Hypertension Produced by Reduced Uterine Perfusion in Pregnant Rats Is Associated With Increased Soluble Fms-Like Tyrosine Kinase-1 Expression

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Abstract—The balance between proangiogenic and antiangiogenic factors, such as vascular endothelial growth factor, placental growth factor, and soluble fms-like tyrosine kinase-1 (sFlt-1), is altered in preeclampsia, and this dysregulation of angiogenic factors may be important in the pathogenesis of preeclampsia. Although sFlt-1 is elevated in preeclampsia, the mechanisms responsible for increasing this antiangiogenic factor remain unclear. We hypothesized that the hypertension produced by reduced uterine perfusion pressure (RUPP) is associated with increased sFlt-1 expression and decreased plasma vascular endothelial growth factor and placental growth factor concentrations in the pregnant rat. Arterial pressure was increased (130±3 versus 100±2 mm Hg; P<0.01) in the RUPP rats compared with the normal pregnant control rats. Plasma sFlt-1 concentration (660±270 versus 82±26 pg/mL; P<0.05) was increased, whereas plasma free placental growth factor (0.28±0.05 versus 1.7±0.5 pg/mL; P<0.01) and vascular endothelial growth factor (594±34 versus 830±33 pg/mL; P<0.01) concentrations were decreased in the RUPP rats compared with normal pregnant rats. Plasma sFlt-1:placental growth factor (37.2±7.8 versus 8.9±1.6; P<0.02) and sFlt-1:vascular endothelial growth factor (0.86±0.22 versus 0.28±0.06; P<0.05) ratios were increased in the RUPP rats compared with normal pregnant rats. Immunoreactive placental sFlt-1 was increased (1.1±0.1 versus 0.3±0.1; P<0.01) in RUPP rats contrasted with the normal pregnant rats. These findings support our hypothesis that RUPP increases the expression of sFlt-1 and alters the balance of angiogenic factors in the maternal circulation. These data also indicate that the RUPP model of pregnancy-induced hypertension may provide an invaluable model for mechanistic studies into the role of sFlt-1 in the pathogenesis preeclampsia. (Hypertension. 2007;50:1142-1147.)

Key Words: preeclampsia ■ gestation ■ VEGF ■ blood pressure ■ angiogenic

Pregnancy-induced hypertension (PIH), or preeclampsia, is a major obstetric problem and a significant source of maternal and neonatal morbidity and mortality.1 Although PIH continues to affect ≈8% of all pregnancies, the incidence of preeclampsia has seen a 40% increase in recent years.2 Although PIH has been well characterized, with many studies indicating that proteinuria, edema, endothelial cell dysfunction, and insufficient placentation are all hallmarks of this disorder,3,4 the mechanisms underlying the pathogenesis of this dread condition remain obscure. The continuing uncertainties regarding the mechanisms underlying the pathogenesis of preeclampsia are at least in part attributable to the difficulties in performing mechanistic studies in pregnant women.5,6 Thus, the continued characterization and development of animal models for mechanistic research of preeclampsia remains an important endeavor.

Recent studies have reported the existence of an imbalance between proangiogenic and antiangiogenic factors, such as vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and soluble fms-like tyrosine kinase-1 (sFlt-1) in preeclampsia, and these authors have suggested that this dysregulation of angiogenic factors may be important in the pathogenesis of preeclampsia.7–12 In an elegantly designed study reported several years ago, Maynard et al12 reported that exogenous administration of sFlt-1 into pregnant rats via adenovirus-mediated gene transfer resulted in increased arterial pressure and proteinuria and decreased plasma-free VEGF and PIGF concentrations similar to that observed in the preeclamptic patients. Although plasma concentrations of sFlt-1 are increased in preeclamptic patients,7–12 further studies have demonstrated increased amniotic fluid sFlt-1 concentrations and elevated sFlt-1 mRNA in the placenta of preeclamptic women, as well.10,12,13

Although recent data suggest that circulating sFlt-1 concentrations may presage the clinical onset of PIH symptoms,7,14,15 several studies indicate that placental hypoxia and
poor placental perfusion may initiate this imbalance of angiogenic factors. Nevertheless, it remains unclear whether impaired placental perfusion initiates increased sFlt-1, which, in turn, causes endothelial dysfunction resulting in preeclamptic signs, such as hypertension, or whether a pathological rise in sFlt-1 expression and secretion generates inadequate placental development, which is ensued by the classical signs of preeclampasia (endothelial dysfunction, high blood pressure, and proteinuria).9 A recent study has shed some light on the matter and reported that uteroplacental ischemia increases blood pressure and sFlt-1 in the baboon, but these authors did not demonstrate any decrease in circulating VEGF or PI GF in their model.17

Thus, the purpose of the present study was to test the hypothesis that the reduced uterine perfusion pressure (RUPP) in the pregnant rat leads to hypertension and increased plasma and amniotic fluid concentrations and placental expression of sFlt-1, which, in turn, lead to decreased plasma concentrations of PI GF and VEGF. To this end, we used our established model of PIH in which chronic reductions of uterine perfusion pressure lead to endothelial dysfunction and hypertension in the pregnant rat.

Methods

Animals

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 of the experimental procedures executed in this study were in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All of the protocols were approved by the institutional animal care and use committee at the University of Mississippi Medical Center. On day 14 of gestation, rat dams were randomly assigned to either RUPP (n = 18) or normal pregnant (NP; n = 18) control groups.

RUPP Procedure

The RUPP procedure is a well-established model for studying the link between placental ischemia and hypertension in the pregnant rat and has been described in detail previously.5,18–23 In brief, all of the rats undergoing surgical procedures under isoflurane anesthesia (Webster) delivered by an anesthesia apparatus (Vaporizer for Forane Anesthetic, Ohio Medical Products). Eighteen pregnant rats were randomly assigned to either the NP control or the RUPP group. Pregnant rats entering the RUPP group underwent the following clipping procedure at 14 days of gestational age. After a midline incision, the lower abdominal aorta was isolated, and a silver clip (0.100-mm ID) was placed around the aorta above the iliac bifurcation. Silver clips (0.100-mm ID) were also placed on branches of the right and left ovarian arteries that supply the uterus, whereas NP dams underwent a sham procedure. When the clipping procedure resulted in total reabsorption of the fetuses, rats were excluded from data analyses.

Measurement of Mean Arterial Pressure in Chronically Instrumented Conscious Rats

Animals were instrumented, and arterial pressure was determined in both groups of rats at day 19 of gestation as described previously.23 Briefly, on day 18 of gestation, rats were instrumented with carotid catheters of V-3 tubing (SCI) while under isoflurane anesthesia (Webster) delivered by an anesthesia apparatus (Vaporizer for Forane Anesthetic). Catheters were tunneled to the back of the neck and exteriorized after implantation. On day 19 of gestation, rat dams were placed in individual restraining cages for arterial pressure measurements using a pressure transducer (Cobe III Transducer CDX Sema). Mean arterial pressure (MAP) was recorded continually for a 2-hour period after 1 hour of stabilization.

Conceputus Measurements

After the measurement of MAP, the dams were placed under isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for plasma and serum collection. The uterus was exteriorized, the number of viable and resorbed pups was counted and recorded, and the pups and placentae were excised, blotted dry, and weighed. The heaviest and the lightest placentae were collected from each uterine horn, snap frozen in liquid nitrogen, and stored at −80°C until further analyses were performed.

Plasma/Serum/Amniotic Fluid Assays

Blood was collected for subsequent assays into Corvac sterile serum separator tubes (Sherwood Davis) and plasma into BD Vacutainer EDTA containing tubes. Amniotic fluid was aspirated from each conceptus into a syringe with a 23-gauge needle and pooled per dam in a prechilled test tube on ice. Circulating VEGF, sFlt-1, and PI GF concentrations were measured using commercial ELISA kits available from R&D Systems (Quantikine) according to the manufacturer’s directions. To enhance the detection of PI GF plasma, samples were lyophilized to create a 2-fold increase in concentration before executing the assay. All 3 of the assays were validated for use in the rat by performing spike and recovery experiments in rat plasma and serum. Spike and recovery experiments were performed in triplicate in RUPP (n = 4), NP (n = 4), and nonpregnant (n = 3) rat plasma and revealed >90% recovery for sFlt-1, VEGF, and PI GF. Furthermore, the intra-assay and interassay variations (n = 6) for all of the assays were <8% and 12%, respectively.

Western Immunoblot

One placenta per pregnant rat was selected from 9 RUPP and 9 NP dams that were selected randomly from the study cohort. Total soluble protein was extracted in radioimmunoprecipitation assay lysis buffer containing PMSF in dimethyl sulfoxide, sodiumorthovanadate, and a protease inhibitor mixture (Santa Cruz Biotechnology, Inc). Total soluble cellular protein concentration was determined using the bicinchoninic acid method (Pierce Biotechnology). Protein (100 μg) was separated by electrophoresis on 4% to 20% sodium dodecyl sulfate polyacrylamide separating gels (ISC BioExpress) and then transferred to PVDF membranes (Amersham International) and Ponceau stained to assure an even transfer across each gel. The images of the Ponceau-stained membranes were digitized with a flatbed scanner (Hewlett Packard). Gels were also stained in Gel Code Blue (Pierce Biotechnology) to demonstrate thorough transfer of protein from the gel to the membrane.

The membranes were then incubated for 1 hour in Li-COR blocking solution (Li-COR, Inc). Membranes were incubated in Li-COR blocking solution containing a commercially available antibody for sFlt-1 (1:500; AF471, R&D Systems) overnight at 4°C. Membranes were washed and incubated with the appropriate secondary antibodies that fluoresce at 700 and 800 nm (IRDye conjugated affinity-purified antibodies, Rockland Inc). The immunoreactive bands were digitized using the LI-COR Odyssey Infrared Imaging System (Li-COR, Inc). All of the digitized images were quantified using Un-Scan-It gel 6.1 software (Silk Scientific). sFlt-1 was expressed as a ratio to the predominant band observed on the Ponceau stain (≈67 kDa corresponding with albumin) to control for possible variation in loading between samples. The identity of the reported band was confirmed in initial experiments using recombinant sFlt-1 (R&D systems) as a positive control. Western blot immunonoassays were performed in triplicate.

Statistical Analysis and Calculations

All of the data are presented as mean ± SEM, and statistical significance was accepted when P < 0.05. Conceptus data were calculated as the mean per pregnancy, sFlt:PI GF and sFlt-1:VEGF ratio data were log transformed to obtain normal distributions for subsequent statistical analysis. Comparisons between the 2 groups
cohort, the 11% decrease observed presently is similar to our previous findings.23 The number of implantation sites in the uteri was not different between the RUPP and NP groups (15±1 versus 15±1). The number of fetuses at 19 days of gestational age was lower in the RUPP pregnancies compared with the NP pregnancies (6±1 versus 15±1; P<0.05).

**Plasma, Placental, and Amniotic Fluid Angiogenic Factors**

As shown in Figure 1A, plasma sFlt-1 was increased (660±270 versus 82±26 pg/mL; P=0.05) in the RUPP compared with the NP dams at 19 days of gestational age. Similarly, the concentration of sFlt-1 was increased 10% (5800±160 versus 5200±130 pg/mL; P<0.03) in the amniotic fluid of the RUPP rats compared with the NP controls. Figure 1B illustrates that immunoreactive placental sFlt-1, expressed as the ratio of sFlt-1:67-kDa protein, was increased 4-fold (1.1±0.1 versus 0.3±0.1; P<0.01) in the RUPP dams compared with the NP controls.

Figure 2A illustrates the positive correlation observed between plasma sFlt-1 and MAP (r=0.52; P<0.05), whereas Figure 2B illustrates the negative correlation between VEGF and MAP (r=−0.42; P<0.05). Plasma PI GF concentration was decreased 84% (P<0.01; Figure 3A) in the RUPP compared with NP dams. The sFlt-1:PIGF ratio was increased (P<0.02; Figure 3B) in the RUPP compared with the NP dams. Similarly, Figure 4 shows that plasma-free VEGF was decreased (P<0.01; Figure 4A) and the sFlt-1:VEGF ratio was increased (P<0.05; Figure 4B) in the RUPP versus the NP control dams.

**Discussion**

The present study reveals several interesting and novel findings regarding the relationship between RUPP and the increased expression of sFlt-1 in the late-gestation pregnant rat. Foremost, we report that circulating sFlt-1 concentration is increased in the plasma and amniotic fluid of RUPP dams compared with NP controls. Furthermore, we also demonstrate that immunoreactive sFlt-1 is increased in the placenta of the RUPP dams when contrasted with the NP dams. Lastly, we found that plasma-free VEGF and free PI GF are decreased and the sFlt-1:VEGF and sFlt-1:PIGF ratios are increased in the RUPP dams compared with NP controls. Thus, the present study is the first to report elevated plasma sFlt-1 concentrations and increased placental sFlt-1 expression in a robust, reproducible, and well-characterized animal model of placental ischemia-induced hypertension.
Our results show that maternal plasma and amniotic fluid concentrations of sFlt-1 were increased along with increased levels of immunoreactive sFlt-1 in the placenta. Although the circulating concentrations of sFlt-1 that we observed in the RUPP rat model of preeclampsia are not as high as those reported in preeclamptic humans, the fold increase reported previously between NP-mild preeclamptic (2-fold increase) and NP-severe preeclamptic (4-fold increase) is quite similar to what we report in the present work (3-fold increase). Furthermore, these findings are in agreement with a previous study that reported an increased amniotic fluid concentration of sFlt-1 in preeclamptic patients. Likewise, the present findings, together with our previous studies demonstrating renal and endothelial dysfunction, are in accordance with many studies in humans reporting elevated plasma sFlt-1 concentrations in preeclamptic patients.

These data demonstrate that RUPP is a stimulus for placental production and secretion of sFlt-1. Indeed, previous authors have suggested that the primary source of circulating sFlt-1 in preeclamptic humans derives from the uteroplacental unit. Our findings are also in agreement with a similar study performed recently in the baboon, which used a model of reduced uteroplacental perfusion pressure and reported increased circulating sFlt-1 concentrations, along with increased sFlt-1 mRNA in the placenta. Viewed together, these studies suggest that the increase in circulating sFlt-1 observed in preeclampsia may be a consequence of aberrant placental perfusion.

In accord with our previous findings, we found that MAP was increased and fetal weight was decreased in the present cohort of RUPP dams. These findings reiterate the robust nature of this model of placental ischemia. Moreover, these data share strong similarities with the observations reported in preeclamptic women indicating hypertension and intrauterine growth restriction. We also show that plasma-free VEGF and free PlGF are decreased in the RUPP dams compared with the NP controls. Both of these observations are in agreement with the data reported previously by Maynard et al in humans with preeclampsia. Furthermore, it appears from these findings and those described above that decreased free VEGF and PlGF are a result of increased circulating concentrations of sFlt-1.

In contrast with the work reported by Maynard et al using sFlt-1 delivered via an adenovirus, we report in the present study that RUPP-induced sFlt-1 elevation is associated with growth restriction in the fetus. Interestingly, these findings are in agreement with a recent report using sFlt-1 overexpression in pregnant mice, which reports preeclamptic-like symptoms and growth-restricted fetuses. Viewed together, it remains unclear to what extent increased concentrations of sFlt-1 might contribute to the restricted growth often observed in preeclamptic pregnancies.

Hypoxia has been proposed to have an important role in the regulation of sFlt-1 in the placenta. Our findings that a focal reduction in uterine perfusion pressure and reduced uteroplacental blood flow result in increased placental and plasma sFlt-1 are consistent with this hypothesis. Alternatively, a recent study by Bahtiyar et al reported that NO inhibition with N-nitro-L-arginine methyl ester during pregnancy increased plasma sFlt-1 concentrations, whereas hypoxia alone did not increase sFlt-1 concentrations. Interestingly, these authors reported that, despite the increased sFlt-1 observed in the N-nitro-L-arginine methyl ester–treated animals, there was no concomitant decrease in VEGF, whereas only severe hypoxia resulted in decreased PlGF concentrations. In contrast, the present findings show that the increased sFlt-1 observed in the RUPP dams was sufficient to reduce circulating concentrations of free plasma VEGF and PlGF, much like what has been reported in the human literature regarding preeclampsia. Moreover, in contrast to the findings reported in our previous studies, we found that MAP was increased and fetal weight was decreased in the present cohort of RUPP dams. These findings reiterate the robust nature of this model of placental ischemia. Moreover, these data share strong similarities with the observations reported in preeclamptic women indicating hypertension and intrauterine growth restriction. We also show that plasma-free VEGF and free PlGF are decreased in the RUPP dams compared with the NP controls. Both of these observations are in agreement with the data reported previously by Maynard et al in humans with preeclampsia. Furthermore, it appears from these findings and those described above that decreased free VEGF and PlGF are a result of increased circulating concentrations of sFlt-1.

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**Figure 3.** Plasma-free PI GF was decreased (A), whereas the plasma sFlt-1:PI GF ratio was increased (B) in RUPP pregnant rats (n=18) compared with NP controls (n=18) at day 19 of pregnancy. Data are expressed as mean±SEM; *P<0.05.

**Figure 4.** Plasma-free VEGF was decreased (A), whereas the plasma sFlt-1:VEGF ratio was increased (B) in RUPP pregnant rats (n=18) compared with NP controls (n=18) at day 19 of pregnancy. Data are expressed as mean±SEM; *P<0.05.
by Bahtiyar et al., it appears that there may be several factors that could be responsible for the discordance between the findings. One such difference is in the type of hypoxia in the 2 models as first described by Kingdom and Kaufmann. The study by Bahtiyar et al. discusses preplacental hypoxia rather than the uteroplacental hypoxia observed in the present study and in preeclampsia. Furthermore, there may be other factors in addition to decreased NO and hypoxia in the RUPP model that may act in concert to stimulate sFlt-1 overexpression and result in decreased VEGF and PIGF reported in the present study. Although the identity of these moieties remains unclear at the present, previous and ongoing work in our laboratory suggest that hypoxia-inducible factor-1α, cytokines such as tumor necrosis factor-α, and the angiotensin II receptor subtype 1 autoantibody are all likely candidates to potentiate the effects of placental ischemia on sFlt-1 expression and secretion in the RUPP model of preeclampsia.

Perspectives
Although there is increasing evidence supporting a role for antiangiogenic factors in the pathogenesis of preeclampsia, the sequence of events leading to the increase of these factors has remained unclear. The present study, which relies on data gathered by using a well-characterized and robust animal model of preeclampsia, provides further evidence that placental ischemia is a primary factor in the pathogenesis of preeclampsia by initiating the generation and secretion of sFlt-1 from the placenta. Although the present study does not shed light on the mechanisms underlying the production and secretion of sFlt-1 as a result of placental ischemia, there are several intriguing possibilities for molecules that mediate this pathway. Hypoxia-inducible factor-1α appears to be a likely candidate, because it is a central mediator of responses to hypoxia. Furthermore, the renin-angiotensin system, either through angiotensin II or angiotensin II receptor type 1 autoantibody, may also be a contributing factor, although this appears to be more contentious based on recent findings in the human literature. Alternatively, the present work suggests that VEGF supplementation may hold potential as a possible intervention in preeclamptic pregnancies. Lastly, we feel that the present work provides a platform from which other studies may be launched to elucidate underlying mechanisms and to develop efficacious interventions for preeclampsia.

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Disclosures
None.

References


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