Timing of ischemic insult alters fetal growth trajectory, maternal angiogenic balance, and markers of renal oxidative stress in the pregnant rat

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Preeclampsia and hypertensive disorders of pregnancy continue to be a major obstetric problem and a significant source of maternal and neonatal morbidity and mortality in pregnancies throughout the world (38, 42). Early delivery of the fetus is often indicated to prevent the progression of preeclampsia and to mitigate immediate maternal and fetal risk. Further, preeclampsia may account for up to 15% of all preterm births (37) and significantly contribute to the short- and long-term health burden generated by low-birth weight and/or preterm deliveries (13). Despite recent advancements that have identified a number of biomarkers that are associated with and may even help predict the onset of preeclampsia (5), numerous unanswered questions remain.

Preeclampsia has historically been termed a “disease of many theories,” and the wide range of risk factors and variability in the manifestations has been a persistent difficulty in the identification and treatment of patients. Recent clinical and experimental evidence suggests that a disruption of normal circulating angiogenic balance, characterized by the ratio of proangiogenic (e.g., vascular endothelial growth factor; VEGF) and antiangiogenic factors (e.g., soluble VEGF receptor-1; sFlt-1), is strongly linked to incidence and development of preeclampsia (1, 30, 33, 44). Similarly, many studies report that oxidative stress plays a role in preeclampsia (14, 15), as well as several models of preeclampsia and pregnancy-induced hypertension (8, 27, 36) but that this may not be the case in intrauterine growth-restricted pregnancies (12).

Nevertheless, previous studies have identified a relationship between increased uterine artery resistance (UAR), early-onset (<34-wk gestation) preeclampsia, and fetal growth restriction (2, 16, 18, 39, 45). Indeed, a large number of women who develop preeclampsia also have increased uterine artery resistance and angiogenic imbalance, while another subset presents with increased UAR and fetal growth restriction, but not hypertension (16, 18, 41, 46). Thus, it remains unclear why some women with increased UAR go on to develop preeclampsia, while others do not. One possible explanation for these disparate findings is that the development of angiogenic imbalance is closely linked to the extent of the relative ischemia present in the utero-placental unit. Indeed, this has been proposed as an underlying reason for increased incidence of preeclampsia and exacerbated angiogenic imbalance in twin vs. singleton pregnancies (7). These observations suggest that the extent of the ischemia experienced by the utero-placental unit can be viewed as a function of the maternal supply and the fetal demand for nutrients. To this end, we set out to test the hypothesis that 5 days of reduced utero-placental perfusion pressure (RUPP) initiated on day 12 of gestation, several days before the substantial increases in fetal growth and uterine blood flow that begin around day 15 of gestation, would not result in the hypertension or angiogenic imbalance compared with RUPP initiated when fetuses are closer to the exponential phase of the growth curve (11, 43) on day 14 of gestation.

MATERIALS AND METHODS

Animals. Studies were performed in timed-pregnant Sprague-Dawley rats purchased from Charles River (Wilmington, MA). Animals were housed in a temperature-controlled room (23°C) with a 12:12-h light-dark cycle. The beginning of gestation, day 0, is noted at the first sign of coitus. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Dams assigned to the RUPP d17 (n = 12) underwent the RUPP procedure from day of gestation (d) 12 through 17. Additionally, dams assigned to the RUPP d19 (n = 12) groups underwent the RUPP procedure from day 14 through d19. Additional timed-pregnant dams were designated as normal pregnant controls (NP d17 (n = 10), and
NP d19 (n = 10)) and were used as matched controls for the respective RUPP-treated groups. An additional cohort of rats was evaluated to determine whether the degree of blood pressure reduction distal to the clip was similar between the RUPP clipped rats on day 12 (n = 5) and d14 (n = 5) of gestation.

Reduced uterine perfusion pressure 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 (3, 19, 23). In brief, silver clips were placed on the lower abdominal aorta [0.203-mm inner diameter (ID)] above the iliac bifurcation and also on branches (0.100-mm ID) of both the right and left ovarian arteries supplying the uterus on day 12 or day 14 of pregnancy (term = 21 days). A subset of the animals underwent a sham surgery, which included the midline incision and suture. After observing no differences in the angiogenic factors and blood pressures, these animals were grouped with the normal pregnant rats.

Measurement of mean arterial pressure in acutely instrumented rats. On d12 and d14, the rats were induced with 4% isoflurane and maintained at 2% for the duration of the procedure. To measure the difference in blood flow superior and inferior of the RUPP clip, the carotid and femoral arteries were cannulated with catheters made from V-1/V-3 tubing (0.280/0.58 mm ID) (Scientific Commodities, Lake Havasu City, AZ) and connected to a pressure transducer (Cobe III Transducer CDX, Sema, Birmingham, AL), as described previously (3, 4, 19, 23). Further, 10 units of heparin were injected in both the carotid and femoral catheters to prevent coagulation. A baseline arterial pressure was measured for 20 min. The RUPP clip was then introduced superior to the iliac bifurcation, and the pressures of the cannulated femoral and carotid arteries were measured for 30 min, flushing the catheter with 0.9% saline solution every 10 min.

Measurement of mean arterial pressure in chronically instrumented conscious rats. Animals were instrumented on either d15 or d17 of gestation, and arterial pressure was determined in both groups of rats 2 days later, as described previously (3, 20, 21).

Conceptus measurements and serum collection. After the measurement of mean arterial pressure (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, as reported previously (3, 20, 21). Blood was collected for subsequent assays into Corvac sterile serum separator tubes (Sherwood-Davis, St. Louis, MO). Fetal weight, placental weight, number of resorptions, and each fetus’s uterine position were recorded in the manner described previously (19). Briefly, uterine position was determined by numbering the implantation closest to the ovary as number one and then numbered in ascending order toward the cervix (Fig. 1). This was repeated on each uterine horn, and weight values were averaged when applicable to yield a mean weight for each position in the uterus. Resorptions at the implantation site were not included in the mean weight analysis. All tissues that were collected for subsequent analysis were flash frozen in liquid nitrogen and stored at −80°C.

Enzyme-linked immunosorbant assays. Plasma concentrations of free VEGF and sFlt-1 (R&D Systems, Minneapolis, MN) were measured using commercial ELISA kits available from R&D Systems (Quantikine, Minneapolis, MN), according to the manufacturer’s directions, as described previously (8, 19, 21).

Endothelial tube formation assay. Angiogenic balance was also assessed in the serum of pregnant rats in vitro using the method of Maynard et al. (34) with the following modifications. Phenol-red free growth factor reduced Matrigel (BD Biosciences, San Jose, CA) was pipetted into each well of a 24-well cell culture-treated plate and incubated at 37°C for a minimum of 30 min to solidify. Primary human umbilical vascular endothelial cells [American Type Culture Collection (ATCC), Manassas, VA] were washed twice with serumless vascular cell growth media (ATCC) and plated at 50,000 cells/ml of serumless media. The cells were then treated with a 5% serum from the respective rat treatment groups and incubated for 8 h. Total number of tubule formations per frame was assessed at 40× optical zoom with a digital inverted compound microscope and ImageJ analysis software (National Institutes of Health, Bethesda, MD). Total tube count was assessed by at least two individual investigators who were blinded to the identity of the experimental groups. Values from each observer were averaged to obtain final counts.

Measurement of oxidative stress. Oxidative stress was assessed by measuring total antioxidant capacity and malondialdehyde. Total antioxidant capacity was assessed in kidney tissue by measuring Trolox-equivalent antioxidant capacity (TEAC) assay kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s directions, as previously described (26, 40). Additionally, malondialdehyde was measured in kidney tissue by using a thiobarbituric acid reactive species (TBARS) assay (Cayman Chemical), according to the manufacturer’s directions, as previously described (26).

Statistical analysis and calculations. All data are presented as means ± SE, and statistical significance was accepted when P < 0.05. sFlt-1/VEGF data were square root transformed. Conceptus data were calculated as mean per pregnancy. Comparisons between the groups were made with a t-test for independent samples, Pearson product moment correlation, or two-way between-subjects ANOVA and post hoc tests were employed when indicated. Statistical calculations were made with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Acute effects of the RUPP clip on MAP. To determine whether our clips reduced blood pressure distal to the clipping site to the same extent at both d12 and d14 of gestation, we measured blood pressure before, during, and for 30 min after the placement of the abdominal aorta clip. Resting blood pressures were not different between d12 and d14 rats. Figure 2 illustrates that there was no difference in the reduction of perfusion pressure distal to the RUPP clip on either d12 or d14. The change in MAP 10, 20, or 30 min after the clipping procedure...
Fig. 2. Femoral mean arterial pressure (MAP) measured immediately before and after the clipping procedure in rats on day 12 (●) and day 14 (■) of pregnancy. MAP measured distal to the clip was not different between pregnant rats on day 12 and day 14 of gestation before, immediately, or after 10, 20, and 30 min of flow restriction. Data were analyzed by an unpaired t-test and are expressed as mean ± SE.

Chronic effects of RUPP on blood pressure during late gestation. Figure 3 illustrates that 5 days after the clipping procedure, MAP was increased in both the RUPP d17 and the RUPP d19 groups compared with gestational age-matched NP rats (d17: 110 ± 3 vs. 100 ± 1 mmHg, P < 0.05; d19: 120 ± 3 vs. 96 ± 3 mmHg, P < 0.001). Further, the magnitude of blood pressure increase was greater in the RUPP d19 compared with the RUPP d17 rats (Fig. 3, P < 0.05).

Similar to our previous work (19), there was a significant negative correlation between MAP and fetal number in the d19 (r = −0.43). In contrast, no relationship was observed between MAP and fetal number in the d17 (r = −0.136) animals.

Conceptus morphometrics. RUPP d19 fetal (2.30 ± 0.03 g, P < 0.05) and placental (0.44 ± 0.05 g vs. 0.49 ± 0.05 g, P < 0.05) weights were decreased compared with the NP d19 controls. The RUPP d17 fetal weight was decreased compared with the NP d17 controls (1.43 ± 0.04 g vs. 1.82 ± 0.15; P < 0.05), but placental weights were not different. Further, fetal weight percentage change from NP respective controls (Fig. 4A) was decreased in the RUPP d17 (P < 0.05) vs. RUPP d19. Placental weight change from NP controls (Fig. 4B) was decreased in the RUPP d19 (P < 0.05) vs. RUPP d17.

In addition to the mean fetal weight, we also examined fetal weight at different positions in the uterine horn at days 17 and 19 of gestation. Figure 5 shows when fetal weight is plotted relative to uterine position, there are profound treatment (RUPP vs. NP) and timing (d17 vs. d19) differences in fetal size in late gestation. Although the slopes of the fetal size relative to uterine position curve were different between d17 and d19 rats, only in the d19 rats was a treatment effect observed (P < 0.05). There were no observed effects of timing of placental ischemia on the total number of implantation sites observed at necropsy.

Effects of placental ischemia on maternal angiogenic balance. The RUPP d19 groups showed a decrease in free VEGF levels compared with the NP control (531 ± 22 vs. 795 ± 72 pg/ml, P < 0.05, Fig. 6A), but RUPP and NP d17 groups showed no difference. Free VEGF concentration was also higher in the d19 NP compared with the NP d17 (535 ± 99 vs. 795 ± 72 pg/ml, P < 0.05). Circulating sFlt-1 concentrations were higher in the RUPP d19 vs. NP d19 (33 ± 5 vs. 16 ± 1 pg/ml, P < 0.05), and no difference was observed between RUPP d17 and NP d17. The RUPP d19 group had a higher sFlt-1:VEGF compared with the NP control (1.3 ± 0.2 vs. 0.7 ± 0.0, P < 0.05, Fig. 6B).
Further evidence of the differences in angiogenic balance between the different periods of placental ischemia was shown by use of an in vitro endothelial cell tube formation assay. Figure 7 illustrates that the RUPP animals in the days 14–19 group had decreased endothelial tube formation compared with the gestational age-matched NP controls. There was no evidence of angiogenic imbalance in the days 12–17 RUPP group.

Measurement of oxidative stress. The kidneys of the RUPP d19 groups showed a significant decrease in TEAC concentration compared with the NP d19 control (0.37 ± 0.04 vs. 0.20 ± 0.02 mM; \( P < 0.05 \); Fig. 8A), but RUPP d17 and NP d17 groups showed no difference. Malondialdehyde (i.e., TBARS) was significantly higher in the RUPP d19 kidneys compared with the NP d19 group (9.30 ± 1.24 vs. 4.48 ± 1.06; \( P < 0.05 \); Fig. 8B), and no difference was shown between RUPP d17 and NP d17 groups (Fig. 8B).

**DISCUSSION**

The present study reveals several important findings regarding the relationship between placental ischemia and angiogenic imbalance. Foremost, we report that RUPP beginning on day 12 of gestation alters the trajectory of fetal growth to a greater extent than RUPP initiated on day 14 of gestation. Second, we report that 5 days of placental ischemia has different effects on the balance of angiogenic factors (i.e., sFlt-1 and VEGF), depending on the time at which the RUPP is initiated. Next, this was observed using two independent measures of angiogenic balance (ELISA and tube formation assay). Finally, markers of increased oxidative stress (i.e., TEAC and malondialdehyde) were observed in the RUPP d19 group compared with the NP d19 group, and not in the RUPP d17 group vs. NP d17. Furthermore, these data do not support our hypothesis that hypertension would only occur in the RUPP d14 group, suggesting the presence of alternative mechanisms for placental ischemia-induced hypertension.

While numerous studies (6, 9, 10, 17, 24, 47) have reported various effects and gestational timing of increased unilateral or bilateral uterine artery resistance, to our knowledge, none have specifically reported the different effects of timing (from days 12–17; and days 14–19 of gestation) of ischemic induction and maternal blood pressure and angiogenic balance in the gravid
relative ischemia is an important factor that may be responsible for the exacerbation of angiogenic imbalance and preeclampsia in twin vs. singleton pregnancies. It has also been reported that women carrying pregnancies in which the placental attachment occurs toward the cervical end (e.g., placenta previa) of the uterus (which receives a greater proportion of uterine blood flow) have a reduced incidence of preeclampsia (25). Further, recent evidence points to increased incidence of placental dysfunction in pregnancies carrying male fetuses (35), which are known to grow at a faster rate than female fetuses in many species (22).

We also found that angiogenic imbalance only occurred when the ischemic insult began on d14 of pregnancy, a time that is much closer to the late-gestation surge in fetal growth. This is similar to a previous report that demonstrated the importance of relative ischemia in preeclamptic pregnancies by showing that angiogenic imbalance was greater in preeclamptic twin pregnancies compared with preeclamptic singleton pregnancies (7). Thus, the present findings support the notion that the amount of relative ischemia may be important to the determination of angiogenic balance in late gestation. Indeed, we observed that the extent of fetal growth restriction was different in the RUPP d12–d17 and the RUPP d14–d19 groups compared with respective controls. Moreover, we also found that the fetal size position curves were different between the two RUPP groups as well. One potential interpretation of these data is that there may be differences in fetal-placental adaptations, such that the fetal growth trajectory is constrained in a manner to minimize the disruption of the balance between maternal-fetal supply and demand of nutrient. Alternatively, there may be differences in the regional hemodynamics and blood flow distribution among the fetuses and pups due to time of gestation as well as RUPP. What remains unclear is whether or not there are different placental adaptations to RUPP at these two different time points. Further studies are planned to evaluate this possibility.

Another important observation in the present study is that there was a modest increase in blood pressure in the RUPP d12–d17 groups despite no increase in plasma sFlt-1 and no concomitant decrease in unbound VEGF. This is strikingly similar to clinical observations in which patients with increased UAR present with a wide range of clinical characteristics that may or may not include angiogenic imbalance, hypertension, fetal growth restriction, and/or other hallmarks of the preeclamptic syndrome (42). Although it remains speculative at this point, the observations in the d12–d17 group from the present study may be more representative of gestational hypertension than preeclampsia and may suggest the presence of alternative mechanisms linking impaired placental perfusion and variations in the severity of hypertension during pregnancy. Moreover, there are many alternative pathways and candidate molecules that may be considered for playing a role in the progression from placental impairment to pregnancy-induced hypertension and preeclampsia. Several of these pathways include, but are not limited to, inflammatory cytokines (32), AT1-auto-antibodies (31), and novel signaling molecules, such as placentally derived microRNA’s miR210 and miR518c (28).

Furthermore, we found that markers of renal oxidative stress were altered in the d19 RUPP compared with d19 NP but were not different between the d17 RUPP and NP, respectively.
Specifically, we report that TEAC was decreased and TBARS were increased in kidney homogenates due to RUPP at d19 but not in the RUPP d17, compared with respective controls. Although these observations are in agreement with previous work demonstrating placental (increased 8-isoprostanate and TBARS) and renal (decreased renal SOD activity) oxidative stress in the RUPP rat at d19 (26, 40), they do identify additional markers of oxidative stress in the kidney of the RUPP rat at d19. When viewed in concert with the angiogenic balance data, the presence of oxidative stress in the RUPP d19 kidneys, but not the RUPP d17 kidneys, further demonstrates the importance of the timing of ischemic insult in this model.

Perspectives and Significance

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 remains unclear. The present study, which relies on data gathered by employing a well-characterized and robust animal model of hypertension during mild preeclampsia and intrauterine growth restriction, provides further evidence that placental ischemia is a primary factor in the pathogenesis of RUPP-induced hypertension. Furthermore, our results reveal the existence of alternative pathways for the development of RUPP hypertension in early utero-placental blood flow restriction. Although the present study does not elucidate the mechanisms underlying the angiogenic imbalance-independent progression of RUPP-induced hypertension, it allows for further questions and investigation targets to reveal this mechanism.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


