1.-Arginine supplementation abolishes the blood pressure and endothelin response to chronic increases in plasma sFlt-1 in pregnant rats

Sydney R. Murphy,1 Babbette LaMarca,2 Kathy Cockrell,1 Marietta Arany,1 and Joey P. Granger1

Departments of 1Physiology and Biophysics and 2Obstetrics and Gynecology, University of Mississippi Medical Center, Jackson, Mississippi

Submitted 21 June 2011; accepted in final form 2 November 2011

PREECLAMPSIA (PE), a pregnancy-specific disease of the maternal vasculature, occurs in 5–10% of pregnancies in the United States (4, 21). Although PE is the most common complication during pregnancy, the mechanisms in the genesis of the disease have yet to be fully elucidated. Reduction in placental perfusion, resulting in placental ischemia and release of soluble placental factors, which act on the maternal endothelium and result in alterations in renal function, proteinuria, and hypertension, is the accepted theory (9, 10, 24, 25, 30). These soluble placental factors are believed to contribute to an imbalance of angiogenic factors (1, 13, 15, 17, 18, 20, 23, 30) weighted toward an antiangiogenic state. This idea is supported by ample data showing that women with PE have increased placental and circulating levels of the antiangiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) and reductions in the proangiogenic factors VEGF and placental growth factor (PIGF) (14, 17). sFlt-1 acts to bind and sequester VEGF and PIGF, leading to an angiogenic imbalance postulated to be involved in the pathogenesis of the disease. In support of this hypothesis are studies showing that adenovirus administration or infusion of sFlt-1 into normal pregnant (NP) rats (8, 18) produces significant hypertension associated with reductions in circulating levels of VEGF, significant proteinuria, and endothelial dysfunction, as marked by a ~3.5-fold increase in renal cortical preproendothelin (prepro-ET) mRNA expression (19). Furthermore, treatment with a selective endothelin (ET) type A (ETa) receptor antagonist abolished the hypertension, suggesting that the enhanced ET-1 production may play an important role in mediating the elevations in mean arterial pressure (MAP) during PE, via ETa receptor activation.

Several lines of evidence suggest that the vascular effects of VEGF on vascular tone and angiogenesis are related to nitric oxide (NO). VEGF is a known proangiogenic factor that stimulates NO production and endothelial NO synthase (NOS) protein levels (22). Data from Sandrim et al. (27) indicate that women with PE have reduced plasma and whole blood nitrite levels, which negatively correlate with the potent VEGF inhibitor sFlt-1. In addition, chronic inhibition of NOS results in significant elevations in blood pressure, alterations in renal hemodynamics, and glomerular damage (11) similar to the effects noted in patients with PE. More recently, Facemire et al. (7) demonstrated that inhibition of VEGF receptor (VEGFR) type 2 (VEGFR2) results in hypertension through impaired NO synthesis, suggesting that inhibition of VEGF leads to a reduced NO synthesis. Furthermore, chronic NOS inhibition in pregnant rats has been shown to increase plasma levels of ET-1 (7). However, whether this angiogenic imbalance reduces NO bioavailability and contributes to the enhanced ET-1 production and elevations in blood pressure in response to sFlt-1-induced hypertension in pregnant rats remains unclear. Therefore, the purpose of this study was to determine if a reduction in NO levels stimulates the enhanced ET-1 production and mediates the hypertension in response to sFlt-1 infusion into pregnant rats. To achieve this goal, we examined the blood pressure responses to chronic NOS inhibition in NP and sFlt-1 hypertensive pregnant rats. We also compared prepro-ET mRNA expression in control and experimental groups supplemented with the NO substrate L-Arg.

METHODS

Timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in a temperature-controlled (23°C) room with a 12:12-h light-dark cycle. All experimental procedures were carried out in...
accordance with National Institutes of Health guidelines for the use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Chronic infusion of sFlt-1 into NP rats. NP rats were placed on a low nitrite/nitrate diet (custom AIN-76 diet, 0.8% NaCl and 0.5% KCl) beginning on day 10 of gestation. sFlt-1 (recombinant mouse VEGFR type 1/Flt-1Fc chimera) was infused at a rate of 3.7 
\[\mu g \cdot kg^{-1} \cdot day^{-1}\] for 6 days (in sterile saline) beginning on day 13 of gestation via osmotic minipump (model 2001, Alzet Scientific, Palo Alto, CA) into NP rats. This infusion rate has been shown to increase plasma sFlt-1 concentrations approximately threefold and decrease free VEGF by 30% (8), comparable to levels observed in the reduced uterine perfusion pressure animal model of pregnancy-induced hypertension. NP control rats were fitted with a vehicle-filled osmotic minipump. To determine whether NO production was reduced, blood pressure responses were measured in NP and sFlt-1 hypertensive pregnant rats treated with the NOS inhibitor N\textsuperscript{3}-nitro-L-arginine methyl ester (t-NAME, 100 mg/l for 4 days in drinking water) beginning on day 15 of gestation. On day 17 of gestation, animals were placed in metabolic cages for 24-h urine collection. Rats were also surgically instrumented with arterial catheters for arterial pressure measurements on day 18. On day 19 of gestation, MAP was measured, blood samples were collected, kidneys, placentas, and aortas were harvested, and litter size and pup weights were recorded.

Measurement of arterial pressure in chronically instrumented conscious rats. Arterial pressure was determined in all groups of rats on day 19 of gestation. Pregnant rats were catheterized on day 18 under a short-acting anesthetic, with isoflurane delivered by an anesthesia apparatus. A catheter (V-3 tubing, SCI, Lake Havasu City, AZ) 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 CDX III transducer, Sema) and recorded continuously for 20-min periods after 30 min of stabilization. Rats were anesthetized using isoflurane delivered by an anesthesia apparatus for blood and tissue collection.

Determination of plasma sFlt-1 levels. Circulating sFlt-1 concentrations were measured using a commercial ELISA kit (Quantikine, R & D Systems) according to the manufacturer’s directions. The assay displayed a sensitivity level of 9.8 pg/ml and inter- and intra-assay variations of <10%.

Isolation of glomeruli. Kidneys were hemisected, and the cortex was isolated from the medulla. The cortex was gently homogenized with rough chopping and pressed through a 150-\(\mu\)m stainless steel sieve. The material passing through the sieve was washed with chilled HBSS. The procedure was repeated using a 100- and a 75-\(\mu\)m sieve. The glomeruli were collected by washing the underside of the 75-\(\mu\)m sieve into a sterile petri dish. Glomeruli were then transferred to a glass test tube and allowed to settle for 5–10 min. The supernatant was removed, and the glomeruli were resuspended in 1 ml of HBSS.

Determination of renal cortical prepro-ET mRNA levels. Isolated glomeruli were incubated for 30 min on ice with 4 \(\mu\)M diaminofluorescein in HBSS. NO production was determined as the percent change in fluorescence from isolated glomeruli after 1 \(\mu\)M L-Arg was achieved in the chamber solution. An average of 10 individual glomeruli from each rat were studied.

Determination of renal cortical prepro-ET mRNA levels. Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen) after the cortex was crushed in liquid nitrogen with a mortar and pestle. The isolation procedure was then performed as outlined in the instructions provided by the manufacturer. Genomic DNA was digested with DNase I following instructions outlined by Ambion. RNA was quantified spectrophotometrically using an Eppendorf BioPhotometer. cDNA was synthesized from 5 \(\mu\)g of RNA with SuperScript II reverse transcriptase (Invitrogen) using the following primers (supplied by custom primers from Invitrogen): prepro-ET forward 1 (CTAGGTCTAAGCGATCTCTTG) and prepro-ET reverse 1 (TCTTTGTCTGCTTGGG). Real-time PCR was performed using the Bio-Rad SYBR Green Supermix and iCycler using a nested forward primer prepro-ET forward 2 (CTAGGTCTAAGCGATCTCTTG) and the reverse primer outlined above. A RT-PCR primer control kit (Invitrogen) was used to amplify \(\beta\)-actin transcripts as control. Levels of mRNA expression were calculated using the mathematical formulas for the cycle threshold (\(\Delta\Delta Ct\)) method recommended by Applied Biosystems (Applied Biosystems User Bulletin No. 2, 1997).

Statistical analysis. Values are means \(\pm\) SE. Differences between control and experimental groups were analyzed using unpaired t-tests. Blood pressure comparisons for multigroup and multifactorial analyses were performed using ANOVA with Tukey’s post hoc test. Data were considered statistically different at \(P < 0.05\).

RESULTS

Plasma sFlt-1 in NP and sFlt-1-infused pregnant rats. Infusion of sFlt-1 into NP rats at a rate of 3.7 \(\mu g \cdot kg^{-1} \cdot day^{-1}\) (5 days) via osmotic minipump results in a threefold elevation in plasma levels of sFlt-1 compared with NP rats: 2,807 \(\pm\) 671 vs. 735 \(\pm\) 34 pg/ml (\(P < 0.05\); Fig. 1). In association with the increased plasma levels of sFlt-1, MAP was increased significantly (116 \(\pm\) 2 vs. 103 \(\pm\) 1 mmHg, \(P < 0.05\)).

Effect of chronic NOS inhibition on blood pressure in NP and sFlt-1 hypertensive pregnant rats. Chronic NOS inhibition with t-NAME (100 mg/l in drinking water) significantly increased MAP (\(\sim 26\) mmHg) in NP pregnant rats (\(P < 0.05\); Fig. 2). The blood pressure response to t-NAME increased sixfold and was therefore attenuated in sFlt-1 hypertensive pregnant rats compared with untreated controls (129 \(\pm\) 4 vs. 122 \(\pm\) 3 mmHg).

![Fig. 1. Plasma levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and mean arterial pressure (MAP) in normal pregnant (NP) and sFlt-1 hypertensive pregnant (NP + sFlt-1) rats. MAP significantly increased in response to sFlt-1 infused for 5 days via osmotic minipump (3.7 \(\mu g \cdot kg^{-1} \cdot day^{-1}\)). Values are means \(\pm\) SE.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00319.2011)
NO production from isolated glomeruli of NP and sFlt-1 hypertensive pregnant rats. Figure 3 presents the change in fluorescence, indicating an increase in glomerular NO production, from isolated glomeruli from NP and sFlt-1 hypertensive pregnant rats in the presence and absence of NOS inhibition with L-NAME (1 μM). Glomeruli isolated from NP rats showed an increase in NO production, as indicated by an increase in fluorescence over a 10-min period after incubation with L-Arg. Glomeruli from sFlt-1 hypertensive pregnant rats showed a 70% reduction in NO production. Treatment with L-NAME significantly reduced glomerular NO production in glomeruli from NP rats to a level similar to that in glomeruli from sFlt-1-infused rats. No alterations in NO production were noted in glomeruli isolated from sFlt-1 hypertensive pregnant rats treated with L-NAME.

MAP in NP and sFlt-1 hypertensive pregnant rats supplemented with 2% L-Arg. Supplementation with 2% L-Arg in drinking water had no effect on the blood pressure response in NP rats (109 ± 5 mmHg) but significantly reduced MAP in sFlt-1 hypertensive pregnant rats (109 ± 3 mmHg, P < 0.05) compared with untreated controls (103 ± 1 vs. 117 ± 2 mmHg, P < 0.05; Fig. 4).

Prepro-ET measurements in cortex of NP and sFlt-1 hypertensive pregnant rats. As previously reported by our laboratory (19), prepro-ET mRNA expression was increased ~3.5-fold (P < 0.05) in the renal cortex of sFlt-1 hypertensive pregnant rats (Fig. 5). Supplementation with L-Arg abolished the sFlt-1-induced enhanced prepro-ET mRNA expression (P < 0.05).

DISCUSSION

PE, or pregnancy-induced hypertension, results in a state of nonhomeostasis of the maternal vascular endothelial lining (9, 10, 26). However, the exact mechanisms leading to the hypertension has remained elusive. Recent data suggest an angiogenic imbalance in the etiology of the disease (1, 13, 15, 17, 18, 20, 23, 30). Fivefold higher placental and circulating levels of sFlt-1 in women with PE than in normotensive pregnant women (17, 28) are associated with reductions in circulating VEGF and PI GF (15, 17, 30). However, the specific mechanisms linking increased sFlt-1 to hypertension are unclear. In a recent study, our laboratory found renal cortical prepro-ET mRNA expression to be elevated ~3.5-fold in response to sFlt-1-induced hypertension in pregnant rats (19). Moreover, treatment with a selective ETA receptor antagonist significantly reduced the hypertension, suggesting that the enhanced ET-1 production in response to chronic increases in plasma levels of sFlt-1 may, via ETA receptor activation, mediate the hypertension in this model. However, the mechanisms by which increases in plasma sFlt-1 lead to an increased ET-1 production are not known. Data suggest that the mitogenic and proliferative actions of VEGF on the endothelium are NO-mediated (31); therefore, a reduction in bioavailable NO may play an important role in the hypertensive response to infusion of sFlt-1 into NP rats. Thus, in the present study, we investigated...
the hypothesis that reductions of NO, due to increased plasma levels of sFlt-1, lead to an enhanced ET-1 production. Furthermore, we hypothesized that supplementation with L-Arg, a substrate for NO production, may reduce the enhanced ET-1 production.

To test this hypothesis, NO production was determined in response to chronic increases in plasma sFlt-1. Thus, NP and sFlt-1 hypertensive pregnant rats were treated with the NOS inhibitor l-NAME (100 mg/l po for 4 days). Upon chronic NO inhibition, the pressure response to sFlt-1 infusion was significantly attenuated. The lack of a pressure increase in response to treatment with l-NAME in sFlt-1 hypertensive pregnant rats may be attributed to a reduction in NO synthesis or a decrease in NO bioavailability. Recently, Facemire and colleagues (7) showed that treatment with a specific antibody aimed at VEGFR2 increased blood pressure and reduced renal endothelial and neuronal NOS expression. Furthermore, l-NAME administration abolished the difference in blood pressure between the vehicle- and anti-VEGFR2-treated groups. Utilizing a diaminofluorescein method to stain isolated glomeruli, we determined that NO production was significantly reduced ~70% in sFlt-1 hypertensive pregnant rats compared with NP rats. In addition, pretreatment with l-NAME inhibited NO production in NP rats to a level similar to that in glomeruli from sFlt-1 hypertensive pregnant rats. Since VEGF is known to promote fenestration and endothelial cell survival in the adult (16), it can be suggested that the antiangiogenic targeting of VEGF may have a greater effect in tissue beds with a higher VEGFR density, such as the choroid plexus of the brain, the liver, or the glomerulus of the kidney. Therefore, depressed NO production within the glomerulus may have a greater physiological impact in mediating the through reductions in hemodynamics in response to increased plasma levels of sFlt-1 in pregnant rats.

A role of a reduced NO synthesis in PE is further supported by data showing that L-Arg supplementation reduces the hypertension in response to placental ischemia in pregnant rats and in pregnant women (3, 29). Our study shows that L-Arg supplementation attenuates the blood pressure response and abolishes the sFlt-1-enhanced prepro-ET mRNA expression in response to sFlt-1 infusion in pregnant rats. These data are consistent with previous studies suggesting a link between a reduced NO production and an enhanced ET-1 production (6, 12). In summary, we found that L-Arg supplementation attenuates the sFlt-1-induced hypertension by mechanisms that are likely involved in the suppression of ET-1 production, suggesting that reductions in NO may possibly be the driving force behind the enhanced ET-1 production.

**Perspectives and Significance**

PE, which affects 5–10% of all pregnancies in the United States, is a multisystemic disorder of pregnancy that is associated with hypertension, proteinuria, and endothelial dysfunction. Although PE is one of the leading causes of maternal and perinatal morbidity and mortality, the pathophysiological mechanisms underlying the hypertension during PE are unknown. While sFlt-1, an endogenous antagonist of VEGF and PGF, has been identified in the etiology of PE, the mechanisms whereby enhanced sFlt-1 production leads to endothelial dysfunction, enhanced ET-1 production, and hypertension remain unclear. However, previous data from our laboratory have shown that reductions in uterine perfusion pressure during pregnancy significantly increase circulating levels of sFlt-1, decrease VEGF (8), reduce glomerular filtration rate (2), and lead to alterations in vascular function (5). However, it remains enigmatic as to how this antiangiogenic state leads to elevations in MAP and PE. On the basis of previous data from our laboratory, supplementation of the reduced uterine perfusion pressure rats model of PE with exogenous VEGF lowers blood pressure and improves renal function in a dose-dependent manner. Therefore, we hypothesize that an increase in circulating sFlt-1 levels leads to reductions in VEGF and a reduction in NO production through a decrease in NO synthesis and activity. Furthermore, increases in oxidative stress may contribute further to decreased NO bioavailability and vascular damage. In addition, increases in circulating sFlt-1 lead to enhanced ET-1 production and elevations in MAP via ETA receptor activation within the renal cortex. Supplementation with L-Arg during pregnancy has been shown to have beneficial effects (29), with few to no known adverse effects and, therefore, should be considered for therapeutic administration to prevent PE.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant HL-51971.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

S.R.M. and J.P.G. are responsible for conception and design of the research; S.R.M., K.C., and M.A. performed the experiments; S.R.M. analyzed the data; S.R.M., B.L., and J.P.G. interpreted the results of the experiments; S.R.M. prepared the figures; S.R.M. drafted the manuscript; S.R.M., B.L., and J.P.G. edited and revised the manuscript; J.P.G. approved the final version of the manuscript.

**REFERENCES**