the pregnant rat.14 In the present study, we demonstrate that sera obtained from pregnant rats with chronic RUPP enhanced endothelin production by endothelial cells. Six hours after exposure to RUPP serum, cell medium endothelin concentration was 100% higher than cell medium exposed to sera from normal pregnant rats. Likewise, 18 hours after exposure to RUPP serum, cell medium endothelin concentration was >140% higher than cell medium exposed to normal pregnant rat serum. These data suggests that circulating agent(s) produced in response to placenta ischemia may play a role in stimulating endothelin production in the maternal vasculature. However, we cannot rule out the possibility that RUPP sera may inhibit endothelin metabolism.

Several lines of evidence support the hypothesis that the ischemic placenta contributes to endothelial cell activation/dysfunction of the maternal circulation by enhancing the synthesis of factors, such as inflammatory cytokines, vascular endothelial growth factor receptor antagonists (sflt1), and agonistic autoantibodies to the AT₁ receptor.9–11 However, the relative importance of these factors in linking placenta ischemia with endothelial cell activation/dysfunction and abnormal blood pressure regulation in women with preeclampsia is unclear. In the present study, we focused on the potential role of AT₁ receptor activation in mediating the enhanced endothelin production induced by serum from pregnant rats exposed to chronic reductions in uterine perfusion.

We have reported previously that AT₁ receptor activation by Ang II is a potent stimulus for endothelin production, and ET₁ receptor activation plays a major role in mediating chronic Ang II-induced hypertension in rats.20 To determine the role of AT₁ receptor activation in mediating the enhanced endothelin production induced by serum from pregnant rats exposed to chronic reductions in uterine perfusion, we examined the effect of sera from pregnant rats with chronic reductions in uterine perfusion in the presence of the AT₁ receptor antagonist losartan. AT₁ receptor antagonism had no effect on endothelin concentration of cells exposed to sera from normal pregnant rats. In sharp contrast, AT₁ receptor antagonism significantly reduced endothelin concentration of cells exposed to sera from pregnant rats with chronic reductions in uterine perfusion. This effect of the AT₁ receptor antagonist to inhibit RUPP sera-induced increases in cell medium endothelin concentration does not appear to be a nonspecific effect of Losartan, because the AT₁ receptor antagonist did not attenuate TNF-α–induced increases in endothelin production.
During normal pregnancy, plasma renin activity and Ang II increases, whereas in women with preeclampsia, plasma renin activity, angiotensinogen, and Ang II concentrations are suppressed.\textsuperscript{16,17} However, the vascular sensitivity to Ang II is markedly enhanced in preeclamptic women.\textsuperscript{16,17} Chronic RUPP in pregnant rats result in hypertension that is also associated with normal plasma renin activity but enhanced vascular responsiveness to exogenous Ang II.\textsuperscript{18} Moreover, the increase in arterial pressure in RUPP rats is significantly blunted in RUPP rats pretreated with an Ang II receptor antagonist.\textsuperscript{18} Collectively, these data suggest that AT\textsubscript{1} receptor activation may play an important role in mediating the endothelial dysfunction and hypertension in response to reductions in uterine perfusion in pregnant rats.

Although renin activity and Ang II levels are not elevated in preeclampsia, recent studies have found that the IgG fraction from preeclamptic women contains an angiotensin-I receptor autoantibody that stimulates the AT\textsubscript{1} receptor, suggesting that these antibodies could contribute to the pathogenesis of preeclampsia.\textsuperscript{9} We reported recently that the hypertension in the RUPP rats is associated with significant elevations in AT\textsubscript{1} receptor agonistic antibodies.\textsuperscript{18} Whereas chronic reductions in uterine perfusion in pregnant rats result in a hypertensive state that closely resembles preeclampsia in women, the role of AT\textsubscript{1} receptor autoantibodies in contributing to the hypertension in this rat model of preeclampsia is unknown. Moreover, additional studies will be required to explore the possibility that AT\textsubscript{1} receptor autoantibodies serve as the serum factor that mediates AT\textsubscript{1} receptor–induced endothelin production in endothelial cells.

**Perspectives**

Preeclampsia, which affects 5% to 10% of all pregnancies in the United States, is a multisystemic disorder of pregnancy that is associated with hypertension and endothelial dysfunction.\textsuperscript{2} Despite being one of the leading causes of maternal and perinatal morbidity and mortality, the pathophysiological mechanisms underlying the hypertension during preeclampsia are unknown. The initiating event in preeclampsia is thought to be reduced uteroplacental perfusion, which leads to widespread dysfunction of the maternal vascular endothelium. Although a number of studies have reported that sera from preeclamptic women cause endothelial cell activation, it has been unclear whether the presence of these circulating agent(s) in preeclamptic patients was directly related to reductions in uterine perfusion. In this study, we report that sera isolated from pregnant rats with chronic RUPP enhance endothelin production by endothelial cells. Moreover, we report that the enhanced endothelin production induced by sera from pregnant rats exposed to chronic reductions in uterine perfusion is, in part, mediated by AT\textsubscript{1} receptor activation.

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