Hypertension Produced by Placental Ischemia in Pregnant Rats Is Associated With Increased Soluble Endoglin Expression

Jeffrey S. Gilbert, Sara A.B. Gilbert, Marietta Arany, Joey P. Granger

Abstract—Recent clinical studies indicate that an excess of angiostatic factors, such as soluble endoglin (sEng), is related to the occurrence of preeclampsia. Although recent clinical studies report that sEng is increased in preeclamptic women, the mechanisms underlying its overexpression remain unclear. Evidence suggests that hypoxia and induction of heme oxygenase-1 have opposing effects on sEng expression, the former stimulatory and the latter inhibitory. Hence, we hypothesized that placental ischemia because of reduced uterine perfusion pressure (RUPP) in the pregnant rat would increase sEng expression and decrease heme oxygenase-1. Mean arterial pressure was obtained via arterial catheter, and serum and placental proteins were measured by Western blot. Mean arterial pressure was increased (132 ± 3 mm Hg versus 102 ± 2 mm Hg; P < 0.001), and fetal (2.35 ± 0.05 g versus 1.76 ± 0.08 g; P < 0.001) and placental weight were decreased (0.47 ± 0.04 g versus 0.58 ± 0.03 g; P < 0.01) in the RUPP compared with normal pregnant controls. Serum sEng (0.10 ± 0.02 arbitrary pixel units [apu] versus 0.05 ± 0.01 apu; P < 0.05) and placental endoglin (4.7 ± 2.3 apu versus 1.45 ± 0.42 apu; P < 0.05) were increased along with placental hypoxia inducible factor-1α (1.42 ± 0.25 apu versus 0.68 ± 0.09 apu; P < 0.05) expression in the RUPP versus the normal pregnant dams. Placental HO-1 (1.4 ± 0.3 apu versus 2.5 ± 0.1 apu; P < 0.05) expression decreased in the RUPP compared with normal pregnant dams. The present findings support our hypothesis that placental ischemia because of RUPP increases the expression of sEng and shifts the balance of angiogenic factors in the maternal circulation toward an angiostatic state. The present study provides further evidence that placental ischemia is a strong in vivo stimulus of angiostatic factors during pregnancy. (Hypertension. 2009;53[part 2]:399-403.)

Key Words: preeclampsia ■ gestation ■ endoglin ■ blood pressure

Preeclampsia is a major obstetric problem and a significant source of maternal and neonatal morbidity and mortality in contemporary pregnancies. In recent years, the incidence of preeclampsia has increased. Despite the thorough characterization of the markers that constitute the preeclamptic syndrome, such as proteinuria, endothelial cell dysfunction, and insufficient placentation, the mechanisms underlying the genesis and progression of the hypertension associated with preeclampsia remain unclear.

Recent clinical studies indicate that angiostatic factors, such as soluble fms-like tyrosine kinase (sFlt) and soluble endoglin (Eng; sEng), may play an important role in the development and progression of preeclampsia. Eng is a component of the transforming growth factor-β (TGF-β) receptor complex and is a hypoxia-inducible protein associated with cellular proliferation and NO signaling. sEng, on the other hand, has been shown to be antiangiogenic, because it is thought to impair TGF-β binding to cell surface receptors. In the pregnant rat, elevations of circulating sEng potentiate the effects of increased plasma sFlt-1 to produce a preeclampsia-like syndrome, including the development of hemolysis, elevated liver enzymes, and low platelets, reduced fetal growth, severe hypertension, and nephritic range proteinuria. In addition, Venkatesha et al have shown that sEng inhibits in vitro endothelial cell tube formation to a similar extent as sFlt-1. Thus, there is compelling experimental evidence that compliments clinical observations that sEng is an important factor in the pathogenesis of preeclampsia.

Although recent data suggest that circulating sEng concentrations may presage the clinical onset of preeclamptic symptoms, the mechanisms underlying the increased expression of sEng remain unclear. Whether impaired placental perfusion initiates increased sEng, which, in turn, causes endothelial dysfunction resulting in preeclamptic signs, such as hypertension, or whether a pathological rise in sEng production occurs independent of placental ischemia remains unknown.
Recent studies have reported that the Eng gene is regulated by hypoxia inducible factor-1α (HIF-1α) and that both are increased in the hypoxic and preeclamptic placenta.\textsuperscript{11,12} Interestingly, the inducible isofrom of heme oxygenase (HO), HO-1 is reportedly decreased in the preeclamptic placenta.\textsuperscript{13} HO-1 is an important enzyme with a variety of roles; perhaps most important in the context of the present study is its role as an antioxidant and in protecting against vasoconstriction.\textsuperscript{14} Moreover, a recent study has shown that induction of HO-1 in vitro results in a decrease of sEng and sFlt-1 expression from trophoblast cells.\textsuperscript{15} Although these reports are intriguing, it remains to be determined whether the altered expressions of HO-1, HIF-1α, Eng, and sEng are sequela of placential ischemia or possibly attributable to another genetic or environmental factor associated with preeclampsia.

Hence, the purpose of the present study was to test the hypothesis that placential ischemia produced by reduced uterine perfusion pressure (RUPP) in the pregnant rat leads to increased plasma and placental expression of sEng, along with increased placental HIF-1α expression and decreased HO-1 expression in the placenta. To this end, we used our previously characterized and established model of RUPP hypertension, in which chronic RUPPs lead to endothelial dysfunction and hypertension in the pregnant rat.

### Methods and Apparatus

#### 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-hour light:dark cycle. All of the experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for the use and care of animals. All of the protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee. On day 14 of gestation, rat dams were randomly assigned to either RUPP (n = 12) or normal pregnant (NP; n = 10) control groups.

#### RUPP Procedure

The RUPP procedure is a well-established model for studying the link between placential ischemia and hypertension in the pregnant rat and has been described in detail previously.\textsuperscript{16–21}

#### Measurement of Mean Arterial Pressure in Chronically Instrumented Conscious Rats

Animals were instrumented on day 17 of gestation, and arterial pressure was determined in both groups of rats at day 19 of gestation, as described previously.\textsuperscript{16–21}

#### Conceptus Measurements and Serum Collection

After the measurement of mean arterial pressure, the dams were placed under isoflurane anesthesia, and a midline ventral incision was made to isolate the abdominal aorta for plasma and serum collection, as reported previously.\textsuperscript{16–21} Blood was collected for subsequent assays into Corvac sterile serum separator tubes (Sherwood Davis). Pups and placentas were excised and weighed.

#### Protein Extraction and Quantitation

As described previously,\textsuperscript{21} total soluble protein was extracted in radioimmunoprecipitation assay lysis buffer containing phenylmethylsulfonyl fluoride in dimethyl sulfoxide, sodium orthovanadate, and a protease inhibitor mixture (Santa Cruz Biotechnology, Inc). Total soluble cellular protein concentration was determined using the bicinchoninic acid method (Pierce Biotechnology).

![Figure 1](http://hyper.ahajournals.org/content/400/6/140/F1.large.jpg) **Figure 1.** Blood pressure (A) and fetal weight (B) during late gestation. Resting mean arterial pressure (MAP) was increased (P < 0.001; A) in the RUPP dams (n = 12) when contrasted with the NP dams (n = 10). RUPP fetuses (n = 12) were 25% lighter (P < 0.05) than fetuses from NP fetal dams (n = 10; B).

#### Western Immunoblot

Western immunoblotting was performed on protein extracts from placential tissue, as described previously by Gilbert et al.\textsuperscript{21} As published previously,\textsuperscript{21} the 67-kDa band corresponding with albumin observed on Ponceau S-stained membranes was used as a loading control. Western immunoblotting was performed on serum samples in the same manner, with the following exceptions. Briefly, serum samples were processed using the Proteoseek spin column kit (Pierce Biotechnology) according to the manufacturer’s directions. Protein (100 μg for placenta samples and 20 μL for serum samples) was separated by electrophoresis on 4% to 12% sodium dodecyl sulfate polyacrylamide separating gels (NuPAGE, Invitrogen).

The membranes were incubated for 1 hour in Li-COR blocking solution (Li-COR, Inc). To measure Eng and sEng in the placenta and serum, membranes were incubated in Li-COR blocking solution containing a commercially available antibody for Eng (1:500; Santa Cruz Biotechnology; M-20) that identified bands at ~65 kDa and ~100 kDa, corresponding with the truncated sEng and full-length Eng, respectively. HIF-1α antibody (1:500; Novus Biologicals) or HO-1 (1:2000; StressGen) as set overnight on a rocker at 4°C. Membranes were washed and incubated with the appropriate secondary antibodies that fluoresced at 700 and 800 nm, respectively (IRDye conjugated affinity purified antibodies; Rockland, Inc).

#### Statistical Analysis and Calculations

All of the data are presented as means±SEM. Western immunoblot data are presented as the ratio of target protein:Ponceau-stained membrane and reported as arbitrary pixel units (apu). Conceptus data were calculated as mean per pregnancy. Comparisons between 2 groups were made with a t test for independent samples, a Welch’s correction for unequal variances was applied when indicated, and statistical significance was accepted when P < 0.05. Statistical calculations were made with GraphPad Prism 4.00 for Windows (Graph-Pad Software).

### Results

#### Blood Pressure During Late Gestation

Mean arterial pressure (132±3 mm Hg versus 102±2 mm Hg; P < 0.001; Figure 1A) was increased in the RUPP dams when contrasted with the NP dams.

#### Conceptus Morphometrics

RUPP fetuses were 25% lighter than fetuses from NP fetal dams (2.35±0.05 g versus 1.76±0.08 g; P < 0.001; Figure 1B). Placental weight was decreased 19% (0.47±0.04 g versus 0.58±0.03 g; P < 0.01) in the RUPP compared with NP controls. The number of implantation sites in the uteri was not different between the RUPP and NP groups (15±1 versus...
The number of viable fetuses at 19 days' gestation age was lower in the RUPP pregnancies compared with the NP pregnancies (6 ± 1 versus 15 ± 1; P<0.05).

Effect of Placental Ischemia on Expression of sEng and Eng in Serum and Placenta

Figure 2 illustrates that the truncated form of Eng, sEng (P<0.05), was increased in the serum of RUPP dams (n=12) compared with the NP dams (n=10). sEng blot data are expressed relative to the 67-kDa band corresponding with albumin on Ponceau-stained membranes.

Figure 2. Effect of placental ischemia on expression of serum sEng. Circulating sEng was increased in the serum of RUPP dams (n=12) compared with the NP dams (n=10). sEng blot data are expressed relative to the 67-kDa band corresponding with albumin on Ponceau-stained membranes.

Effect of Placental Ischemia on Expression of Placental HIF-1α

Figure 4 shows that placental HIF-1α expression was increased (P<0.05) in the RUPP versus the NP dams.

Effect of Placental Ischemia on Expression of Placental HO-1

Figure 5 illustrates the differences observed between RUPP and NP rats with respect to the expression of HO-1 in the placenta. Placental HO-1 expression was decreased (P<0.05) in the RUPP compared with NP dams.

Discussion

The present study reveals several interesting and novel findings regarding the relationship among placental ischemia, hypertension, and alterations in angiogenic factors in the late-gestation pregnant rat. Foremost, we report that circulating sEng expression is increased in the serum and placenta of pregnant rats with ischemic placentas when compared with...
pregnant control rats with nonischemic placentas. We also demonstrate that immunoreactive HIF-1α is increased, whereas immunoreactive HO-1 is decreased in the placenta of the RUPP dams with placental ischemia when contrasted with the NP control dams. Hence, the present study is the first to report elevated serum sEng concentrations and increased placental HIF-1α expression in a reproducible and well-characterized animal model of hypertension that spontaneously arises after placental ischemia.

Our results show that maternal serum levels of sEng were increased along with increased levels of immunoreactive sEng in the placenta. Although the fold increase in circulating levels of sEng that we observed between the RUPP rat model of preeclampsia and NP rats (2.0-fold increase) are not as high as those reported in severely preeclamptic humans (2.5-fold increase), it is similar to the increase reported previously between NP and moderate preeclamptic (2.0-fold increase) women. Furthermore, the present findings are in agreement with our previous observations regarding sFlt-1 expression that suggest the RUPP model has robust similarities to moderate preeclampsia.

Previous studies have reported that the increase in sEng and sFlt-1 presage the onset of preeclamptic symptoms, but the mechanism underlying the increased serum levels remained unclear. The present data suggest that RUPP and placental ischemia during pregnancy are a stimulus for placental production and secretion of sEng. Indeed, previous authors have suggested that the primary source of circulating sEng in preeclamptic humans derives from the uteroplacental unit. Although the study performed by Venkatesha et al demonstrated that sEng plays a role in the pathogenesis of several manifestations of the preeclamptic syndrome (eg, hemolysis, elevated liver enzymes, and low platelets, as well as hypertension and proteinuria), their study did not reveal any potential mechanisms for the pathological increase of sEng in preeclamptic pregnancies. Taken together, these studies suggest that the increase in circulating sEng observed in preeclampsia may be a consequence of aberrant placental perfusion and may contribute to the pathogenesis of several characteristics of the preeclampsic syndrome.

In accord with our previous findings, we found that mean arterial pressure was increased and fetal weight was decreased in the present cohort of RUPP dams. These findings reiterate the robust nature of this model of hypertension associated with placental ischemia. Moreover, these data agree with previous findings that adenoviral overexpression of sEng and sFlt-1 results in fetal growth restriction in the pregnant rat.

In the present study we show that HIF-1α is increased along with sEng in the placenta of pregnant rats with reduced uterine perfusion. Although the preeclamptic placenta has been shown to express increased levels of sEng, HIF-1α, and sFlt-1, under in vitro culture conditions and using trophoblast cells there appears to be a disconnect between HIF-1α and sEng. This may indicate that factors elaborated by other parts of the placenta or elsewhere in the mother play an important role in the regulation of sEng and in vivo. Viewed together with our previous findings that a reduction in uteroplacental blood flow results in increased placental sFlt-1, the present data suggest that an untimely and excessive decrease in placental pO₂ may underlie the pathological overexpression of HIF-1α and placental and serum sEng.

We also report that HO-1 expression is reduced in the RUPP placenta. Previous studies have reported conflicting data, suggesting that HO-1 expression may be decreased² or increased in the placenta of preeclamptic women and decreased because of hypoxia in vitro. One study has also reported that, whereas HO-1 may not be induced by hypoxia, enzyme activity may be decreased.² Interestingly, previous work has indicated that HO-1 induction may be suppressed by factors such as interferon-γ.² Moreover, Cudmore et al have reported recently that induction of HO-1 negatively regulates sEng and sFlt-1 expression in vitro. Viewed in concert, it appears that there may be enough evidence to hypothesize a role for decreased expression or activity of HO-1 in the pathological overexpression of antiangiogenic molecules in the setting of placental ischemia during preeclampsia. Further studies are planned to investigate this possibility.

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 sEng from the placenta.

Although the present study does not elucidate the mechanism underlying the production and secretion of sEng as a result of placental ischemia, our data do reveal several intriguing possibilities for mediation of this pathway. Clearly, it appears that HIF-1α is involved and is a likely candidate to be a molecule playing a central regulatory role. Suppression of HO-1 expression, possibly because of elevated cytokines, such as interferon-γ, may play a permissive role in the overexpression of sEng and sFlt-1, because it is a central mediator of responses to hypoxia. Alternatively, the present work suggests that stimulation of TGF-β signaling may hold potential as a possible intervention in preeclamptic pregnancies. In summary, the present work provides a platform from which other studies may be launched to elucidate underlying mechanisms and develop efficacious interventions for preeclampsia.

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Disclosures

None.

References

Gilbert et al

Placental Ischemia and Soluble Endoglin 403


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