Restoring Placental Growth Factor-Soluble fms-like Tyrosine Kinase-1 Balance
Reverses Vascular Hyper-reactivity and Hypertension-in-Pregnancy

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Running Title: Angiogenic-Antiangiogenic Imbalance in Hypertensive Pregnancy

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List of Abbreviations: ACh, acetylcholine; BP, blood pressure; [Ca\textsuperscript{2+}], intracellular free Ca\textsuperscript{2+} concentration; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; HELLP, hemolysis elevated liver enzymes and low platelets; HTN-Preg, hypertension-in-pregnancy; HUVECs, human umbilical vein endothelial cells; IUGR, intrauterine growth restriction; L-NAME, N\textsubscript{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; pEC\textsubscript{50}, (−log EC\textsubscript{50}) drug concentration producing half-maximal response; PE, preeclampsia; p-eNOS, phospho-eNOS; PG\textsubscript{I2}, prostacyclin; Phe, phenylephrine; PI GF, placental growth factor; Preg, pregnant; RUPP, reduced uteroplacental perfusion pressure; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase-1; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; VSM, vascular smooth muscle

Key Words: angiogenesis, endothelium, nitric oxide, vascular smooth muscle, calcium, hypertension, preeclampsia
ABSTRACT

Preeclampsia (PE) is a pregnancy-related hypertensive disorder (HTN-Preg) with unclear mechanism. An imbalance between anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and angiogenic placental growth factor (PIGF) has been observed in PE, but the vascular targets and signaling pathways involved are unclear. We assessed the extent of sFlt-1/PIGF imbalance and vascular dysfunction in a rat model of HTN-Preg produced by reduction of uteroplacental perfusion pressure (RUPP), and tested if inducing a comparable sFlt-1/PIGF imbalance by infusing sFlt-1 (10 µg/kg/day) in day-14 pregnant (Preg) rats cause similar increases blood pressure (BP) and vascular reactivity. Using these guiding measurements, we then tested if restoring sFlt-1/PIGF balance by infusing PIGF (20 µg/kg/day) in RUPP rats would improve BP and vascular function. On gestational day 19, BP was in Preg+sFlt-1 and RUPP > Preg, and in RUPP+PIGF < RUPP rats. Plasma sFlt-1/PIGF ratio was increased in Preg+sFlt-1 and RUPP, and was reduced in RUPP+PIGF rats. In isolated endothelium-intact aorta, carotid, mesenteric and renal artery, phenylephrine (Phe)- and high KCl-induced contraction was in Preg+sFlt-1 and RUPP > Preg, and in RUPP+PIGF < RUPP. The differences in vascular reactivity to Phe and KCl between groups were less apparent in vessels treated with the NOS inhibitor L-NAME or guanylate cyclase inhibitor ODQ or endothelium-denuded, suggesting changes in endothelial NO-cGMP pathway. In Phe precontracted vessels, acetylcholine (ACh)-induced relaxation was in Preg+sFlt-1 and RUPP < Preg, and in RUPP+PIGF > RUPP, and was blocked by L-NAME or ODQ treatment or endothelium-removal. Western blots revealed that aortic total eNOS and activated p-eNOS were in Preg+sFlt-1 and RUPP < Preg, and in RUPP+PIGF > RUPP. ACh-induced vascular nitrate/nitrite production was in Preg+sFlt-1 and RUPP < Preg, and in RUPP+PIGF > RUPP. Vascular relaxation to the exogenous NO donor sodium nitroprusside was not different among groups. Thus, a tilt in the angiogenic balance towards anti-angiogenic sFlt-1 is associated with decreased vascular relaxation and increased vasoconstriction and BP. Restoring the angiogenic/anti-angiogenic balance using PIGF enhances endothelial NO-cGMP vascular
relaxation and decreases vasoconstriction and BP in HTN-Preg rats, and could be a new approach in the management of PE.
INTRODUCTION

Normal pregnancy is associated with adaptive hemodynamic and vascular changes including increased plasma volume and cardiac output and decreased vascular resistance, such that blood pressure (BP) remains unchanged or is slightly decreased (59, 71). Also, changes in vasodilator substances such as nitric oxide (NO), prostacyclin (PGI2), endothelium-derived hyperpolarizing factor (EDHF), and vascular endothelial growth factor (VEGF) contribute to the vascular adaptations during normal pregnancy (75). These hemodynamic and vascular changes lead to redistribution of blood flow in different maternal tissues and ensure adequate blood supply to the growing fetus.

In 5-8% of pregnancies in the United States, ~8 million pregnancies worldwide, women develop PE (74). PE is manifested as hypertension-in-pregnancy (HTN-Preg) and often proteinuria. If untreated, PE could progress to eclampsia with severe hypertension, cerebral edema and seizures. PE causes 15-20% of maternal deaths (8, 58, 63), and is often associated with intrauterine fetal growth restriction (IUGR), accounting for 10-15% of preterm births (2, 63).

Although PE is a major cause of maternal and fetal morbidity and mortality, the mechanisms involved are not fully understood. Clinical observations in PE women have shown reduction in brachial artery flow-mediated vasodilation, pointing to endothelial dysfunction as an underlying mechanism (55, 79). Because of the difficulty to perform mechanistic studies in pregnant women, animal models of HTN-Preg have been developed (31, 40). Studies in a rat model of reduced uteroplacental perfusion pressure (RUPP) have shown some of the features of PE including HTN, endothelial dysfunction and increased vascular reactivity (11, 19, 31). Also, studies in a rat model of gestational hypoxia have shown PE-like manifestations (81), supporting that placental ischemia/hypoxia could be an initiating event in HTN-Preg.

In search of the intermediary mechanisms that link localized RUPP to systemic endothelial and vascular dysfunction, studies have proposed that placental ischemia/hypoxia could trigger the release of various bioactive factors in the maternal circulation (19, 34, 36, 38, 65, 80).
Because of the marked fetal growth and placental vascularization during pregnancy, studies have focused on possible changes in angiogenic factors such as VEGF and placental growth factor (PIGF) in PE. VEGF gene expresses a family of proteins including VEGF-A, -B, -C, -D and PIGF (29). VEGF-A, VEGF-B and PIGF bind to tyrosine kinase receptor Flt-1 (VEGFR-1) and VEGF-A binds to VEGFR-2 (Flk-1 or KDR) to promote development of placental vasculature (29). Measurements of circulating levels of VEGF in PE have not been consistent, with studies showing decreased (56), unchanged (46, 48) or even increased levels in PE (7, 26, 72). In comparison, PIGF has only 1/10th the affinity for VEGFR-1 compared to VEGF, but its levels are ~40 times higher than VEGF during normal pregnancy (33). PIGF has four alternatively spliced mRNA species (PIGF 1-4), and the circulating levels of its predominant isoform PIGF-1 is decreased in PE (6, 72). In contrast with angiogenic factors, soluble fms-like tyrosine kinase-1 (sFlt-1, sVEGFR-1) is an anti-angiogenic factor expressed as an alternatively spliced variant of VEGFR-1 that lacks both the transmembrane and cytoplasmic domains. sFlt-1 binds to VEGF and PIGF in the circulation and inhibits their action on cell surface VEGFR-1 (39). Circulating levels of sFlt-1 are 10-fold higher in pregnant than non-pregnant women, remain almost stable during the first and second trimester, then show an increase after the 36th week of gestation and throughout the third trimester (65). In PE, the increase in sFlt-1 levels is more pronounced (6, 29, 45, 72), and the sFlt-1/PIGF ratio is higher in the circulation of PE than normal pregnant women from the second trimester onwards (45, 51). These observations have led to the suggestion that an imbalance between angiogenic and anti-angiogenic factors could be an intermediary mechanism linking RUPP to HTN-Preg and PE.

Some studies have tested the effects of angiogenic factors in animal models of HTN-Preg. One study showed that infusion of recombinant VEGF lowered BP and improved renal function in rats with placental ischemia-induced HTN (21). Also, during the preparation of this manuscript, a recently published study showed that infusion of recombinant PIGF reduced BP and improved glomerular filtration rate in RUPP rats (68). However, the vascular mechanisms underlying the relationship between improving the angiogenic
balance and decreasing BP in HTN-Preg are unclear. The present study was designed to test the hypothesis that restoring the sFlt-1/PIGF balance reverses HTN-Preg by improving vascular function and the vascular contraction and relaxation mechanisms.

**METHODS**

**Animals.** We used control normal Preg rats to assess the desirable sFlt-1/PIGF balance and favorable vascular mechanisms. We used a RUPP rat model to assess the extent of sFlt-1/PIGF imbalance and vascular dysfunction in HTN-Preg. We then tested if inducing a comparable sFlt-1/PIGF imbalance by infusing sFlt-1 in Preg rats was sufficient to increase BP and impair vascular function to levels similar to those in RUPP rats. Using these guiding measurements, we then tested if restoring the sFlt-1/PIGF balance by infusing PIGF would improve BP and vascular function in HTN-Preg RUPP rats.

Time-pregnant (day 11) Sprague-Dawley rats (12 weeks of age, Charles River Laboratories, Wilmington, MA) were housed in the animal facility and maintained on *ad libitum* standard rat chow and tap water in 12 h light-dark cycle. On gestational day 14, some of the rats were infused intravenously (IV) via a jugular vein catheter (PE-50) and osmotic minipump with murine recombinant sFlt-1 (VEGF-R1/Flt-1 Fc Chimera, R&D Systems, Minneapolis, MN) 10 µg/kg/day for 5 days (Preg+sFlt-1); a dose that was previously shown to be sufficient to cause HTN, proteinuria and glomerular endotheliosis (48). Previous studies projected a high homology between murine and rat VEGFR-1 (4, 41), and other laboratories used murine recombinant sFlt-1 in similar rat models of PE and did not report any immune response or secondary effects (25, 48). Other day 14 Preg rats underwent surgical procedure to reduce uteroplacental perfusion pressure (RUPP) by banding the lower abdominal aorta above the iliac bifurcation and the main uterine branches of the ovarian arteries as previously described (3, 11, 19, 50). Briefly, the abdominal aorta near the iliac bifurcation was carefully dissected free of surrounding tissue and perivascular fat and separated from the vena cava. A blunt plastic rod (OD, 0.3 mm) was placed parallel to the aorta, and a 4-0 silk braided ligature was
knotted twice around both the aorta and the adjacent plastic rod. Once taut, the rod was carefully removed from the knotted ligature, thus creating a constrictive band (ID, 0.3 mm) and reducing blood flow through the aorta. This procedure has been shown to reduce uterine perfusion pressure in the gravid rat by ~40% (14). Since compensation of blood flow to the placenta occurs through an adaptive increase in ovarian blood flow (54), a blunt plastic rod (OD, 0.1 mm) was used to place a ligature band (ID, 0.1 mm) on the main uterine branches of both the right and left ovarian arteries. RUPP rats in which the banding procedure resulted in maternal death or total reabsorption of the pups were excluded from data analyses.

RUPP rats were either nontreated or simultaneously infused via a jugular vein catheter and osmotic pump with recombinant PIGF-1 (MBS696135, MyBiosource) 20 µg/kg/day for 5 days (RUPP+PIGF). A lower dose of PIGF (10 µg/kg/day) was also used, but was not sufficient to decrease BP in RUPP rats. In other control experiments, some day 14 Preg rats were infused IV for 5 days with sFlt-1 plus PIGF (Preg+sFlt-1+PIGF). Norm-Preg rats were sham operated. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the guidelines of American Physiological Society and the Harvard Medical Area Standing Committee on Animals.

**Blood Pressure (BP).** On day 19 of pregnancy, rats were anaesthetized with isoflurane, a PE-50 catheter was inserted in the carotid artery, and exteriorized at the back of the neck. The rats were allowed to recover from anesthesia for at least 1 h. The carotid arterial catheter was connected to a pressure transducer attached to an amplifier and pressure recorder (Living System Instrumentation, Burlington, VT). BP in conscious rats was recorded for 1 min, every 20 min, over a period of 1 h and the average BP was measured (50).

**Plasma sFlt-1 and PIGF.** After measuring BP, blood samples were collected via the arterial catheter into sterile heparin tubes (Tyco Healthcare, Mansfield, MA). Plasma was separated by blood centrifugation at 2000×g for 10 min, and stored at -80°C for later use. Plasma sFlt-1
levels were measured using rat sFlt-1 ELISA microplate kit (MBS2602003, MyBiosource) according to the manufacturer instructions, with 0.05 ng/ml sFlt-1 sensitivity, intra-assay and inter-assay precision, and coefficient of variability (CV) <12 %. The ELISA kit would detect complexed sFlt-1 that was bound to PIGF and possibly VEGF in rat plasma. Plasma PIGF levels were measured using rat PIGF ELISA microplate kit (MBS703282, MyBiosource) according to the manufacturer instructions, with <0.78 pg/ml PIGF sensitivity, intra-assay and inter-assay precision, and coefficient of variability (CV) <10 %. The ELISA kit would detect both free PIGF and complexed PIGF that was bound to sFlt-1 in rat plasma. Absorbance was measured at 450 nm on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

**Tissue Preparation.** On day 19 of pregnancy, rats were euthanized by inhalation of CO₂, the uterus was excised, and the litter size and individual pup weight were recorded. Because different tissues may have different blood flow requirement, the pregnancy-associated changes in vascular function could vary in different vascular beds. In this respect, a large vasodilator effect in one vascular bed could be balanced by minimal effect in other vascular beds. We have recently shown region-specific differences in the cephalic, thoracic and abdominal arteries of non-pregnant (62) and pregnant rats (47). Regional differences in vasodilation and blood flow are particularly important during pregnancy as they could have significant implications on the cardiovascular system and the health of mother and fetus. To test for potential differences in the vascular responses in various vascular beds, we used four blood vessels with specific roles and functions; namely the thoracic aorta as a conduit vessel, the carotid artery supplying the cephalic circulation, the mesenteric artery with specific role in the splanchnic circulation, and the renal artery with specialized role in the kidney and the regulation of renal function, plasma volume and BP. The thoracic aorta, carotid artery, mesentery and mesenteric arterial arcade and the kidney and renal vessels were rapidly excised and placed in Krebs physiological solution. With the aid of a dissection microscope, the aorta, carotid,
mesenteric and renal artery were carefully cleaned of fat and connective tissue and cut into 3
mm wide rings for vascular function studies. For endothelium-intact segments extreme care
was taken to minimize injury to the endothelium. For endothelium-denuded segments, the
endothelium was removed by scraping the vessel interior five times around the tip of forceps
(for aorta) or around thin tungsten wire (for carotid, mesenteric and renal artery). Different
segments from each vascular bed were used for different experimental protocols. The
remainder of the vessels was used to measure nitrate/nitrite production or stored at -80°C for
Western blots analysis.

Isometric contraction and relaxation. Vascular rings were suspended between two wire
hooks, one hook was fixed at the bottom of a tissue bath and the other hook was connected to a
Grass force transducer (FT03, Astro-Med Inc., West Warwick, RI). Arterial rings were stretched
under 2 g (aorta), 1 g (carotid artery), or 0.5 g basal tension (mesenteric and renal artery) and
allowed to equilibrate for 45 min in a temperature controlled, water-jacketed tissue bath, filled
with 50 ml Krebs solution bubbled with 95% O₂ 5% CO₂ at 37°C. These basal tensions
produced maximal potassium chloride (KCl) contraction, and further increases in basal tension
did not cause further contraction to KCl (62). The changes in contraction/relaxation were
recorded on a Grass polygraph (Model 7D, Astro-Med Inc., West Warwick, RI).

After tissue equilibration, a control contraction to 96 mM KCl was elicited. Once maximum
KCl contraction was reached the tissue was rinsed with Krebs 3 times, 10 min each. Vascular
segments were stimulated with increasing concentrations of phenylephrine (Phe, 10⁻⁹ to 10⁻⁵
M), concentration-contraction curves were constructed, and the maximal Phe contraction was
measured. The individual Phe concentration-response curves were further analyzed using
non-linear regression, sigmoidal dose-response curves were fitted using the least squares
method, and the effective concentration that produced half the maximal contraction (EC₅₀) was
measured and presented as pEC₅₀ (-log M). To investigate endothelial function, the vessels
were precontracted with Phe (6x10⁻⁷ M), increasing concentrations (10⁻⁹ to 10⁻⁵ M) of
acetylcholine (ACh) were added and the % relaxation of Phe contraction was measured. To test the role of endothelium-dependent NO, parallel contraction and relaxation experiments were performed in endothelium-intact vessels pretreated with the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 3x10^{-4} M) or the guanylate cyclase inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10^{-5} M) for 15 min. Phe contraction and ACh relaxation were also measured in endothelium-denuded vessels. Endothelium removal was determined by the absence of vasodilator responses to ACh. To test the ability of VSM to respond to vasodilators, the relaxation of Phe-precontracted vascular rings to the exogenous NO donor sodium nitroprusside (SNP) was measured. Individual ACh and SNP concentration-relaxation curves were further analyzed using non-linear regression, sigmoidal dose-response curves were fitted using the least squares method, and ACh and SNP pEC_{50} were calculated.

Nitrate/Nitrite (NOx) Production. Endothelium-intact aortic or mesenteric arterial segments were incubated in normal Krebs for 30 min to measure basal NO production then stimulated with ACh (10^{-8} to 10^{-5} M) for 30 min and the incubation solution was assayed for the stable end product of NO, NO_{2}^{-} using Griess reagent (Promega, Madison, WI) (11, 50).

Western Blots. Samples of endothelium-intact aorta were prepared; each sample containing arteries pooled from 4 rats per group. Arteries were homogenized in a homogenization buffer containing 20 mM 3-[N-morpholino] propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 μM leupeptin, 5.5 μM pepstatin, 2.15 μM aprotinin and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, using a 2-ml tight-fitting homogenizer (Kontes Glass Co., Vineland, NJ). The tissue homogenate was centrifuged at 10,000×g for 2 min at 4°C, and the supernatant was collected. If the supernatant contained floating debris, centrifugation was repeated at least two times to obtain a clear supernatant. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). Protein extracts (20 μg) were combined with an equal volume of 2X Laemmli loading buffer,
boiled for 5 min, and size fractionated by electrophoresis on 8% SDS-polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane by electroblotting. Membranes were incubated in 5% nonfat dried milk in phosphate buffered saline (PBS)-Tween for 1 h and then with rabbit polyclonal anti-eNOS antibody (NOS3 H-159, sc-8311) or anti-phospho-eNOS (p-eNOS) antibody (p-NOS3 Ser\textsuperscript{1177}, sc-21871-R) (1:800; Santa Cruz Biotechnology, Dallas, TX) for 24 h at 4°C. Membranes were washed 5 times 15 min each in PBS-Tween then incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 2 h. Membranes were washed another 5 times 15 min each in PBS-Tween, and the immunoreactive bands were detected using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The blots were subsequently stripped then reprobed for β-actin (1:3000). The reactive bands were analyzed by optical densitometry and ImageJ (National Institutes of Health, Bethesda, MD). The densitometry values represented the pixel intensity normalized to β-actin to correct for loading (49, 50).

**Solution and Drugs.** Krebs solution contained in mM 120 NaCl, 5.9 KCl, 25 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 11.5 dextrose, 2.5 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, at pH 7.4, and bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. High KCl (96 mM) was prepared as normal Krebs but with equimolar substitution of NaCl with KCl. Stock solutions of Phe, ACh, SNP, and L-NAME (10\textsuperscript{-4} M, Sigma-Aldrich, St. Louis, MO) were prepared in distilled water. Stock solution of ODQ (10\textsuperscript{-4} M, Enzo Life Sciences, Farmingdale, NY) was prepared in diemethylsulfoxide (DMSO). The final concentration of DMSO in the experimental solution was <0.1%. PBS was used in the Western blot experiments and contained in mM: 137 NaCl, 2.7 KCl, 8 Na\textsubscript{2}HPO\textsubscript{4}, 2 KH\textsubscript{2}PO\textsubscript{4}, at pH 7.4. All other chemicals were of reagent grade or better.

**Statistical Analysis.** Data from different groups were analyzed and presented as means±SEM with “n” representing the number of rats per group (Preg n=7, Preg+sFlt-1 n=9,
RUPP n=6, RUPP+PIGF n=7). For vascular reactivity experiments, individual concentration-
contraction or concentration-relaxation curves were constructed, sigmoidal curves were fitted
to the data using the least squares method, and the pEC$_{50}$ values (−log EC$_{50}$, drug
concentration evoking half-maximal response) were measured using Prism (v.5.01; GraphPad
Software, San Diego, CA). Data were first analyzed using ANOVA with multiple classification
criteria [rat group (Preg vs. Preg+sFlt-1, RUPP and RUPP+PIGF), condition of vascular
endothelium (denuded vs. intact), vessel treatment (treated with L-NAME or ODQ vs.
nontreated control tissues). When a statistical difference was observed, the data were further
analyzed using Bonferroni’s post-hoc test for multiple comparisons. Student’s unpaired t-test
was used for comparison of two means. Differences were considered statistically significant if
P < 0.05.

RESULTS

Effect of altering sFlt-1/PIGF balance on maternal and fetal parameters

On gestational day 19, plasma sFlt-1 levels were higher in Preg+sFlt-1, RUPP and
RUPP+PIGF than Preg rats, and not different in RUPP+PIGF versus RUPP rats (Fig. 1A).
Plasma PIGF was deceased in Preg+sFlt-1 and RUPP versus Preg rats, and was increased in
RUPP+PIGF versus RUPP to levels not different from those in Preg rats (Fig. 1B). The sFlt-
1/PIGF ratio was increased in Preg+sFlt-1 and RUPP versus Preg rats, and was restored in
RUPP+PIGF to levels lower than in RUPP rats and not different from Preg rats (Fig. 1C).

On gestational day 19, BP was increased in Preg+sFlt-1 and RUPP versus Preg rats, and
was reduced in RUPP+PIGF versus RUPP to levels not significantly different from those in
Preg rats (Fig. 1D). In other control experiments, BP was not significantly different in
Preg+sFlt-1+PIGF (107.3±2.7 mmHg) versus Preg rats (101.0±6.0 mmHg).

Maternal body weight was not significantly different in Preg (333.8±9.2 gram), Preg+sFlt-1
(310.3±5.2 gram), RUPP (303.3±20.6 gram) and RUPP+PIGF rats (320.7±13.6 gram). The
litter size (Fig. 1E) and average pup weight (Fig. 1F) were reduced in Preg+sFlt-1 and RUPP
versus Preg, and were increased in RUPP+PIGF versus RUPP to levels not significantly different from control Preg rats. Other control experiments showed no significant difference in average pup weight in Preg+sFlt+PIGF (2.5±0.1 gram) versus Preg rats (1.87±0.4 gram).

**Effect of altering sFlt-1/PIGF balance on KCl-induced vascular contraction**

High KCl is known to stimulate Ca^{2+} influx (53). In isolated endothelium-intact vessels of all animal groups, KCl (96 mM) caused rapid followed by maintained contraction over a 10 min time course. KCl contraction was greater in the aorta (Fig. 2A), carotid (Fig 2B), mesenteric (Fig. 2C) and renal artery (Fig. 2D) of Preg+sFlt-1 and RUPP versus Preg rats. KCl contraction was reduced insignificantly in the aorta, and significantly in the carotid, mesenteric and renal artery of RUPP+PIGF versus RUPP rats, and to levels not significantly different from the respective arteries of control Preg rats (Fig. 2A-D). No significant differences in KCl contraction could be observed in endothelium-denuded aorta (Fig. 2E), carotid (Fig 2F), mesenteric (Fig. 2G) and renal artery (Fig. 2H) among the different animal groups. Other control experiments showed that KCl-induced contraction was not different in aorta (1.77±0.39 versus 1.68±0.12), carotid (0.48±0.09 versus 0.50±0.02), mesenteric (0.43±0.13 versus 0.60±0.04) or renal artery (0.56±0.04 versus 0.51±0.03 gram) of Preg+sFlt-1+PIGF versus Preg rats, respectively.

**Effect of altering sFlt-1/PIGF balance on Phe-induced vascular contraction**

In endothelium-intact vessels, Phe caused a concentration-dependent contraction that was greater in the aorta (Fig. 3A), carotid (Fig. 3B), mesenteric (Fig. 3C) and renal artery (Fig. 3D) of Preg+sFlt-1 and RUPP versus Preg rats. Phe contraction was reduced in the aorta, carotid, mesenteric and renal artery of RUPP+PIGF versus RUPP, and to levels not significantly different from the respective arteries of control Preg rats (Fig. 3A-D). To test if the enhanced contraction in Preg+sFlt-1 and RUPP versus Preg rats, and its reduction in RUPP+PIGF versus RUPP are due to changes in α-adrenergic receptor sensitivity, Phe
contraction was presented as % of maximum and Phe EC$_{50}$ was calculated. Phe contraction as % max was not significantly different between the different animal groups in any of the vessels tested (Fig. 3E-H, Table 1). Assuming that KCl contraction is mainly due to Ca$^{2+}$ entry through voltage-gated channels (53), then any changes in Phe contraction relative to KCl could be due to additional activation of other Ca$^{2+}$ channels (37, 57) or Ca$^{2+}$-sensitization pathways (13, 64). Phe response as % of KCl contraction was not significantly different between the different animal groups in any of the vessels tested (Fig. 3I-L, Table 1). Other control experiments showed that maximal Phe (10$^{-5}$ M)-induced contraction was not different in the aorta (1.78±0.39 versus 1.71±0.05), carotid (0.57±0.13 versus 0.43±0.02), mesenteric (0.43±0.08 versus 0.44±0.05), or renal artery (0.46±0.09 versus 0.41±0.02 gram) of Preg+sFlt-1+PIGF versus Preg rats, respectively.

**Effect of endothelium blockade or removal on Phe contraction**

We tested the role of endothelial NO-cGMP pathway in the differences in contraction among the different animal groups and found that in endothelium-intact aorta (Fig. 4A), carotid (Fig. 4B), mesenteric (Fig. 4C) and renal artery of Preg rats (Fig. 4D), treatment with the NOS inhibitor L-NAME or the guanylate cyclase inhibitor ODQ enhanced Phe contraction in grams. Also, Phe contraction in grams was enhanced in endothelium-denuded versus intact vessels of Preg rats (Fig. 4A-D, Table 1). Phe contraction in grams was enhanced to a less extent by L-NAME in the aorta (Fig. 4E) and by endothelium removal in the carotid artery (Fig. 4F), and was not affected by blockers of endothelial NO-cGMP or endothelium-removal in the mesenteric (Fig. 4G) or renal artery (Fig. 4H) of Preg+sFlt-1 rats, suggesting that endothelial function is compromised in Preg+sFlt-1 rats. Phe contraction in grams was also enhanced to a less extent by endothelium blockers or removal in the aorta (Fig. 4I), and by endothelium removal in the carotid (Fig. 4J) and mesenteric artery (Fig. 4K), and was not affected by endothelium blockers or removal in renal artery of RUPP rats (Fig. 4L, Table 1), suggesting that endothelial function is also compromised in RUPP rats. Importantly, Phe contraction in
grams was enhanced by endothelium blockers or removal in the aorta (Fig. 4M), carotid (Fig. 4N), mesenteric (Fig. 4O) and renal artery (Fig. 4P) of RUPP+PIGF rats to levels that closely mimic those observed during similar treatment of respective vessels from control Preg rats, suggesting restored functional endothelium. Of note, the differences in vascular reactivity between groups were less apparent in vessels treated with NO-cGMP blockers and in endothelium-denuded vessels (Table 1), suggesting that the differences in vascular reactivity are likely due to differences in the endothelial NO-cGMP pathway. Also, measurement of Phe contraction in L-NAME or ODQ treated or endothelium-denuded vessels as fold change of the control contraction in nontreated intact vessels, showed greater fold change in Phe contraction in Preg compared with Preg-sFlt-1 or RUPP rats, and in RUPP+PIGF versus RUPP (Table 1), supporting differences in endothelial NO-cGMP activity among the different animal groups.

When Phe contraction was presented as %max and EC50 was calculated the Phe response did not change significantly by L-NAME or ODQ treatment or endothelium removal in the aorta, carotid and renal artery of the different animal groups (Fig. 5, Table 1), suggesting little change in sensitivity to Phe. Phe was more potent in inducing contraction in ODQ treated mesenteric vessels of all animal groups and in L-NAME treated mesenteric vessels of Preg-sFlt-1 rats (Fig. 5, Table 1)

When the Phe response was presented as % of KCl contraction, treatment with L-NAME or ODQ enhanced Phe contraction in the aorta, carotid, mesenteric and renal artery of Preg rats (Fig. 6A-D, Table 1). Only treatment with L-NAME enhanced Phe contraction as % of KCl contraction in mesenteric artery (Fig. 6G), and endothelium NO-cGMP blockers or removal did not affect the Phe response in the aorta (Fig. 6E), carotid (Fig. 6F) or renal artery of Preg+sFlt-1 rats (Fig. 6F). Also, only ODQ enhanced Phe contraction as % of KCl contraction in the aorta (Fig. 6I), but endothelium NO-cGMP blockers or removal did not affect Phe response in the carotid (Fig. 6J), mesenteric (Fig. 6K) or renal artery of RUPP rats (Fig. 6L).

On the other hand, treatment with L-NAME or ODQ or endothelium removal enhanced Phe contraction as % of KCl in the aorta, carotid, mesenteric and renal artery of RUPP+PIGF rats
(Fig. 6M-P, Table 1). Of note, the enhancement of Phe contraction as % of KCl in RUPP+PIGF was more pronounced and exceeded that in RUPP rats in carotid artery treated with L-NAME or endothelium-denuded, and in mesenteric artery treated with ODQ (Table 1). Also, measurement of the changes in Phe contraction in L-NAME or ODQ-treated or endothelium-denuded vessels as fold change of the control contraction in nontreated intact vessels showed greater fold change in Phe contraction as % of KCl in Preg compared with Preg-sFlt-1 and RUPP, and in RUPP+PIGF versus RUPP rats (Table 1), supporting differences in endothelial NO-cGMP among the different animal groups.

Effect of altering sFlt-1/PIGF balance on ACh-induced vascular relaxation

In endothelium-intact vessels, ACh-induced relaxation was reduced in the aorta (Fig. 7A), carotid (Fig 7B), and renal artery (Fig. 7D) of Preg+sFlt-1 and RUPP versus Preg rats, suggesting that sFlt-1 infusion in Preg rats causes reduction in endothelium-dependent relaxation similar to that in RUPP rats. ACh relaxation was enhanced in RUPP+PIGF versus RUPP and to levels similar to those in control Preg rats, suggesting that restoring sFlt-1/PIGF balance improves endothelium-dependent relaxation in HTN-Preg rats. Maximal ACh relaxation was similar in the mesenteric artery of all animal groups (Fig. 7C). ACh concentration-response curve was also shifted to the right, and ACh was less potent in the aorta, carotid, mesenteric, and renal artery of Preg+sFlt-1 and RUPP versus Preg rats, suggesting impaired endothelium-dependent relaxation. Importantly, the sensitivity shift in ACh relaxation was corrected in RUPP+PIGF versus RUPP rats and to levels comparable to those in control Preg rats (Table 1). ACh relaxation was completely blocked by the NOS inhibitor L-NAME or guanylate cyclase inhibitor ODQ in the aorta, carotid and renal artery of all animal groups (Fig. 8). ACh relaxation was only partially blocked by L-NAME and ODQ in the mesenteric artery of all groups. ACh relaxation was abolished by endothelium removal in all vessels tested from all animal groups (Fig. 8), supporting a role of endothelial NO-cGMP relaxation pathway.
Effect of altering sFlt-1/PlGF balance on vascular eNOS levels and NO production

Western blot analysis revealed that total eNOS (Fig. 9A) and activated p-eNOS (Fig. 9B) were reduced in aortic tissue homogenate of Preg+sFlt-1 and RUPP versus Preg rats, and were enhanced in RUPP+PIGF versus RUPP and to levels similar to those in control Preg rats. Further analysis of p-eNOS/total eNOS ratio showed that it was not significantly different between Preg (0.64±0.26), Preg+sFlt-1 (1.03±0.32), RUPP (1.13±0.59) and RUPP+PIGF rats (0.89±0.31), suggesting that the observed differences in p-eNOS are mainly due to changes in eNOS expression. In endothelium-intact aortic rings (Fig. 9C), nitrate/nitrite production under 10^{-9} M ACh was lower in Preg+sFlt-1 and RUPP versus Preg rats and higher in RUPP+PIGF versus RUPP. A less distinct trend in nitrate/nitrite production under 10^{-9} M ACh was observed in mesenteric artery rings, and the differences in nitrate/nitrite production under 10^{-9} M ACh were not significant among the different groups (Fig. 9D). ACh caused concentration-dependent increases in nitrate-nitrite production that were reduced in aorta and mesenteric artery of Preg+sFlt-1 and RUPP versus Preg rats, and was enhanced in RUPP+PIGF versus RUPP and to levels similar to those in control Preg rats (Fig. 9C, 9D). These data suggest that the observed effects of sFlt-1 and PIGF treatment involve long-term changes in eNOS expression which in turn affect the extent of eNOS phosphorylation and activation and NO production.

Effect of altering sFlt-1/PlGF balance on endothelium-independent relaxation

In endothelium-denuded vessels, the exogenous NO donor sodium nitroprusside (SNP) caused concentration-dependent relaxation that was not significantly different in the aorta (Fig. 10A), carotid (Fig. 10B), mesenteric (Fig. 10C) or renal artery (Fig. 10D) of all the animal groups (Table 1), supporting that the decreased responsiveness to ACh in Preg+sFlt-1 and RUPP rats and their recovery in RUPP+PIGF rats are due to changes in endothelium-derived relaxing factors rather than the ability of VSM to relax.
DISCUSSION

The present study assessed the extent of sFlt-1/PlGF imbalance and vascular dysfunction in HTN-Preg RUPP rats, and showed how inducing a comparable sFlt-1/PlGF imbalance by infusing sFlt-1 in Preg rats was sufficient to increase BP and vascular contraction and decrease endothelium-dependent relaxation, eNOS expression/activity and NO production to levels similar to those in RUPP rats. Using these guiding measurements, the present study then demonstrated that restoring sFlt-1/PlGF balance by infusing PlGF in HTN-Preg RUPP rats reversed the decrease in endothelial NO-cGMP and the increases in vascular contraction and BP and brought them to levels comparable to those in control Preg rats.

sFlt-1/PlGF imbalance and increased vasoconstriction in HTN-Preg

Inadequate trophoblast invasion of spiral arteries and the ensuing uteroplacental insufficiency are important events in the pathogenesis of PE (17, 31, 63). Studies in RUPP and hypoxic pregnant rats have supported placental ischemia as an initiating mechanism of HTN-Preg and IUGR (11, 19, 31, 81). In agreement, the present study showed increased BP and decreased litter size and pup weight in RUPP vs. Preg rats. We assessed the extent of vascular dysfunction in RUPP rats and found increases in contraction to the \( \alpha \)-adrenergic receptor agonist Phe in four major systemic vessels with relevance to PE. The increased aortic contraction is important as it supplies all the vascular beds in the systemic circulation. The increased carotid artery contraction could affect the cephalic circulation and in turn contribute to the cerebral manifestations in PE. The increased contraction in the mesenteric vascular bed, which constitute a major portion of peripheral resistance (9), could contribute to the increased BP. The increased renal vasoconstriction could contribute to the changes in glomerular filtration rate, plasma volume and protein excretion in PE. These observations support changes in vascular reactivity as an important mechanism of the increased BP and other manifestations in HTN-Preg RUPP rats (50, 53).
PE has also been postulated to involve angiogenic imbalance with a tilt towards anti-
angiogenic factors (19, 38, 39, 45, 48). The observed increase in plasma sFlt-1 levels and sFlt-
1/PIGF ratio in RUPP rats is consistent with reports that circulating sFlt-1 and the sFlt-1/PIGF 
ratio are elevated in PE women (6, 45, 51, 72, 73), and in RUPP and other animal models of 
HTN-Preg (18, 19, 23, 60, 67). The sFlt-1/PIGF ratio can be modulated by not only an increase 
in sFlt-1, but also a decrease in PIGF. The present observation that plasma levels of PIGF are 
decreased in RUPP vs. Preg rats is consistent with reports that PIGF levels are decreased in 
PE women (6, 51, 72) and in RUPP and DOCA-salt rat models of HTN-Preg (1, 19).

We assessed the pressor and vascular effects of inducing a sFlt-1/PIGF imbalance 
comparable to that in RUPP rats and found that infusing sFlt-1 in Preg rats was associated 
with increases in BP. This is consistent with reports that Preg rats treated with sFlt-1 develop 
HTN, proteinuria, and marked glomerular endotheliosis (25, 48). The increase in BP in 
Preg+sFlt-1 rats was associated with increases in Phe contraction, suggesting that circulating 
sFlt-1 enhances vasoconstrictor stimuli and may function as a link between RUPP and the 
vascular hyper-reactivity in HTN-Preg. If this is the case, then restoring sFlt-1/PIGF balance 
should reverse the increase in BP and vascular contraction in HTN-Preg. In the present study, 
infusing PIGF in RUPP rats improved the sFlt-1/PIGF ratio, reduced BP, and reversed the 
increases in Phe contraction to levels similar to those in control Preg rats, providing evidence 
for a role of PIGF in regulating sFlt-1/PIGF balance, BP and vascular function in HTN-Preg.

sFlt-1/PIGF balance and mechanisms of vascular contraction in HTN-Preg

The increases in vascular contraction in RUPP and Preg+sFlt-1 rats and their reversal in 
RUPP+PIGF rats appear to involve changes in Ca$^{2+}$ entry, as high KCl, a known activator of 
voltage-gated Ca$^{2+}$ channels (VGCCs) (53), caused greater contraction in RUPP and 
Preg+sFlt-1 vs. Preg and RUPP+PIGF rats. While these findings point to increased vascular 
Ca$^{2+}$ influx through VGCCs in HTN-Preg (50, 53), Phe may also activate Ca$^{2+}$ influx through 
receptor- and store-operated Ca$^{2+}$ channels (53). Nevertheless, the increased vascular
contraction to both Phe and KCl with potentially different signaling mechanisms in Preg+sFlt-1 and RUPP rats and the reversal of this increase in RUPP+PlGF rats could be explained by common changes in endothelium-dependent relaxation pathways. Phe contraction was enhanced in endothelium-denuded vessels of Preg and RUPP+PlGF rats, but to a less extent in Preg+sFlt-1 and RUPP rats. Also, the differences in Phe and KCl contraction among the different rat groups were observed in intact vessels, but were absent when the endothelium was removed. Thus it is plausible that the enhanced vascular contraction in RUPP and Preg+sFlt-1 rats is due to reduction in endothelium-derived relaxing factors, and the reduced vascular hyper-reactivity in RUPP+PlGF rats could be due to improved endothelial function, making it necessary to further examine endothelial function in the different animal groups.

**sFlt-1/PlGF balance and mechanisms of vascular relaxation in HTN-Preg**

ACh caused less relaxation in the aorta, carotid and renal artery and was less potent in all vessels of RUPP and Preg+sFlt-1 vs. Preg rats, suggesting that sFlt-1/PlGF imbalance is an important factor in the reduced endothelium-dependent relaxation in HTN-Preg. These data are in agreement with previous studies showing a decrease in ACh relaxation or sensitivity to ACh in the aorta (11) and mesenteric artery of HTN-Preg RUPP rats (50, 78). ACh relaxation was improved in the aorta, carotid and renal artery and ACh was more potent in all vessels of RUPP+PlGF vs. RUPP rats, suggesting that restoring sFlt-1/PlGF balance reverses the decrease in endothelium-dependent relaxation mechanisms in HTN-Preg.

The endothelium releases vasodilator substances such as NO, PGI₂, and EDHF. NO diffuses into VSM where it increases cGMP, which in turn causes relaxation by decreasing VSM [Ca^{2+}], and Ca^{2+} sensitivity of the contractile proteins (27, 42). Our previous studies in the aorta, carotid and renal artery of virgin rats showed that ACh relaxation was associated with increased NO production and was inhibited by L-NAME, suggesting a role of endothelial NO-cGMP (62). In contrast, in the mesenteric artery, a large portion of ACh relaxation remained in the presence of L-NAME and the COX inhibitor indomethacin, and was abolished by the K⁺
channel blocker TEA, suggesting a role of EDHF (49). The enhanced vascular contraction in
RUPP and Preg+sFlt-1 vs. Preg rats appears to involve reduction in endothelial NO-cGMP
because the NOS inhibitor L-NAME and guanylate cyclase inhibitor ODQ enhanced Phe
contraction markedly in Preg rats but to a less extent in RUPP and Preg+sFlt-1 rats,
suggesting that an endothelial NO-cGMP pathway that reduces contraction during pregnancy
is compromised in HTN-Preg rats. Of note, blocking endothelial NO-cGMP pathway did not
affect the sensitivity to Phe in the aorta, carotid and renal artery, while Phe was more potent in
inducing contraction in ODQ-treated mesenteric artery of all animal groups (Fig. 5, Table 1),
suggesting increased sensitivity to changes in cGMP in the mesenteric circulation. The present
data also suggest that sFlt-1 may downregulate endothelial NO-cGMP in HTN-Preg because:
1) In all animal groups, ACh relaxation was abolished by endothelium removal in all vessels
tested, and was blocked by L-NAME or ODQ, completely in the aorta, carotid and renal artery
and partially in the mesenteric artery. 2) Total eNOS and activated p-eNOS were reduced in
the aorta of Preg+sFlt-1 and RUPP rats vs. Preg rats. 3) ACh-induced NO production was
reduced in aorta and mesenteric artery of Preg+sFlt-1 and RUPP rats vs. Preg rats. 4) Vascular relaxation to the NO donor SNP was not different in Preg+sFlt-1 and RUPP rats vs.
Preg rats, further suggesting a role of endothelial NO-cGMP in the observed changes in
vascular relaxation rather than differences in the ability of VSM to relax.

Our experiments with PlGF to counterbalance sFlt-1 in RUPP rats provide evidence that
the reduced BP and vascular contraction in RUPP+PIGF involve improvement in endothelial
NO-cGMP because: 1) Endothelium-removal or treatment with L-NAME or ODQ enhanced
Phe contraction in RUPP+PIGF to levels similar to those in RUPP rats. 2) ACh-induced
relaxation was enhanced in RUPP+PIGF vs. RUPP and to levels similar to those in control
Preg rats. 3) Total eNOS and p-eNOS were augmented in the aorta of RUPP+PIGF vs. RUPP
rats. 4) ACh nitrate/nitrite production was enhanced in the aorta and mesenteric artery of
RUPP+PIGF vs. RUPP rats. 5) Vascular relaxation to the NO donor SNP was not different in
RUPP+PIGF vs. RUPP rats, suggesting that the differences are not related to differences in
the ability of VSM to relax, and further suggesting a role of endothelial NO-cGMP in the observed changes in vascular relaxation.

The changes in the vascular relaxation pathways are particularly important in the mesenteric circulation as it contributes substantially to BP regulation and could be one of the main sites altered in HTN-Preg. Similar to the observations in the aorta, carotid and renal artery, ACh was less potent in causing relaxation in the mesenteric artery of RUPP and Preg+sFlt-1 vs. Preg rats and more potent in RUPP+PIGF vs. RUPP rats. Also, similar to the observation in the aorta, ACh-induced nitrate/nitrite production was reduced in RUPP and Preg+sFlt-1 vs. Preg rats and enhanced in RUPP+PIGF vs. RUPP rats. However, in contrast with other vascular beds, maximal ACh relaxation was not different in the mesenteric artery of the different animal groups. Also, in contrast with the complete blockade of ACh relaxation in the aorta, carotid and renal artery by the NOS inhibitor L-NAME and guanylate cyclase inhibitor ODQ, ACh relaxation of the mesenteric artery was only partially inhibited by blockade of the NO-cGMP pathway. These differences emphasize how the changes in angiogenic factors during pregnancy could affect different signaling pathways in different vascular beds. In this respect, EDHF has been suggested as an important vasodilator in mesenteric arteries (16) and may explain the observed L-NAME and ODQ-resistant component of ACh-induced relaxation. However, we have previously shown that blockade of K+ channels with tetraethylammonium (TEA) or apamin+TRAM-34 caused similar inhibition of ACh-induced relaxation in mesenteric microvessels of both Preg and RUPP rats, suggesting an intact and equally active EDHF-dependent relaxation pathway (49).

The effects of antiangiogenic and angiogenic factors on the NO-cGMP pathway could be due to their general effects on angiogenesis and in turn endothelial cell development and signaling, as well as direct effects on endothelial cell signaling. Studies in human umbilical vein endothelial cells (HUVECs) cocultured with hypoxic human trophoblasts have shown an increase in sFlt-1 and a decrease in VEGF in cell-conditioned medium that are associated with endothelial cell dysfunction and decreased eNOS synthesis (82). VEGF has been shown to
regulate angiogenesis, endothelial cell proliferation, and vascular permeability (29, 65). VEGF also increases [Ca^{2+}], Ca^{2+}/calmodulin, eNOS activity and PGI2 (10, 24, 66). Also, in HUVECs, VEGF induces Akt activation, eNOS Ser^{1177} phosphorylation and Ca^{2+}-independent NO generation, and blockade of VEGFR leads to decreased Akt activity and eNOS phosphorylation (10). Likewise, PIGF promotes endothelial cell growth, vasculogenesis, and placental development (65). PIGF also dilates uterine and mesenteric artery via VEGFR-1 and endothelium-derived vasodilators (44, 52). Also, a dimeric ligand comprising VEGF and PlGF-1 causes activation and tyrosine phosphorylation of VEGFR, endothelial cell migration, in vitro tube formation, Ca^{2+} mobilization, eNOS phosphorylation and NO production in HUVECs, and induces L-NAME sensitive vasorelaxation in rat aorta (12). Furthermore, PIGF causes vasodilation and increases cGMP production in the pulmonary vasculature of perfused piglet lungs (28), supporting a role of PIGF in activating the NO-cGMP vasodilation pathway.

**Other observations/considerations**

The observed antagonism between sFlt-1 and PIGF in the setting of sFlt-1/PlGF imbalance, increased BP and vascular hyper-reactivity in RUPP rats could involve sFlt-1 sequestering of endogenous PIGF and VEGF, which would in turn lead to a decrease in plasma free PIGF and VEGF. In the present study, we used PIGF to restore sFlt-1/PlGF balance in RUPP rats. A previous elegant study used recombinant VEGF infusion at 90 or 180 µg/kg/day to restore angiogenic balance, and showed that it lowered BP and improved renal function in rats with placental ischemia-induced HTN (21). We used PIGF instead of VEGF because: 1) Most studies show decreased PIGF levels in PE (6, 45, 72), while measurements of VEGF have not been consistent with studies showing decreased (56), not changed (46, 48) or even increased levels in PE (7, 26, 72). 2) In PE, there is an increase in circulating sFlt-1 that binds to VEGF, thus total (bound and unbound) VEGF levels could be elevated or not changed in most forms of PE and PE-like models. On the other hand, the angiogenic imbalance is induced because the free, unbound form of VEGF is decreased, due to the
sequestering, neutralizing effect of sFlt-1 (5). 3) VEGF may stimulate sFlt-1 production in the placenta through an action on VEGFR-2 (15). VEGF levels are controlled at the maternal-fetal interface, partly through feedback modulation of sFlt-1, in order to prevent damage to the placenta or fetus by excess VEGF (15), and dysregulation of this feedback mechanism may complicate the measurement of angiogenic and anti-angiogenic levels. 4) PlGF is specific for VEGFR-1 and its soluble form sFlt-1, while VEGF also binds to VEGFR-2, causing increased vascular permeability and edema (43) and could promote cancer partly through its angiogenic effects (61).

Also, while preparing this manuscript, a recently published study showed that infusion of recombinant human PlGF at 180 μg/kg/day abolished placental ischemia-induced HTN-Preg in rats (68). We used a smaller dose 20 μg/kg/day of PlGF because 1) Using our guiding measurements of sFlt-1 and PlGF levels in Preg, RUPP and Preg+sFlt-1 rats, we aimed to reduce the sFlt-1/PlGF ratio to levels that closely resemble those in control Preg rats. 2) The present dose of PlGF was sufficient to counterbalance sFlt-1 because the sFlt-1/PlGF ratio was decreased in RUPP+PlGF vs. RUPP rats, and to levels similar to those in control Preg rats. 3) The present dose of PlGF was sufficient to block the effects of sFlt-1 as BP was reduced in RUPP+PlGF rats and in Preg+sFlt-1+PlGF rats to levels similar to those in control Preg rats. A lower dose of PlGF (10 μg/kg/day) was not sufficient to decrease BP in RUPP rats. 4) PlGF reversed the increases in Phe contraction and the decreases in ACh relaxation, eNOS expression/activity and NO production in RUPP rats to levels similar to those in control Preg rats. 5) We avoided higher doses of PlGF as excess PlGF may induce microvascular abnormalities (32) or developmental anomalies (69). 6) The angiogenic/anti-angiogenic balance is tightly controlled by feedback mechanisms, and excess PlGF could drive a feedback increase in sFlt-1 to maintain the sFlt-1/PlGF ratio.

The present study showed that blocking the endothelial NO-cGMP relaxation pathway by endothelium removal or treatment with the NOS inhibitor L-NAME or guanylate cyclase inhibitor ODQ enhanced Phe contraction not only in grams but also as % of KCl contraction.
Assuming that KCl contraction is mainly due to Ca\(^{2+}\) influx (53), then the enhanced Phe contraction in endothelium-denuded and NO-cGMP-blocked vessels is likely due to activation of Ca\(^{2+}\)-sensitization pathways such as protein kinase C (PKC) or Rho-kinase (13, 64). While Phe contraction as % of KCl was not different in endothelium-intact vessels from the different groups (Fig. 3), the enhancement of Phe contraction by endothelium removal or NO-cGMP blockade was more apparent in Preg than Preg+sFlt-1 or RUPP and in RUPP+PIGF than RUPP rats (Fig. 8, Table 1). The differences in Ca\(^{2+}\) sensitization pathways were not observed in endothelium-intact vessels because they were likely obscured by increased NO-cGMP, and only with endothelium removal or blockade of NO-cGMP the enhanced contraction mechanisms could be manifested. This is supported by reports that NO and cGMP reduce [Ca\(^{2+}\)]\(_i\) (42), and cGMP-dependent protein kinase causes phosphorylation and inhibition of myosin light chain kinase and Ca\(^{2+}\) sensitization pathways (64). Whether the differences in the Ca\(^{2+}\) sensitization mechanisms of VSM contraction are due to changes in sFlt-1 or PIGF is unclear and needs to be further examined.

In addition to endothelium-derived vasodilators, the endothelium releases contracting factors such as endothelin-1 (ET-1). Studies have shown an association between sFlt-1/PIGF imbalance and the levels of ET-1 in PE (77). Also, we have shown increases in ET-1 induced vasoconstriction in mesenteric vessels of RUPP rats (50), and whether the vascular responses to ET-1 are altered with changes in sFlt-1 and PIGF in HTN-Preg should to be tested.

The present data should be interpreted with caution as there are forms of HTN-Preg that may not be represented by the RUPP model such as the Dahl salt-sensitive HTN-Preg rat and the catechol-O-methyltransferase deficient mice (23, 30). Also, other anti angiogenic factors may be released and affect vascular function in PE. Soluble endoglin (sEng) is an anti angiogenic protein that binds transforming growth factor-β1 and inhibits its effects on signaling, eNOS activation and vasodilation (29). Serum sEng levels are higher in PE and HELLP syndrome compared with normal Preg women (76), and in RUPP vs. normal Preg rats (20). The role of sEng in the changes in vascular function in HTN-Preg should be further examined.
Increases in other bioactive factors such as the cytokines TNF-α and interleukin-6, reactive oxygen species, hypoxia inducible factor, angiotensin type 1 receptor autoantibodies have also been observed in PE women and in animal models of HTN-Preg (19, 34-36, 38, 65, 80), and future studies should examine the integrated role of these bioactive factors in the changes in vascular function in HTN-Preg and PE.

**Perspective**

The present study showed that similar to RUPP rats, inducing sFlt-1/PIGF imbalance by infusing sFlt-1 in Preg rats increases BP and vascular contraction and decreases endothelial NO-cGMP-mediated vasodilation, supporting that endothelial dysfunction is a central mechanism in HTN-Preg and that sFlt-1 is an important intermediary factor linking placental ischemia to decreased endothelial function in HTN-Preg. In this respect, counterbalancing sFlt-1 by infusing PIGF enhanced eNOS expression and NO-mediated vasodilation and reduced vascular contraction and BP in HTN-Preg RUPP rats. Currently, delivery of the baby and placenta is the only effective measure to reverse PE. The present findings could be useful in designing new approaches for the management of HTN-Preg and PE. For example, activators of NO-cGMP could counteract the sFlt-1-induced decrease in eNOS expression/activity and NO production in HTN-Preg. This is in line with reports that the phosphodiesterase-5 (PDE-5) inhibitor sildenafil, which enhances and prolongs the effects of cGMP, ameliorates HTN-Preg in RUPP and Dahl salt-sensitive rats (18, 22). Another approach is extracorporeal removal of circulating sFlt-1 from PE patients. Although this procedure decreases the sFlt-1/PIGF ratio and could improve PE symptoms and prolong pregnancy (70), it may require advanced apheresis equipment and highly trained clinical staff that may only be feasible in large medical centers. An alternative approach is to infuse PIGF to restore sFlt-1/PIGF balance and reverse the maternal vascular hyper-reactivity and increased BP in PE. Preterm birth is another undesirable outcome of PE, and could be the only measure to prevent the progress to eclampsia. Our data in RUPP and Preg+sFlt-1 rats support that sFlt-1/PIGF imbalance is
associated with decreased litter size and pup weight. Importantly, RUPP+PlGF rats showed improved litter size and pup weight when compared with RUPP rats, suggesting that PlGF not only counterbalances the effects of sFlt-1 in the maternal circulation, but could also promote fetal growth and development and prolong pregnancy in HTN-Preg and PE. An important question is how PlGF would improve fetal parameters in the face of fixed RUPP. We propose that RUPP is only an initiating event that triggers a cascade of biochemical and pathological events that lead to the different maternal and fetal manifestations associated with preeclampsia. The initial physically-induced reduction in uterine perfusion pressure in the RUPP rat is thought to cause initial placental ischemia/hypoxia which would increase the release of antiangiogenic sFlt-1, cause sFFlt-1/PlGF imbalance and decrease angiogenesis, leading to further RUPP, placental ischemia and a deleterious vicious cycle that would lead to further decreases in angiogenesis and IUGR. Restoring the sFlt-1/PlGF balance would disrupt the vicious cycle, and ameliorate the placental ischemia despite the fixed physical RUPP, leading to improved angiogenesis and IUGR.

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FIGURE LEGENDS

**Fig. 1.** Maternal and fetal parameters in Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. On day 19 of pregnancy, maternal plasma sFlt-1 (A), PlGF (B), sFlt-1/PIGF ratio (C) and BP (D) as well as litter size (E) and average pup weight (F) were compared in Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. Data represent means±SEM, n=6-7. *P<0.05, Preg+sFlt-1 or RUPP vs. Preg. #P<0.05, RUPP+PIGF vs. RUPP.

**Fig. 2.** KCl-induced contraction in blood vessels of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. KCl (96 mM)-induced contraction was compared in endothelium-intact (A-D) and denuded (-Endo) (E-H) aorta, carotid, mesenteric and renal artery of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats, Data represent means±SEM, n=5-9. *P<0.05, Preg+sFlt-1 or RUPP vs. Preg. #P<0.05, RUPP+PIGF vs. RUPP.

**Fig. 3.** Phe-induced contraction in blood vessels of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. Endothelium-intact aortic, carotid, mesenteric and renal artery rings from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were stimulated with increasing concentrations of Phe, the contractile response was measured and presented in grams (g) (A-D) as % of maximum Phe contraction (E-H), or as % of KCl contraction (I-L). Data represent means±SEM, n=5-9. *P<0.05, Preg+sFlt-1 or RUPP vs. Preg. #P<0.05, RUPP+PIGF vs. RUPP.

**Fig. 4.** Effect of endothelium removal or blockade of NO-cGMP pathway on Phe-induced contraction in grams (g). Isolated aortic, carotid, mesenteric and renal artery rings from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were either kept endothelium-intact (open circles), pretreated with the NOS inhibitor L-NAME (3x10^-4 M) (closed circles) or the guanylate cyclase inhibitor ODQ (10^-5 M) (open triangles) for 15 min, or endothelium-denuded (closed triangles). The vessels were stimulated with increasing concentrations of Phe, and the contractile response was presented in grams (g). Data represent means±SEM, n=5-9. *P<0.05, L-NAME
treated vs. nontreated vessels. *P<0.05, ODQ-treated vs. nontreated vessels. † endothelium-denuded (-Endo) vs. intact (+Endo) vessels.

**Fig. 5.** Effect of endothelium removal or blockade of NO-cGMP pathway on sensitivity to Phe. Aortic, carotid, mesenteric and renal artery rings from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were either kept endothelium-intact (open circles), pretreated with the NOS inhibitor L-NAME (3x10\(^{-4}\) M) (closed circles) or guanylate cyclase inhibitor ODQ (10\(^{-5}\) M) (open triangles), or endothelium-denuded (closed triangles). The vessels were stimulated with increasing concentrations of Phe, and the contractile response was presented as % of maximum Phe contraction. Data represent means±SEM, n=5-9. *P<0.05, L-NAME treated vs. nontreated vessels. *P<0.05, ODQ-treated vs. nontreated vessels. † endothelium-denuded (-Endo) vs. intact (+Endo) vessels.

**Fig. 6.** Effect of endothelium removal or blockade of NO-cGMP on Phe-induced contraction relative to control KCl contraction. Aortic, carotid, mesenteric and renal artery rings from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were either kept intact (open circles), pretreated with the NOS inhibitor L-NAME (3x10\(^{-4}\) M) (closed circles) or guanylate cyclase inhibitor ODQ (10\(^{-5}\) M) (open triangles), or endothelium-denuded (closed triangles). After measuring contraction to 96 mM KCl, the vessels were stimulated with increasing concentrations of Phe, and the contractile response was presented as % of KCl contraction. Data represent means±SEM, n=5-9. *P<0.05, L-NAME treated vs. nontreated vessels. *P<0.05, ODQ-treated vs. nontreated vessels. † endothelium-denuded (-Endo) vs. intact (+Endo) vessels.

**Fig. 7.** ACh-induced relaxation in blood vessels of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. Endothelium-intact aortic (A), carotid, (B) mesenteric (C) and renal artery rings (D) from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were precontracted with submaximal concentration of Phe, then stimulated with ACh (10\(^{-9}\)-10\(^{-5}\) M) and the % relaxation of Phe...
contraction was measured. Data represent means±SEM, n=6-9. *P<0.05, Preg+sFlt-1 or RUPP vs. Preg. #P<0.05, RUPP+PIGF vs. RUPP.

**Fig. 8.** Role of endothelial NO-cGMP pathway in ACh-induced vascular relaxation. Isolated aortic, carotid, mesenteric and renal artery rings from Preg (A-D), Preg+sFlt-1 (E-H), RUPP (I-L) and RUPP+PIGF rats (M-P) were either kept endothelium-intact (open circles), pretreated with the NOS inhibitor L-NAME (3x10^-4 M) (closed circles) or the guanylate cyclase inhibitor ODQ (10^-5 M) (open triangles) for 10 min, or endothelium-denuded (closed triangles). The vessels were precontracted with submaximal concentration of Phe, then stimulated with ACh (10^-9-10^-5 M) and the % relaxation of Phe contraction was measured. Data represent means±SEM, n=6-9. *P<0.05, L-NAME treated vs. nontreated vessels. #P<0.05, ODQ-treated vs. nontreated vessels. † endothelium-denuded (-Endo) vs. intact (+Endo) vessels.

**Fig. 9.** Protein amount of eNOS and NO production in blood vessels of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. Aortic tissue homogenate from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were prepared for Western blots using eNOS and p-eNOS antibodies (1:800), and both total eNOS (A) and activated p-eNOS (B) were measured. In parallel experiments, ACh induced nitrate/nitrite (NOx) production was measured in the aorta (C) and mesenteric artery (D) of different animal groups. Data represent means±SEM, n=4-7. *P<0.05, Preg+sFlt-1 or RUPP vs. Preg. #P<0.05, RUPP+PIGF vs. RUPP.

**Fig. 10.** SNP-induced relaxation in blood vessels of Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats. Endothelium-denuded aortic (A), carotid, (B) mesenteric (C) and renal artery rings (D) from Preg, Preg+sFlt-1, RUPP and RUPP+PIGF rats were precontracted with submaximal concentration of Phe, then stimulated with SNP (10^-9-10^-5 M) and the % relaxation of Phe contraction was measured. Data represent means±SEM, n=6-9.
Table 1. Phe contraction, and ACh and SNP relaxation in the aorta, carotid, mesenteric and renal artery of Preg, Preg+sFlt-1, RUPP, and RUPP+PIGF rats.

<table>
<thead>
<tr>
<th>Control</th>
<th>+l-NAME</th>
<th>+ODQ</th>
<th>-Endo</th>
<th>+l-NAME</th>
<th>+ODQ</th>
<th>-Endo</th>
<th>+l-NAME</th>
<th>+ODQ</th>
<th>-Endo</th>
<th>+l-NAME</th>
<th>+ODQ</th>
<th>-Endo</th>
<th>+l-NAME</th>
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<tr>
<td>Phe Maximum</td>
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<tr>
<td>Contraction (g)</td>
<td>1.5±0.18</td>
<td>0.46±0.02</td>
<td>0.44±0.05</td>
<td>0.41±0.02</td>
<td>2.0±0.07</td>
<td>*</td>
<td>0.61±0.05</td>
<td>*</td>
<td>0.62±0.05</td>
<td>*</td>
<td>0.59±0.03</td>
<td>*</td>
<td>2.2±0.09</td>
<td>*</td>
<td>0.58±0.01</td>
</tr>
<tr>
<td>+L-NAME</td>
<td>2.3±0.21</td>
<td>0.78±0.10</td>
<td>0.85±0.10</td>
<td>0.68±0.09</td>
<td>2.4±0.19</td>
<td>*</td>
<td>0.64±0.09</td>
<td>*</td>
<td>0.67±0.06</td>
<td>0.68±0.12</td>
<td>2.8±0.18</td>
<td>*</td>
<td>0.60±0.06</td>
<td>0.85±0.08</td>
<td>0.65±0.18</td>
</tr>
<tr>
<td>+ODQ</td>
<td>2.5±0.26</td>
<td>0.72±0.11</td>
<td>1.0±0.06</td>
<td>0.70±0.11</td>
<td>2.4±0.14</td>
<td>*</td>
<td>0.73±0.07</td>
<td>0.64±0.08</td>
<td>*</td>
<td>0.65±0.10</td>
<td>2.6±0.15</td>
<td>*</td>
<td>0.71±0.07</td>
<td>0.84±0.17</td>
<td>0.58±0.13</td>
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<tr>
<td>+l-NAME + ODQ</td>
<td>2.1±0.21</td>
<td>0.65±0.06</td>
<td>0.98±0.14</td>
<td>0.65±0.02</td>
<td>2.2±0.19</td>
<td>0.75±0.03</td>
<td>0.68±0.08</td>
<td>0.66±0.15</td>
<td>2.7±0.11</td>
<td>*</td>
<td>0.77±0.09</td>
<td>0.85±0.06</td>
<td>0.68±0.07</td>
<td>2.5±0.16</td>
<td>*</td>
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<tr>
<td>ACh (10-8 M)</td>
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</tr>
<tr>
<td>Relaxation (%)</td>
<td>82.6±3.4</td>
<td>76.4±2.2</td>
<td>99.7±0.26</td>
<td>68.6±7.1</td>
<td>61.7±6.4</td>
<td>56.1±6.2</td>
<td>97.6±1.3</td>
<td>57.7±6.3</td>
<td>66.4±4.2</td>
<td>58.8±10.2</td>
<td>93.2±3.2</td>
<td>54.1±7.4</td>
<td>82.2±2.1</td>
<td>81.1±4.1</td>
<td>96.4±2.6</td>
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<tr>
<td>pEC50 (-log M)</td>
<td>7.10±0.08</td>
<td>6.86±0.12</td>
<td>7.51±0.08</td>
<td>6.95±0.16</td>
<td>6.66±0.13</td>
<td>6.09±0.18</td>
<td>6.76±0.14</td>
<td>6.44±0.19</td>
<td>6.28±0.22</td>
<td>6.00±0.19</td>
<td>6.74±0.14</td>
<td>6.47±0.12</td>
<td>7.27±0.06</td>
<td>*</td>
<td>6.47±0.17</td>
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<tr>
<td>SNP (10-7 M)</td>
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<td></td>
</tr>
<tr>
<td>Relaxation (%)</td>
<td>90.6±1.9</td>
<td>89.5±3.1</td>
<td>99.4±0.42</td>
<td>81.6±3.7</td>
<td>91.3±2.8</td>
<td>91.3±1.1</td>
<td>99.7±0.28</td>
<td>90.8±2.7</td>
<td>89.7±3.4</td>
<td>91.4±1.7</td>
<td>99.6±0.32</td>
<td>82.6±4.9</td>
<td>93.3±1.3</td>
<td>89.7±1.5</td>
<td>97.2±1.2</td>
</tr>
<tr>
<td>pEC50 (-log M)</td>
<td>8.4±0.17</td>
<td>8.3±0.10</td>
<td>8.8±0.11</td>
<td>7.9±0.12</td>
<td>8.5±0.12</td>
<td>8.7±0.23</td>
<td>9.1±0.09</td>
<td>8.0±0.08</td>
<td>8.5±0.16</td>
<td>8.4±0.08</td>
<td>9.0±0.10</td>
<td>7.9±0.18</td>
<td>8.5±0.10</td>
<td>8.5±0.07</td>
<td>8.8±0.12</td>
</tr>
</tbody>
</table>

Data represent means±SEM, n=5-9.

* P<0.05, Preg+sFlt-1 and RUPP vs. control Preg.

† P<0.05, RUPP+PIGF vs. RUPP

‡ P<0.05, L-NAME or ODQ-treated or endothelium-denuded (-Endo) vessels vs. control nontreated intact (+Endo) vessels of the same animal group.
Plasma Factors, Blood Pressure, Litter Size and Fetal Weight

**Fig. 1**

**A**
Plasma sFlt-1 (pg/ml)

- **B**
Plasma PlGF (pg/ml)

- **C**
Plasma sFlt-1/PlGF Ratio

- **D**
Blood Pressure (mmHg)

- **E**
Litter Size (No of Pups)

- **F**
Pup weight (g)
Fig. 2

KCl-Induced Contraction

<table>
<thead>
<tr>
<th>Condition</th>
<th>Aorta</th>
<th>Carotid</th>
<th>Mesenteric</th>
<th>Renal</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Endo</td>
<td><img src="#" alt="Graph A" /></td>
<td><img src="#" alt="Graph B" /></td>
<td><img src="#" alt="Graph C" /></td>
<td><img src="#" alt="Graph D" /></td>
</tr>
<tr>
<td>-Endo</td>
<td><img src="#" alt="Graph E" /></td>
<td><img src="#" alt="Graph F" /></td>
<td><img src="#" alt="Graph G" /></td>
<td><img src="#" alt="Graph H" /></td>
</tr>
</tbody>
</table>

- **Legend**:
  - Preg
  - Preg+sFlt-1
  - RUPP
  - RUPP+PIGF

*Significant differences compared to control*
Effect of Endothelium Blockade or Removal on Phe Contraction (g)

Fig. 4

Preg

Aorta

Carotid

Mesenteric

Renal

Preg + sFlt-1

RUPP

RUPP + PI GF

Phe Contraction (g)

log [Phe] (M)
Fig. 5  Effect of Endothelium Blockade or Removal on Phe Contraction (% Max)

Preg

Aorta

Carotid

Mesenteric

Renal

Preg+sFlt-1

RUPP

RUPP+PIGF

-Endo

L-NAME

Control

ODQ

Phe Contraction (% Max)

log [Phe] (M)
Fig. 6  
Effect of Endothelium Blockade or Removal on Phe Contraction (% 96KCl)
Endothelium-Dependent ACh-Induced Relaxation

Aorta

% Relaxation of Phe Contraction

B

Carotid

% Relaxation of Phe Contraction

C

Mesenteric

% Relaxation of Phe Contraction

D

Renal

% Relaxation of Phe Contraction

Fig. 7

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

Aorta Carotid Mesenteric Renal Endothelium-Dependent ACh-Induced Relaxation

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

Aorta Carotid Mesenteric Renal Endothelium-Dependent ACh-Induced Relaxation

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

Aorta Carotid Mesenteric Renal Endothelium-Dependent ACh-Induced Relaxation

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)

-10 -9 -8 -7 -6 -5
0
20
40
60
80
100
log [ACh] (M)
Endothelium-Mediated Relaxation Pathways

Fig. 8

- **Aorta**
- **Carotid**
- **Mesenteric**
- **Renal**

**Preg**
- Aorta (A)
- Carotid (B)
- Mesenteric (C)
- Renal (D)

**Preg+sFlt-1**
- Aorta (E)
- Carotid (F)
- Mesenteric (G)
- Renal (H)

**RUPP**
- Aorta (I)
- Carotid (J)
- Mesenteric (K)
- Renal (L)

**RUPP+PIGF**
- Aorta (M)
- Carotid (N)
- Mesenteric (O)
- Renal (P)

Graphs show the percentage relaxation of Phe contraction (% Relaxation of Phe Contraction) as a function of log [ACh] (M). Different curves represent control, L-NAME, ODQ, and Endo treatment conditions.
Fig. 10

Endothelium-Independent Relaxation

Aorta

B Carotid

C Mesenteric

D Renal

Log [SNP] (M)

% Relaxation of Phe Contraction