

A Maternally Sequestered, Biopolymer-Stabilized Vascular Endothelial Growth Factor (VEGF) Chimera for Treatment of Preeclampsia

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Background—Preeclampsia is a hypertensive syndrome that complicates 3% to 5% of pregnancies in the United States. Preeclampsia originates from an improperly vascularized and ischemic placenta that releases factors that drive systemic pathophysiology. One of these factors, soluble fms-like tyrosine kinase-1, is believed to sequester vascular endothelial growth factor (VEGF), leading to systemic endothelial dysfunction and hypertension. With the goal of targeting soluble fms-like tyrosine kinase-1 while simultaneously preventing fetal exposure to VEGF, we fused VEGF to elastin-like polypeptide, a biopolymer carrier that does not cross the placental barrier (ELP-VEGF).

Methods and Results—ELP-VEGF restored in vitro endothelial cell tube formation in the presence of plasma from placental ischemic rats. Long-term administered ELP-VEGF in pregnant rats accumulated in maternal kidneys, aorta, liver, and placenta, but the protein was undetectable in the pups when administered at therapeutic doses in dams. Long-term administration of ELP-VEGF in a placental ischemia rat model achieved dose-dependent attenuation of hypertension, with blood pressure equal to sham controls at a dose of 5 mg/kg per day. ELP-VEGF infusion increased total plasma soluble fms-like tyrosine kinase-1 levels but dramatically reduced free plasma soluble fms-like tyrosine kinase-1 and induced urinary excretion of nitrate/nitrite, indicating enhanced renal nitric oxide signaling. ELP-VEGF at up to 5 mg/kg per day had no deleterious effect on maternal or fetal body weight. However, dose-dependent adverse events were observed, including ascites production and neovascular tissue encapsulation around the minipump.

Conclusions—ELP-VEGF has the potential to treat the preeclampsia maternal syndrome, but careful dosing and optimization of the delivery route are necessary. (*J Am Heart Assoc.* 2017;6:e007216. DOI: 10.1161/JAHA.117.007216.)

Key Words: elastin-like polypeptide • maternally sequestered drug delivery • preeclampsia/pregnancy • soluble fms-like tyrosine kinase-1 • therapy • vascular endothelial growth factor

Preeclampsia is a pregnancy hypertensive disorder that normally presents after the 20th week of gestation and complicates 3% to 5% of pregnancies in the United States.¹ Over the past 25 years, the annual incidence of preeclampsia has risen by over 10%.^{2,3} A principal cause of maternal

mortality⁴ and a chief risk factor for both maternal and fetal morbidity worldwide,⁵ the health risks of preeclampsia persist well beyond the index pregnancy. Women with a history of preeclampsia are at higher risk for developing diabetes mellitus,⁶ stroke,⁷ and cardiovascular⁸ and renal diseases⁹ in later life. This susceptibility to cardiorenal and metabolic diseases post-index pregnancy has been attributed to the pathophysiological mechanisms^{10,11} initiated by the placental ischemia. In addition to the maternal effects, preeclampsia is highly associated with intrauterine growth restriction¹² and developmentally programs the increased risk for cardiovascular disease,^{13,14} stroke,¹⁵ renal disease,¹⁶ and metabolic dysfunction^{17,18} in the adult offspring of preeclamptic women.¹⁹ Since the resolution of preeclampsia is achieved only through parturition of the ischemic placenta,²⁰ a difficult decision often must be made between inducing early delivery in order to halt the progression of the syndrome in the mother and risking premature birth complications to the fetus.²¹ Given the importance of preeclampsia for programming later life cardiovascular risk in both the mother and the fetus,²² the lack of therapies specifically targeting the drivers of the

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Accompanying Figures S1 through S3 are available at <http://jaha.ahajournals.org/content/6/12/e007216/DC1/embed/inline-supplementary-material-1.pdf>

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Clinical Perspective

What Is New?

- A novel therapeutic for preeclampsia was developed by fusing vascular endothelial growth factor to a biopolymer carrier called elastin-like polypeptide.
- Elastin-like polypeptide–vascular endothelial growth factor did not cross the placental barrier when administered to pregnant rat dams, thus reducing the risk of adverse effects on fetal development.
- In a preclinical model of placental ischemia, elastin-like polypeptide–vascular endothelial growth factor chimera blocked maternal hypertension in part by reducing the bioavailability of soluble fms-like tyrosine kinase-1 and increasing renal nitric oxide signaling.

What Are the Clinical Implications?

- Less than 0.5% of all cardiovascular drugs under development target the maternal syndrome of preeclampsia, because of the potential risk of developmental effects to the fetus.
- Fusing drugs to a biopolymer carrier that does not cross the placental barrier is a potential means of reducing risks to the developing fetus.
- The potential clinical benefits of these biopolymer-stabilized therapeutics extend well beyond the index pregnancy. Restoration of angiogenic balance in late-term pregnancy may avert the downstream pathophysiology events that contribute to the rising incidences of later life cardiorenal disease and metabolic dysfunction both in the mother and offspring.

preeclampsia syndrome represents a significant deficit in this field.

Despite the identification of the ischemic placenta as the pathophysiological source of preeclampsia well over a century ago,²³ the cause continues to remain unresolved. However, it is commonly agreed upon that the initiating detrimental events occur at the maternal/fetal interface, where invading fetal cytotrophoblasts fail to convert the maternal spiral arteries into high-capacitance, low-resistance vessels, leading to occlusive arterial lesions in the myometrial segment of the arteries^{24,25} and a reduction in uterine perfusion pressure.^{20,26} Unable to meet the metabolic demands of the developing fetus because of this compromised arterial blood supply, the placenta enters a chronically hypoxic/ischemic state,²⁷ which activates pathways that increase the production of inflammatory cytokines,²⁰ reactive oxygen species,²⁸ and angiogenic imbalance factors including the soluble form of the vascular endothelial growth factor (VEGF) receptor Flt-1 (sFlt-1),²⁹ all of which lead to systemic endothelial dysfunction that is manifested as hypertension.³⁰ Among these, sFlt-1

has been implicated as a major driver of the preeclampsia syndrome because of its high levels in the plasma of preeclamptic mothers³¹ and the fact that exogenously administered sFlt-1 induces a preeclampsia-like syndrome in pregnant rodents.^{32–35}

Strategies to target sFlt-1 have shown preclinical efficacy in combating preeclampsia in rodent models.³⁶ These include administration of exogenous growth factors that bind Flt-1 and sFlt-1, including VEGF and placental growth factor,^{37,38} thereby sequestering sFlt-1 and restoring angiogenic balance and bioavailable VEGF. However, a potential limitation of this approach is that exogenously administered growth factors possesses a brief plasma half-life and are prone to degradation.³⁹ This limitation can be overcome by fusion of growth factors with a biopolymer synthetic protein based on human elastin.^{40,41} Elastin-like polypeptide (ELP) is composed of a repeating 5 amino acid motif that is thermally responsive,^{25,41,42} facilitating purification of ELP and ELP-fusion proteins by thermal cycling.⁴³ Moreover, the genetically encoded ELP is nonimmunogenic,^{44,45} is well suited as a carrier to stabilize therapeutic peptides, and offers protection from clearance and/or degradation.^{46–48} We previously showed that ELP does not cross the placental barrier.⁴⁹ Here we describe a fusion of human VEGF-A₁₂₁ with the ELP carrier. We evaluated the *in vitro* activity, *in vivo* biodistribution, and the therapeutic efficacy and side effects of this novel candidate biologic in a rat model of placental ischemia.

Materials and Methods

The data, analytic methods, and study materials will be made available upon request to other researchers for purposes of reproducing the results or replicating the procedure.

Cloning and Purification of ELP-VEGF

A chimeric protein containing the ELP biopolymer (160 V-P-G-x-G repeats in which the x residue was V, G, or A in a 1:7:8 ratio⁵⁰) fused in-frame with human VEGF-A₁₂₁ was cloned and purified from a recombinant expression system as described in George et al.⁴¹ The clone was confirmed by DNA sequencing, and the protein product was assessed for purity by SDS-PAGE analysis.

Human Umbilical Vein Vascular Endothelial Cell Tube Formation Assay

A 48-well, sterile, and non-tissue-culture-treated plate was coated with 150 μ L of growth factor reduced Matrigel (BD Biosciences) and incubated at 37°C in a humidified incubator

with 5% CO₂ for 30 minutes. Human umbilical vein vascular endothelial cells (American Type Culture Collection) were serum and growth factor starved for 2 to 3 hours before seeding them over Matrigel-coated wells at 30 000 cells/well in basal media containing 0.1 mg/mL of heparin supplemented with either 3% normal rat serum or serum from rats treated with surgical placental ischemia in the absence or presence of 1 nmol/L or 10 nmol/L final concentrations of ELP, VEGF, or ELP-VEGF. The cells were incubated at 37°C in a humidified incubator with 5% CO₂ for 5 hours. At the end of the incubation, the cells were imaged with an inverted microscope using bright field illumination and ×10 magnification. Five nonoverlapping fields per well were imaged, and the tubes between 2 cell nodes were counted for each field, averaged for each well, and expressed relative to untreated wells. The data represent the mean±SE of 3 independent experiments.

Fluorescent Labeling

ELP-VEGF was covalently labeled on exposed primary amines using an NHS-Rhodamine (5/6-carboxy-tetramethyl-rhodamine succinimidyl ester), mixed isomer (Thermo Fisher) as previously described.⁵¹

Animal Use

All protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Timed pregnant Sprague-Dawley rats (Harlan Labs, Indianapolis, IN) were received on gestational day 11, and were acclimated to the animal housing facility. Rats were maintained on a 12:12-hour light-dark cycle, at 23°C constant temperature and were provided food and water ad libitum.

Chronic Biodistribution of ELP-VEGF in Pregnant Rats

As described in George et al,⁴⁹ on gestational day 14 (GD14) at 09:00, Alzet[®] osmotic pumps containing saline- or rhodamine-labeled ELP-VEGF delivered at a dose of 1, 5, and 10 mg/kg per day were inserted in the peritoneal cavity of anesthetized timed-pregnant rats. Blood draws were collected from the lateral tail vein twice daily at 09:00 and 17:00 on GD15 through GD18. On GD19, the rats were euthanized, and amniotic sacs and organs were harvested for whole organ ex vivo fluorescent imaging. Placentae and their associated pups from each experimental rat were dissected from the amniotic sac and imaged using an In Vivo Imaging System (IVIS Spectrum, Perkin Elmer) with 535-nm excitation and 580-nm emission, field-of-

view B, f stop=2, and auto exposure. Maternal heart, liver, kidney, spleen, lungs, aorta, and brain were imaged similarly. Standard curves were prepared from the injected proteins and plated 100 µL per well in a black 96-well plate. The plate of standards was imaged using identical parameters. Mean fluorescence radiant efficiency of each organ or standard was determined using a closely drawn region of interest using Living Image 4.3 software (Perkin Elmer). The mean fluorescence radiant efficiency was averaged for all placentae, pups, and maternal organs from each rat, levels were corrected for autofluorescence determined from tissues of the saline-injected animals, and fluorescence data were fit to the standard curve of the injected protein. Mean standardized fluorescence intensity was calculated for all rats in each group (n=7–9 placentae and pups per rat and 5 rats per group). To generate images for publication, 1 representative image of placentae and pups from each group was opened using Living Image's "load as group" tool, and all images were displayed on the same scale to allow direct comparison of the fluorescence intensity among groups. Rhodamine-labeled ELP-VEGF was detected directly in plasma by measuring fluorescence using a Nanoquant plate and a fluorescence plate reader (Tecan). As was performed in George et al,⁴⁹ plasma data from GD15 to GD19 were fit linearly to determine the average plasma ELP level. All fitting was performed using GraphPad Prism.

Reduced Uterine Perfusion Pressure Surgery

Methods were previously described in George et al and Granger et al.^{49,52,53} Briefly, in timed pregnant Sprague-Dawley rats on GD14, anesthesia was induced and maintained with 3% isoflurane, and carprofen (5 mg/kg) was administered subcutaneously. After performing a midline abdominal incision, both uterine horns were externalized. A single 0.203-mm silver surgical clip was placed on the abdominal aorta just rostral to the iliac bifurcation, so as to bilaterally restrict blood flow from the uterine arteries that originate from their respective common iliac arteries. In order to prevent compensatory blood flow to the placenta, the left and right ovarian arteries each received a 0.100-mm silver surgical clip that was applied just rostral to the first segmental artery. Sham surgeries included isolation of the vessels but no clip placement. Alzet[®] intraperitoneal osmotic pumps containing saline or ELP-VEGF delivered at a dose of 1, 5, or 10 mg/kg per day (n=8–10 rats per group, run in consecutive weekly cohorts of 10 rats per week) were carefully placed within the abdominal cavity. The uterine horns were replaced, and the incisions were closed with 4-0 suture. Animals were returned to their home cages, administered acetaminophen (75 mg/d po, Bio-Serv), and were allowed to recover for 96 hours. Separately, an additional 10 rats (5 sham and 5 reduced uterine perfusion pressure [RUPP] with saline administration)

were subjected to the same surgical protocol in order to confirm the original measurements of mean arterial pressure and sFlt-1 levels. sFlt-1 plasma Western blots and sFlt-1 placental ELISA measurements (described in detail below) were performed separately on these rats. This separate set of rats confirmed high reproducibility of the measurements, and data from these temporally independent rats were combined with data from the original cohort and included in all plots.

Mean Arterial Pressure (mm Hg) Measurement

As described in Granger et al and George et al,^{53,54} on GD18, rats were anesthetized as stated above and implanted with indwelling carotid catheters consisting of V-3 tubing (Scientific Commodities, Lake Havasu City, AZ), which were tunneled under the skin and externalized between the scapulae. On GD19 at ≈07:00 to 07:30, rats were placed in individual restraining cages and acclimatized. Because anesthesia has been reported to affect cardiovascular function and mean arterial pressure measurements in both humans and animal models, blood pressure measurements were conducted without the use of anesthesia, and only after the rodents were acclimatized to the restraint cages. After acquiring a steady baseline for 20 minutes, mean arterial pressure was measured for 30 minutes via Cobe III pressure transducers (CDX Sema), and data were collected and analyzed using receivers, amplifiers, and Lab Chart 7 PowerLab software from AD Instruments.

Plasma and Tissue Collection

Following blood pressure measurement on GD19, rats were anesthetized, urine was drawn from the bladder, and blood was collected by arterial puncture of the abdominal aorta using a butterfly needle. Both plasma and serum were collected using appropriate tubes (BD Vacutainer, Franklin Lakes, NJ) and stored frozen at -80°C until analysis. After exsanguination, fetal numbers were counted, and placental, fetal, maternal kidney, and maternal heart weights were recorded. For the average pup weight measurement, only viable pups were weighed. Pup weights were recorded for all viable pups from each dam and then averaged per rat. The “Fetal Weight” was calculated by averaging all pup weights for the entire treatment group. The resorption rate was calculated for each individual rat by adding the total resorptions of both uterine horns and dividing by the sum of total resorptions plus the total number of viable pups. For an entire treatment group, the reported resorption rate was the average of the resorption rates from each individual rat.

Measurement of Plasma and Placental sFlt-1

Placental protein was extracted from a random placenta from individual rats.⁵³ Briefly, 100 to 150 mg of frozen tissue was

cut and weighed under sterile conditions. Minced tissue fragments were resuspended in 1 mL radioimmunoprecipitation assay buffer in molar: Tris-HCl, 1; NaCl, 5; EDTA, 0.5, sodium deoxycholate, 10%; and NP-40, 10%. Isolation and purification of placental protein was performed using a FastPrep-24™ 5G homogenizer (MP Biomedicals, Solon, OH). Following the 40-s homogenization run, samples were centrifuged at 12,000 g for 1 minute. Placental supernatants were transferred to sterile tubes, and diluted to 1:20 for bicinchoninic assay determination of total protein levels. Bicinchoninic assay protein levels were used to normalize protein levels for ELISAs.

Plasma samples were stored at -80°C until assayed. Undiluted plasma samples (4 μL) were electrophoresed directly on 4% to 12% 15-well gels (Invitrogen Bolt BT Plus). Each well was loaded with a total volume of 30 μL : plasma, 4 μL ; sample buffer, 8 μL ; 10X reducing agent, 3 μL ; and sterile water, 15 μL . Electrophoresis of samples was run at 150 volts, 0.13 amps for 40 minutes. Two gels were run simultaneously to accommodate all the samples. The 2 gels were transferred side by side onto a single nitrocellulose membrane using 1-Step transfer buffer (Thermo Scientific, Rockford, IL) with Pierce G2 Fast Blotter (Thermo Scientific). The membrane was blocked for 1 hour at RT with phosphate buffered saline with Tween 20 (PBST)/5% blotting-grade blocker (Bio-Rad, Hercules, CA), and 0.02 g of bovine serum albumin. A separate blot was carried out independently using plasma from the additional cohort of sham- and RUPP-treated rats. Blocked membranes were incubated with primary anti-sFlt rabbit monoclonal IgG (Abcam 32152) at a 1:1000 dilution overnight at 4°C . Following incubation, membranes underwent six 10-minute washes with fresh PBST on an orbital shaker at room temperature. Incubation with the secondary antibody (Pierce goat anti-rabbit poly horseradish peroxidase) was at 1:10 000. Bio-Rad precision protein streptavidin horseradish peroxidase conjugate (cat# 161-0380) diluted at 1:5000 and added to the secondary antibody incubation in order to visualize the biotinylated molecular weight markers. Incubation occurred for 1 hour at room temperature. Membranes were again washed with six 10-minute washes with fresh PBST. For band visualization, chemiluminescent Thermo Scientific Super Signal West Pico Substrate (Cat# 34080) was added to the membrane in a 1:1 ratio of components. Blot imaging was performed with a Bio-Rad Universal Hood Gel Doc System. Band analysis was performed with ImageJ software.

sFlt-1 levels in isolated placental proteins and plasma were quantitatively measured with a commercial solid-phase sandwich ELISA kit (R&D Systems, Quantikine, Minneapolis, MN) in duplicate, according to the manufacturer's protocols.^{32,53,55,56} Optical density was determined with a microplate reader set to 450 nm, with wavelength correction between 540 and 570 nm. ELISA plates were read immediately after adding stop solution (100 μL /well). All duplicate samples were averaged. The standard curve was fit with a 4-parameter curve fit, and sFlt-1

levels in plasma and tissue samples were extrapolated from this curve. For diluted samples, the concentrations read from the standard curve were multiplied by the dilution factor.

Measurement of Urinary Protein and Nitrate/Nitrite

Urine collected at harvest was used to assess total protein and total nitrate/nitrite. Total protein levels were determined using urine diluted 1:5 by the bicinchoninic assay (Pierce) according to the manufacturer's instructions. A commercially available nitrate/nitrite colorimetric assay kit (Cayman Chemical 780001, Ann Arbor, MI) was used to assess total nitrate/nitrite concentrations. Urine (80 μ L) from each rat diluted 1:10 was assayed in duplicate according to the manufacturer's instructions.

Statistical Analysis

Human umbilical vein vascular endothelial cell tube formation assay was analyzed with 1-way ANOVA with post hoc Dunnett's correction for multiple comparisons of each experimental mean to the control group mean. Ex vivo whole-organ fluorescence data were analyzed using a 2-way ANOVA with treatment parameters for dose and tissue type and post hoc Tukey's multiple comparison. In the efficacy testing assay, preset removal criteria included any RUPP-treated rat with <10% fetal resorption (unsuccessful RUPP surgery) or >90% fetal resorption. This resulted in excluding 4 rats from the RUPP saline group (2 with no fetal resorption and 2 with >90% resorption), 1 rat from the RUPP+1 mg/kg per day ELP-VEGF group (>90% resorption), and 2 rats from the RUPP+5 mg/kg per day group (1 with no fetal resorption and 1 with >90% resorption). In the separate cohort of 10 rats, 1 with no fetal resorption was excluded from the RUPP group. Data from these animals were excluded from all assays, and the final n numbers reported do not include these excluded rats. Mean arterial pressures, plasma and placental sFlt-1 levels, placenta weight, maternal heart and kidney weight, urinary nitrate/nitrite levels, and pup weight were analyzed by 1-way ANOVA with a post hoc Sidak multiple comparisons analysis of the relevant treatment groups. Maternal body weights were analyzed with a 2-way repeated-measures ANOVA with a post hoc Tukey's multiple comparison. A *P* value of <0.05 was considered statistically significant. All statistical comparisons were conducted using GraphPad Prism 6.

Results

ELP-VEGF Retains Full Angiogenic Activity In Vitro

To assess the activity of the ELP-VEGF fusion protein, a tube formation experiment was conducted using human umbilical

vein vascular endothelial cells. As illustrated in Figure 1, human umbilical vein vascular endothelial cells exposed to the serum from RUPP-treated dams exhibited a significantly reduced tube number relative to cells exposed to serum from normal pregnant control rats. This is consistent with data obtained using serum from human preeclamptic patients³¹, and it suggests angiogenic imbalance in the serum of rats exposed to placental ischemia. When serum from RUPP rats was modified by the addition of free VEGF (10 nmol/L), tube formation was partially restored. While treatment with ELP alone had no effect on restoring tube formation capability, ELP-VEGF (10 nmol/L) possessed the same potency as free VEGF in improving endothelial cell tube formation.

Biodistribution of ELP-VEGF in Pregnant Rats

The plasma levels of ELP-VEGF were measured in pregnant Sprague-Dawley rats from GD14 to GD19 with continuous infusion at 1, 5, and 10 mg/kg per day. As shown in Figure 2A, the plasma level at each dose increased linearly over the dosing period, and the slope of the increase was proportional to the dose. On GD19 following 5 days of continuous infusion, maternal organs, placentae, and pups were removed for ex vivo whole organ analysis to determine the biodistribution and placental transfer of ELP-VEGF. Figure 2B shows representative In Vivo Imaging System images of placentae and pups for each treatment dose. These data indicate that ELP-VEGF was present in the placenta, and the placental levels increased with dose. However, little to no ELP-VEGF was detectable over autofluorescence in the pups (quantified in Figure 2C). In the maternal organs, long-term administration of ELP-VEGF led to highest accumulation in the kidney, liver, and aorta, respectively, and the levels in all organs increased with increasing dose (Figure 2C).

ELP-VEGF Ameliorates Hypertension Associated With Placental Ischemia

Induction of placental ischemia via the RUPP procedure induced a marked increase in mean arterial pressure relative to sham-operated rats (Figure 3). When the RUPP rats were administered ELP-VEGF continuously following induction of placental ischemia, a dose-dependent reduction in mean arterial pressure was observed, with the mean arterial pressure being statistically no different from sham saline-treated rats at the 5 mg/kg per day dose. Interestingly, the reduction in blood pressure was not a generic hemodynamic phenomenon induced by ELP-VEGF, as the sham-operated rats showed no decrease in blood pressure at any tested dose. This suggests that ELP-VEGF normalizes blood pressure by specifically antagonizing a factor or factors induced by placental ischemia.

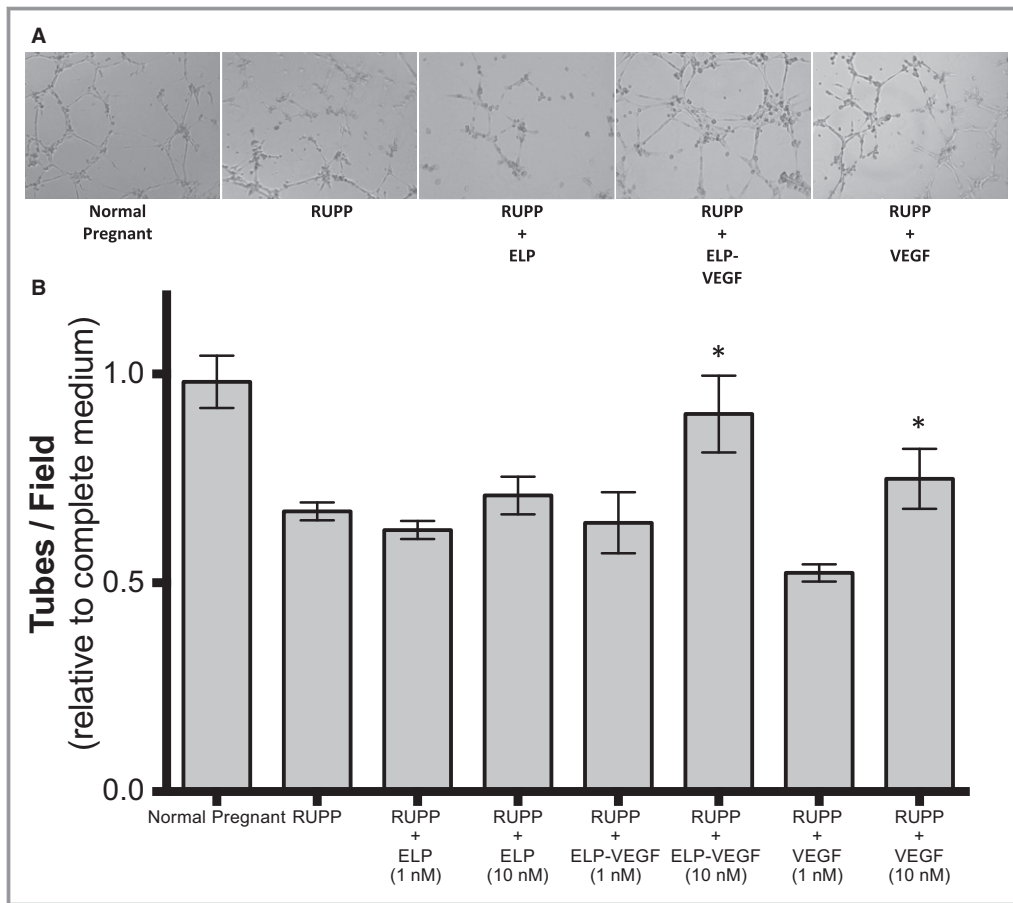


Figure 1. The elastin-like polypeptide–vascular endothelial growth factor fusion protein (ELP-VEGF) retains full activity in vitro. A, Human umbilical vascular endothelial cell tube formation was assessed 5 hours after seeding on growth factor–reduced Matrigel and supplementing the media with either 3% normal rat serum or serum from rats treated with surgical placental ischemia in the absence or presence of 1 nmol/L or 10 nmol/L final concentrations of ELP, VEGF, or ELP-VEGF. B, Average tubes per field were counted for 5 nonoverlapping fields per sample. The data represent the mean \pm SE of 3 independent experiments. *Statistically significant restoration of tube formation to levels found after treatment with normal pregnant serum ($P<0.05$, 1-way ANOVA. RUPP indicates reduced uterine perfusion pressure.

ELP-VEGF Infusion Increases Total sFlt-1 Plasma Levels But Decreases Free Plasma sFlt-1

Increased circulating sFlt-1 is a hallmark of preeclampsia²⁰, and evidence suggests that sFlt-1 plays a causative role in the systemic endothelial dysfunction and renal damage present in preeclampsia^{57,58}. Studies using viral overexpression^{33,57,59–61} or direct infusion of sFlt-1^{32,55,62,63} have demonstrated a critical role for this anti-angiogenic factor for induction of hypertension and renal damage during pregnancy. Previous studies using exogenous VEGF infusion have reported reduced circulating sFlt-1 levels.^{36,64} However, induction of local VEGF expression in the maternal decidua was shown to actually promote increased placental sFlt-1 expression in the cytotrophoblasts,⁶⁵ suggesting a protective role for placental sFlt-1 against maternal overproduction of VEGF.⁶⁶ To assess the effects of long-term

ELP-VEGF infusion on sFlt-1 levels, we measured circulating sFlt-1 by both Western blot and ELISA, and we measured sFlt-1 levels in whole placental extracts by ELISA. Western blots conducted on plasma, loaded in equal volumes directly onto a gel with no prior fractionation or enrichment, revealed 2 bands when probed with an anti-Flt-1 antibody at ≈ 70 and 140 kDa (though exact determination of molecular weight was not done). A third, smaller molecular weight band was present below the albumin smear (Figure 4A). These bands correspond to the report of Rajakumar,⁶⁷ who describes sFlt-1 bands of 145, 100, and 60 kDa in plasma from preeclamptic patients, though considerable species and glycosylation variation could exist. The RUPP surgery leads to a significant increase in plasma sFlt-1, as has previously been reported.^{32,36,53,56,58,68} This was confirmed in a separate cohort of rats, treated entirely independently with the sham or RUPP surgeries, and analyzed in a separate Western blot

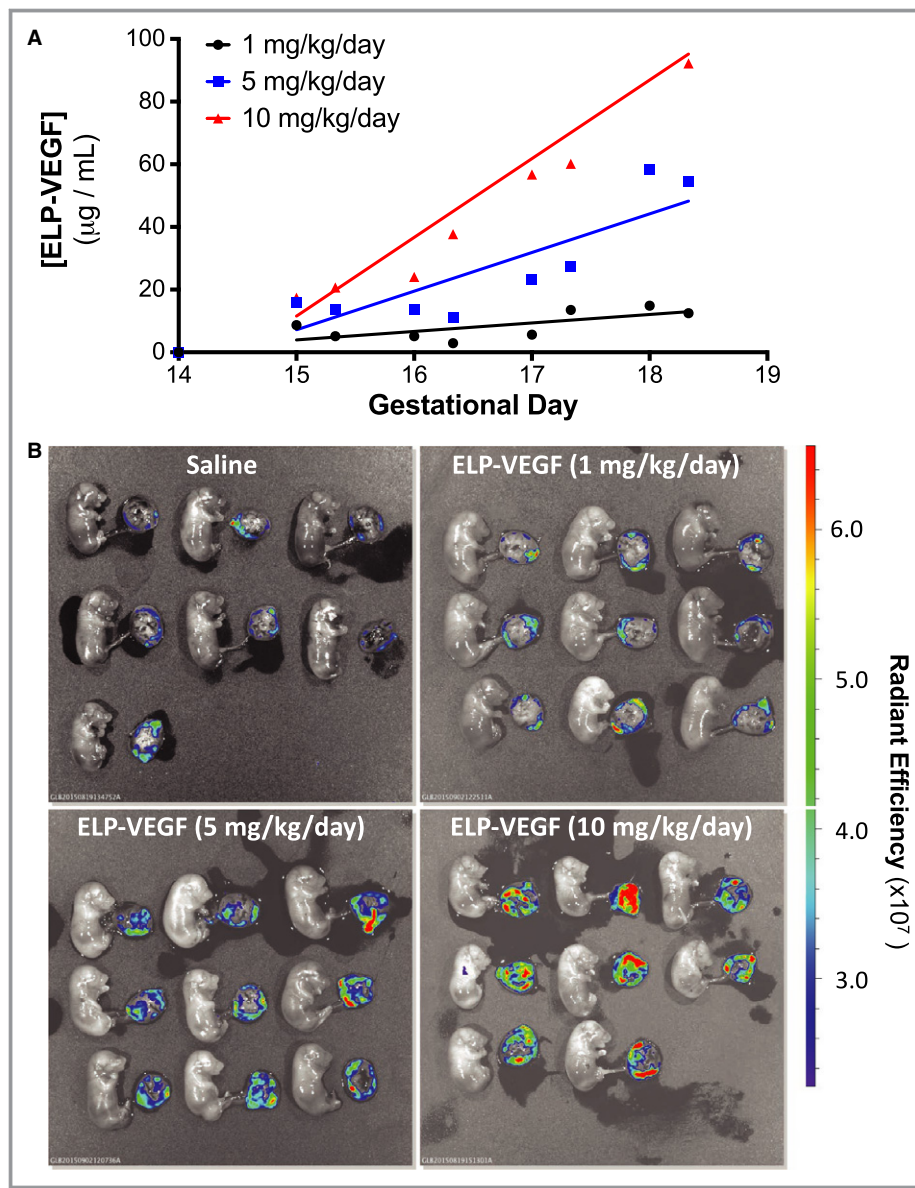


Figure 2. Chronic biodistribution, placental deposition, and fetal exposure to ELP-VEGF in pregnant rats. Rhodamine-labeled ELP-VEGF was administered chronically from gestational day 14 to gestational day 19 (GD14-GD19) at dosages of 1, 5, and 10 mg/kg per day by intraperitoneal minipump. A, Plasma was sampled throughout the experiment, and polypeptide levels were determined. B, GD19 ex vivo fluorescence imaging of pups with corresponding placentae from 1 rat in each treatment group is displayed. C, Fluorescence intensities for maternal organs, placentae, and pups were quantified, corrected for autofluorescence, and fit to standards of the injected proteins. Data represent the mean standardized fluorescence intensity (RFU, relative fluorescence units) \pm SE. *Statistically significant difference among doses ($P < 0.05$, 2-way ANOVA), #ELP-VEGF levels were undetectable. At the 5 mg/kg per day and 10 mg/kg per day doses, kidney levels were significantly higher than in all other organs (not marked). Other statistically significant levels were observed among organs at the 10 mg/kg per day dose (not marked). ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein.

(Figure S1). ELP-VEGF infusion caused a marked induction of total circulating sFlt-1 levels in both sham- and RUPP-treated rats at this time point, which represents a snapshot of plasma

levels at GD19 after 5 days of continuous ELP-VEGF infusion at 5 mg/kg per day (Figure 4A). The 140-kDa band was quantified (Figure 4B, which includes data from the gel shown in Figure 4A

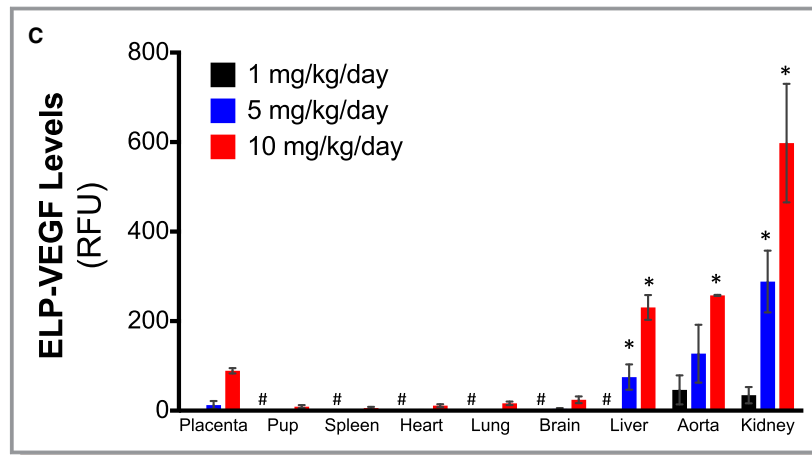


Figure 2. Continued.

and from a gel of the plasma from additional sham and RUPP saline-treated rats run separately, shown in Figure S1). Rats treated with the RUPP surgery alone with saline infusion had higher sFlt-1 levels. Also, a robust induction of total sFlt-1 was present in all ELP-VEGF-infused rats. In addition to Western blotting for total sFlt-1 levels, we also assessed plasma sFlt-1 by ELISA. The ELISA used has been previously reported to be specific for free, non-VEGF-bound, sFlt-1.^{32,53,55,56} We found that spiking the ELISA standard curves with ELP-VEGF reduced the signal, suggesting that ELP-VEGF might bind the Flt-1 in the

assay and prevent binding of 1 or both antibodies, thus confirming this as an assay for free sFlt-1. Again, RUPP surgery resulted in a significant increase in free plasma sFlt-1 (Figure 4C), consistent with previous reports.^{32,36,53,56,58,63,68} In contrast to the Western blot data, the ELISA data showed that ELP-VEGF-treated rats (both sham and RUPP surgical groups) had markedly reduced free sFlt-1 levels (Figure 4C). The same ELISA was used to measure free sFlt-1 in whole placental extracts. The RUPP surgery tended to also increase placental sFlt-1 (Sidak adjusted $P=0.088$, Figure 4D), consistent with previous studies^{32,53,56,58,63}; however, the burden for statistical significance was not reached after correcting for multiple comparisons. The effects of ELP-VEGF treatment were less dramatic in the placenta than in the plasma (Figure 4D). In sham-operated rats, ELP-VEGF induced a small but significant increase in placental sFlt-1, and the protein had no effect on placental sFlt-1 in RUPP rats. Taken together, these assays suggest that ELP-VEGF infusion might induce sFlt-1 production and/or release from the placenta, but since the ELP-VEGF levels are circulating at many times the plasma sFlt-1 level (Figure 2), the ELP-VEGF can bind and sequester the increased sFlt-1. Furthermore, the ELP-VEGF treatment is sufficient to sequester even the increased sFlt-1 levels induced by placental ischemia, suggesting a possible mechanism for the attenuation of placental-ischemia-induced hypertension.

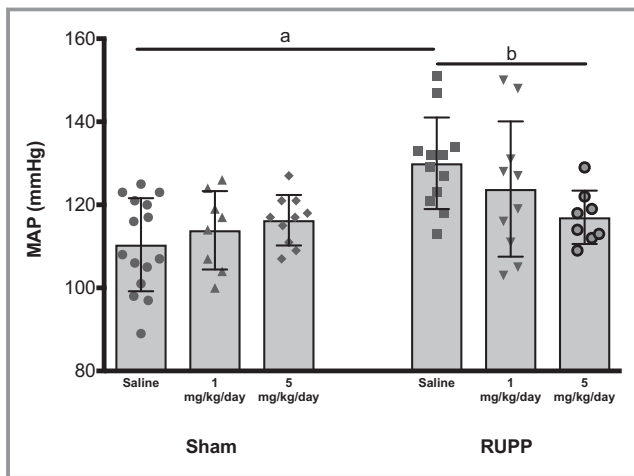


Figure 3. ELP-VEGF ameliorates hypertension associated with reduced uterine perfusion pressure. Gestational day 19 mean arterial pressure (MAP) measurements were acquired from conscious rats treated with saline or ELP-VEGF and exposed to sham or reduced uterine perfusion pressure (RUPP) surgery. Bars represent the mean \pm SD with indicators for individual rats. (Adjusted P values from 1-way ANOVA with post hoc Sidak multiple comparisons: a) $P<0.0001$, b) $P=0.05$). ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein.

ELP-VEGF Treatment Causes Increased Urinary Nitric Oxide Metabolites

Nitric oxide (NO) availability in the kidney is suggested to be an important factor for the impaired renal function associated with preeclampsia.^{69,70} VEGF is also known to be a potent inducer of NO signaling.⁷¹ To assess whether ELP-VEGF infusion affected renal NO levels, we measured urinary excretion of nitrate and nitrite as a surrogate marker. ELP-

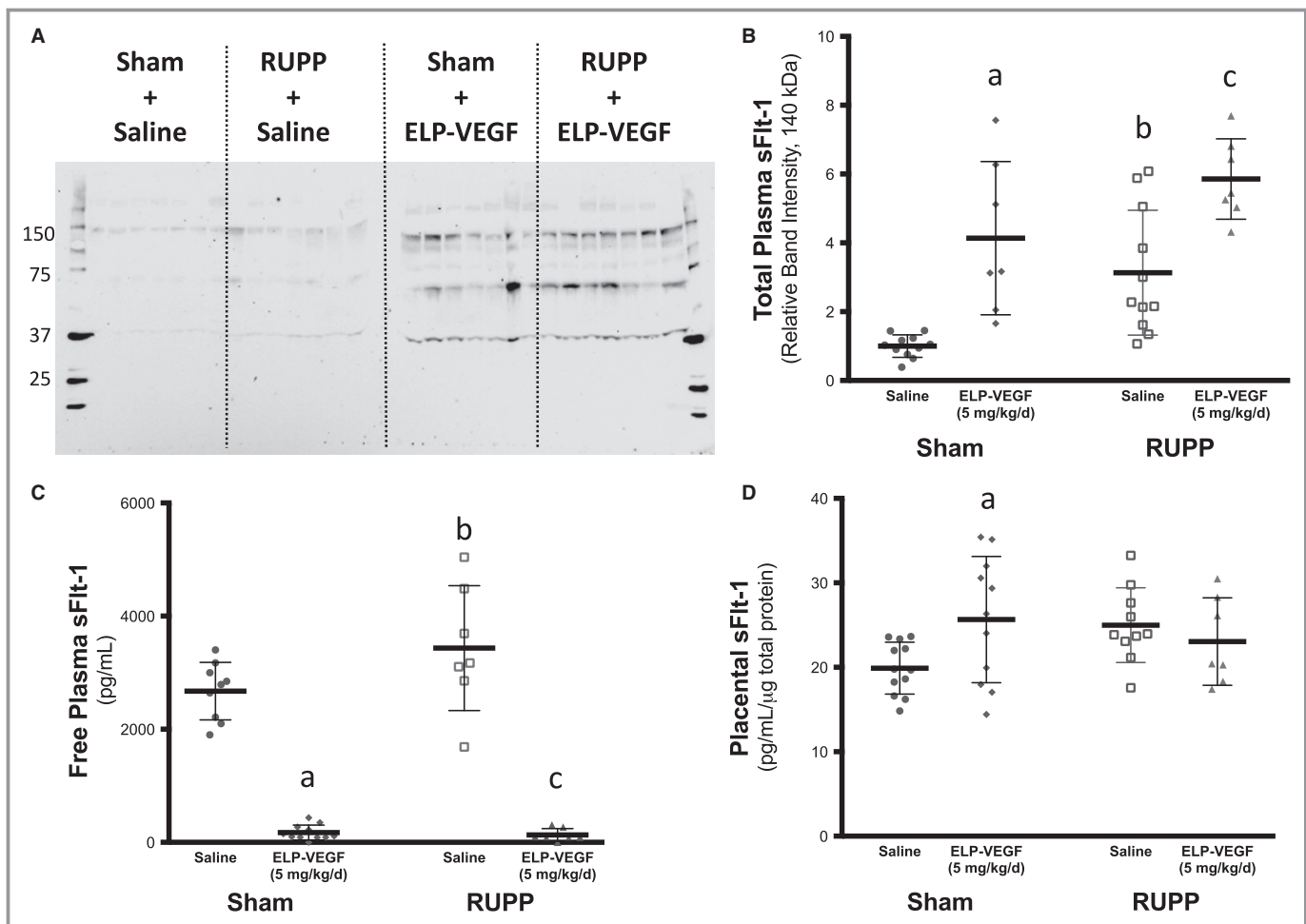


Figure 4. ELP-VEGF infusion increases total (free+bound) soluble fms-like tyrosine kinase-1 (sFlt-1) plasma levels at gestational day (GD) 19, but decreases free (ie, bioavailable) plasma sFlt-1. A, Undiluted plasma samples that were collected on GD19 were electrophoresed and probed for sFlt-1 by Western blotting. A single blot containing plasma from 7 rats per group, run on 2 separate gels and transferred to the same membrane, is shown. B, The 140-kDa band intensity was quantified. Data from the gel in (A) and the gel in Figure S1 are included (Adjusted *P* values from 1-way ANOVA with post hoc Sidak multiple comparisons: a) sham saline vs sham ELP-VEGF $P=0.0004$, b) sham saline vs RUPP saline $P=0.006$, c) RUPP saline vs RUPP ELP-VEGF $P=0.002$). C and D, A solid-phase sandwich ELISA was used to measure free sFlt-1 levels in plasma (C, adjusted *P* values from 1-way ANOVA with post hoc Sidak multiple comparisons: a) sham saline vs sham ELP-VEGF $P<0.0001$, b) sham saline vs RUPP saline $P=0.037$, c) RUPP saline vs RUPP ELP-VEGF $P<0.0001$), and placental extract (D, adjusted *P* values from 1-way ANOVA with post hoc Sidak multiple comparisons: a) sham saline vs sham ELP-VEGF $P=0.038$). All plots are mean \pm SD, and markers represent individual animals. ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein; RUPP, reduced uterine perfusion pressure.

VEGF treatment was associated with large increases in urinary nitrate and nitrite in the sham group and trended toward an increase in the RUPP group (Figure 5). These data suggest that increased NO production in the kidney is induced by ELP-VEGF therapy.

Other Effects of ELP-VEGF Treatment in Pregnant Rats

Maternal body weight was measured daily during the drug infusion period in both sham- and RUPP-operated rats as a first-line assessment of toxicity. As shown in Figure 6A, the administration of ELP-VEGF had no significant effect on

maternal body weight at any dose in the sham-operated rats. RUPP rats (Figure 6B) gained less overall weight than shams, their weight being significantly less than sham saline rats beginning on GD15, and remained smaller throughout the study. This result is expected because of their decreased total fetal and placental mass. Within the RUPP groups, the rats treated with ELP-VEGF at the 5 mg/kg per day dose weighed more than RUPP saline rats on GD18 and GD19. There was no difference in placenta, heart, or kidney weights among any of the groups (Table 1). Total urine protein levels were assessed in terminally collected urine samples, and no significant differences were seen among treatment groups (Figure S2). There was a trend for increased total protein levels in sham

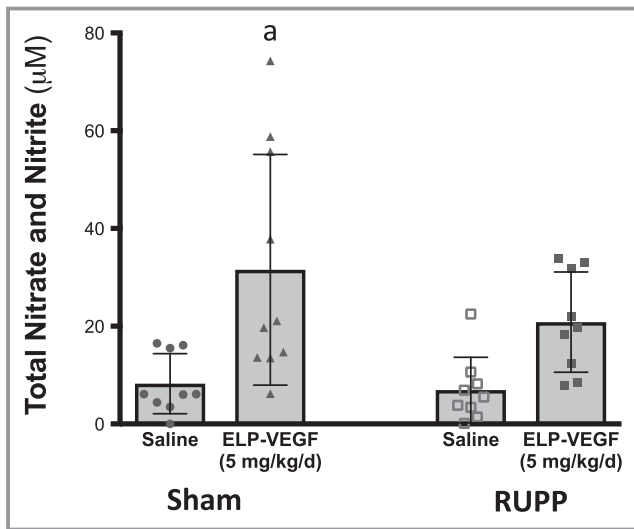


Figure 5. ELP-VEGF induces augmented nitric oxide (NO) production in the kidney. To assess whether ELP-VEGF infusion affected renal NO levels, urinary excretion of nitrate and nitrite as a surrogate marker was measured. Bars are mean±SD, and markers represent individual animals. (Adjusted *P* values from 1-way ANOVA with post hoc Sidak multiple comparisons: a) sham saline vs sham ELP-VEGF *P*=0.003). ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein; RUPP, reduced uterine perfusion pressure.

rats treated with 5 mg/kg per day ELP-VEGF, but it is possible that degradation products of the infused protein itself are being detected in this assay. It has been widely studied that the RUPP surgery induces fetal resorption and intrauterine growth restriction of the surviving pups.^{72–76} Fetal resorption and fetal growth restriction was observed in our study as well (Table 1 and Figure S3), validating the success of the RUPP procedure in our rats. There were no differences in total litter size among the treatment groups, but all RUPP-treated rats had more resorbed fetuses than sham-treated rats did

(Table 1 and Figure S3). There was no effect of ELP-VEGF therapy on resorption rate or fetal weight in any of the treatment groups up to the 5 mg/kg per day dose. This is expected given that the RUPP procedure involves placement of a fixed restrictive clip on the vessels supplying the uterus, and it is unlikely that a pharmacological intervention can overcome the fixed physical blood flow restriction.

Adverse Side Effects of Long-Term Administration of ELP-VEGF

VEGF is a potent angiogenic cytokine and induces vascular permeability.⁷⁷ In addition to the markers of overall health, we also monitored for specific side effects related to prolonged intraperitoneal exposure to ELP-VEGF. Maternal dams treated with ELP-VEGF demonstrated marked ascites presence at the 10 mg/kg per day dose (Table 2). Also, many ELP-VEGF-treated rats presented with a highly vascularized and pulsatile tissue encapsulation on the end of the osmotic pump, the frequency of which increased with increasing dose (Table 2 and Figure 6C). Therefore, the evaluation of the 10 mg/kg per day dose was terminated early, and we concluded that this high dose of ELP-VEGF was beyond the maximum tolerated dose.

Discussion

When developing therapies for preeclampsia, the treatment strategy requires that the health of both patients, the gravid female and the developing fetus, be taken into account. Drug development for preeclampsia is one of the least funded areas of research,⁷⁸ in part because therapeutic agents that may be beneficial to the mother^{79,80} may pose harmful risks to the fetus.^{25,49} Thus, it is imperative that any potential therapy guarantee that fetal exposure to the drug be minimized and

Table 1. Characteristics of Treated Dams

	Sham			RUPP		
	Saline	ELP-VEGF (1 mg/kg per d)	ELP-VEGF (5 mg/kg per d)	Saline	ELP-VEGF (1 mg/kg per d)	ELP-VEGF (5 mg/kg per d)
Fetal weight, g	2.31±0.05	2.33±0.05	2.25±0.08	2.09±0.07	1.88±0.07*	1.99±0.10*
Placental weight, g	0.55±0.01	0.58±0.01	0.55±0.02	0.52±0.02	0.55±0.02	0.52±0.02
Heart weight, g	0.83±0.02	0.87±0.02	0.88±0.02	0.83±0.02	0.84±0.03	0.89±0.04
Kidney weight, g	0.74±0.01	0.72±0.03	0.75±0.02	0.75±0.02	0.75±0.02	0.75±0.02
Viable pups (average per dam)	12.33±2.22	12.00±2.27	11.56±3.40	4.82±1.94*	6.10±3.51*	7.75±4.06*
Resorbed pups (average per dam)	1.25±1.33	0.50±1.07	1.89±2.67	8.91±2.07*	9.10±3.90*	5.50±3.25*
Fetal resorption rate, %	8.08±2.38	3.85±2.91	15.13±4.93	63.82±4.84*	59.38±7.81*	48.02±10.13*

*, Statistically significant difference relative to sham, saline (*p* <0.05, one-way ANOVA).

ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein; RUPP, reduced uterine perfusion pressure.

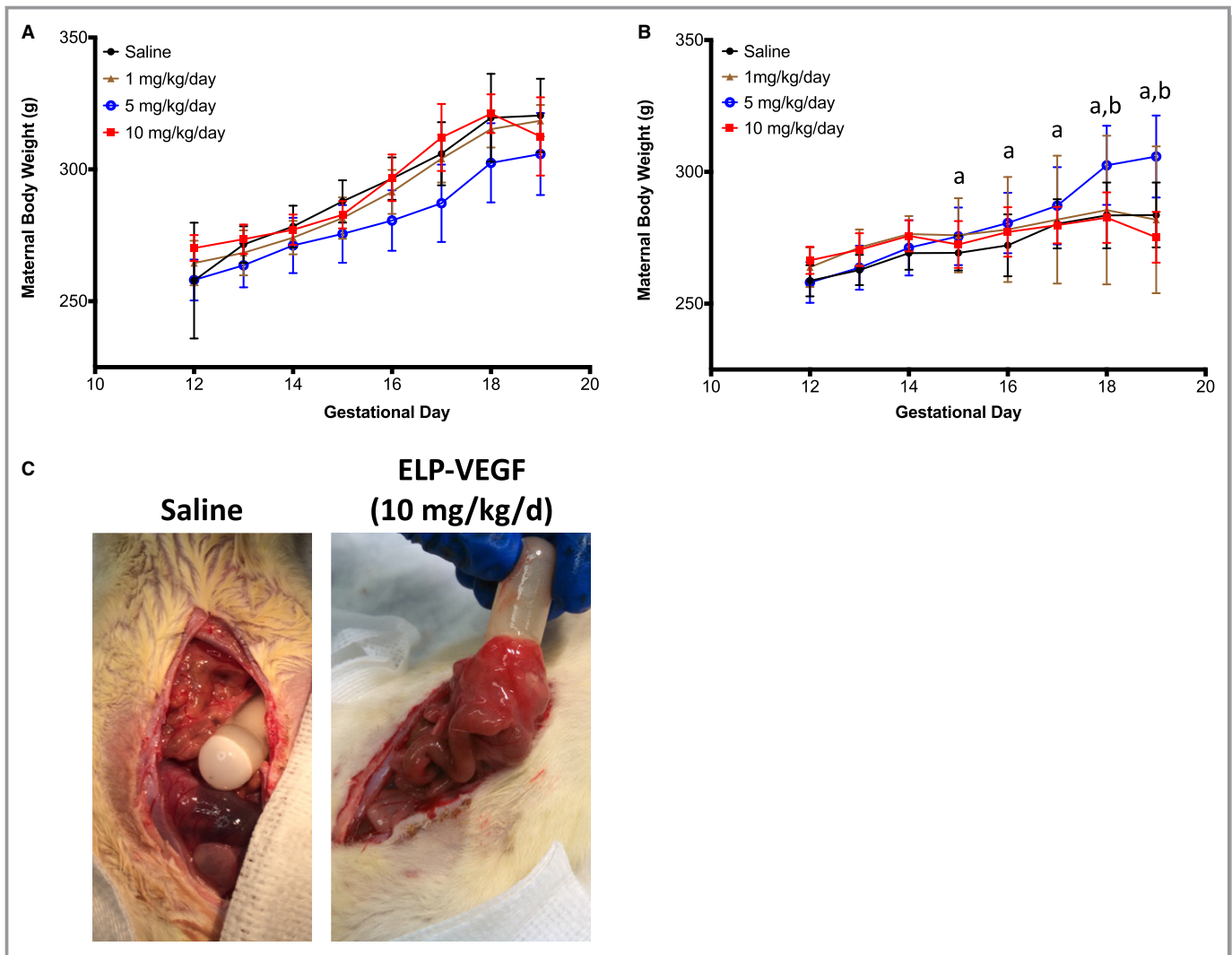


Figure 6. Assessment of adverse effects of ELP-VEGF infusion in pregnant dams. A and B, Maternal body weights were taken daily throughout the treatment protocol in sham-operated (A) and RUPP-operated (B) rats (All plots are mean±SD; a) RUPP rats treated with saline had significantly lower body weight than sham saline rats beginning on gestational day (GD) 15, and remained smaller throughout the experiment; b) RUPP rats treated with ELP-VEGF had significantly increased body weight relative to RUPP saline rats on GD18 and GD19; 2-way repeated-measures ANOVA with post hoc Tukey’s multiple comparison. C, Rats treated with ELP-VEGF at 10 mg/kg per day presented with a highly vascularized and pulsatile tissue encapsulation on the end of the osmotic pump, which was absent in the saline-treated rats. Photos were taken during the GD19 harvest, just before drawing urine and blood samples. ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein; RUPP, reduced uterine perfusion pressure.

Table 2. Frequency of Adverse Events With ELP-VEGF Treatment

	Saline	ELP-VEGF (1 mg/kg per d)	ELP-VEGF (5 mg/kg per d)	ELP-VEGF (10 mg/kg per d)
Tissue encapsulation, n (%)	0/15 (0%)	3/18 (16.7%)	6/18 (33.3%)	4/5 (80%)
Ascites, n (%)	0/15 (0%)	0/18 (0%)	1/18 (5.5%)	4/5 (80%)

ELP-VEGF indicates elastin-like polypeptide–vascular endothelial growth factor fusion protein.

that fetal health is not endangered by maternal therapy. This directive applies especially to VEGF supplementation in the gravid female because of a myriad of adverse fetal effects

from VEGF overexposure that have been observed in animal models.^{81–86} Previous studies using free VEGF-A isoforms, including VEGF-A₁₆₅^{87–89} and VEGF-A₁₂₁,^{36,64,90,91} have

demonstrated therapeutic efficacy in treating the maternal syndrome of preeclampsia. In these previous studies, VEGF was administered *in vitro*,⁸⁸ by chronic infusion,^{36,64} or via adenoviral vector overexpression.^{31,87,89–91} However, David et al raise the concern that adenoviral delivery of VEGF to the mother may risk harming the fetus by breaching the placental barrier.⁸⁹ In our novel study, a biopolymer-stabilized VEGF chimera mitigates this potential risk by preventing fetal exposure to VEGF.

In addition to maternal sequestration, we observed that the hypertensive response characteristically associated with the RUPP surgery^{36,52–54} was ameliorated at the 5 mg/kg per day dose of ELP-VEGF. The therapeutic dose range of ELP-VEGF (1–5 mg/kg per day) is equivalent to a free VEGF dose of 187 to 932 $\mu\text{g}/\text{kg}$ per day because of the differences in molecular weight between the free and ELP-fused cytokine. The efficacy observed at these doses is consistent with previous studies using the free cytokine, which showed normalization of blood pressure at doses in the range of 180 to 400 $\mu\text{g}/\text{kg}$ per day.^{36,64} The ameliorative effect of ELP-VEGF on mean arterial pressure may be the result of 2 possible mechanisms. First, ELP-VEGF could be directly replacing the endogenous free plasma VEGF that is bound to sFlt-1.⁹¹ A previous study noted that insufficient plasma levels of VEGF were the result of placental mRNA posttranscriptional dysregulation, such that while placental VEGF mRNA levels were not affected by placental ischemia,^{92,93} plasma protein levels of free VEGF were significantly blunted.⁹⁰ Therefore, chronic infusion of ELP-VEGF may be compensating for the reduced levels of endogenous free plasma VEGF and directly binding VEGF receptors. Secondly, ELP-VEGF is also likely binding and sequestering sFlt-1, and thus preventing its interaction with free plasma VEGF. By doing so, this would permit the endogenous VEGF to bind to functional VEGF receptors.

Though exogenous VEGF has been shown to ameliorate the hypertensive syndrome associated with preeclampsia, other reports have demonstrated increased plasma or placental sFlt-1 in response to increasing VEGF levels, suggesting a complex regulatory mechanism is at play. VEGF-A has been shown to stimulate the expression of sFlt-1 by regulating the alternative splicing of the Flt-1 gene⁹⁴ in both in vascular endothelial cells and human placental explants.^{65,95} VEGF-A binds to the VEGFR2 receptor, and by protein kinase C (PKC)–MEK signaling, acts on the VEGF-A Response Element on Intron 13 of the VEGFR1 gene.⁹⁴ Premature polyadenylation sites are located near the VEGF-A Response Element on Intron 13, which then trigger alternative splicing of the VEGFR1 gene into sFlt-1 mRNA transcripts.^{96–98} In this study, all rats that received ELP-VEGF at a dose of 5 mg/kg per day had increased total plasma sFlt-1 levels (Figure 4A and B). However, free plasma sFlt-1 as measured by ELISA (Figure 4C) indicated almost complete removal of free sFlt-1 in

the ELP-VEGF-treated rats. Based on these findings, we propose that ELP-VEGF infusion leads to increased sFlt-1 production and release in the placenta, the vascular endothelium, or both. This could be because of the induction of alternate splicing favoring the truncated sFlt-1 mRNA over full-length Flt-1 mRNA. Alternatively or in addition to a splicing mechanism, ELP-VEGF could lead to increased plasma sFlt-1 by inducing release of sFlt-1 from placental extracellular matrix,⁹⁹ where sFlt-1 stores have been shown to be associated with heparan sulfate proteoglycans.¹⁰⁰ However, in spite of the increased total plasma sFlt-1, free sFlt-1 levels are dramatically reduced in ELP-VEGF-treated rats. This suggests that circulating ELP-VEGF is binding with sFlt-1, sequestering it, and preventing it from initiating downstream antiangiogenic pathways that would ultimately manifest as hypertension.^{41,49} This conclusion is supported by the observation of increased urinary nitrate/nitrite, indicating ELP-VEGF-induced enhanced NO production in the kidneys. The effects of ELP-VEGF in the kidney and systemic vascular endothelium¹⁰¹ likely explain its ability to ameliorate placental ischemia-induced hypertension.

Though ELP-VEGF infusion showed promising results with regard to correcting maternal hypertension, we did observe dose-dependent side effects of ascites and vascularized tissue encapsulation around the pumps. From these observations, we conclude that the therapeutic window for use of ELP-VEGF to attenuate RUPP-associated hypertension is very narrow. Also, many of the adverse effects are likely related to the method of delivery. The neovascular tissue encapsulation around the minipump and the ascites production in the peritoneal cavity are very likely related to the angiogenesis and vascular permeability induced by the continuous administration of ELP-VEGF into the peritoneal cavity. Ongoing studies are examining alternative delivery routes, including repeated intravenous dosing and controlled-release subcutaneous dosing. Building on the therapeutic potential of a biopolymer-stabilized VEGF chimera in treating placental ischemia, future studies will also involve evaluation of other therapeutics to target and reduce the bioavailability of sFlt-1. VEGF-B and placental growth factor also bind to sFlt-1,¹⁰² and unlike VEGF-A, do not bind to the main angiogenic receptor VEGFR2, which reduces their risk of adverse effects because of aberrant angiogenesis.^{37,38,65} We are currently testing ELP–placental growth factor and ELP-VEGF-B fusion proteins.

In summary, this study demonstrates the power of ELP fusion to a therapeutic growth factor for achieving sequestration in the maternal circulation. Furthermore, the ELP-VEGF fusion protein was effective at prevention of hypertension in response to placental ischemia. However, the therapeutic dosing window is narrow, consistent with the tight regulation of VEGF levels during pregnancy. Ongoing work includes

evaluation of alternative delivery routes for this ELP-VEGF-A₁₂₁ protein and evaluation of ELP fusions to other VEGF family members that bind Flt-1 and sFlt, but do not possess the strong angiogenic drive of VEGF-A. The ELP drug delivery platform is a promising modality for stabilization of therapeutics in the maternal circulation and preventing their placental transfer.

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Disclosures

Bidwell is owner of Leflore Technologies, LLC, a private company working to translate ELP-fusion technology. Bidwell and George are inventors of patent applications related to the described technology.

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SUPPLEMENTAL MATERIAL

Figure S1. A separate cohort of 10 rats (5 sham and 5 RUPP) were run independently to confirm the measured sFlt-1 effects. Total plasma sFlt-1 was determined by Western blot as described in Figure 4A (four animals per group, chosen at random, were analyzed in order to fit on a single gel). Quantitation of the 140 kDa band was determined and expressed relative to the average level of the sham + saline rats and included in the data presented in Figure 4B.

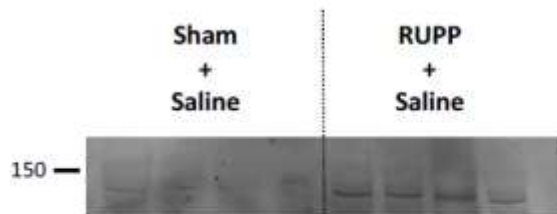


Figure S2. Urine collected at harvest was assessed for total protein content using the bicinchoninic acid (BCA) assay. No significant differences in total urine protein concentration were observed at the time of sacrifice.

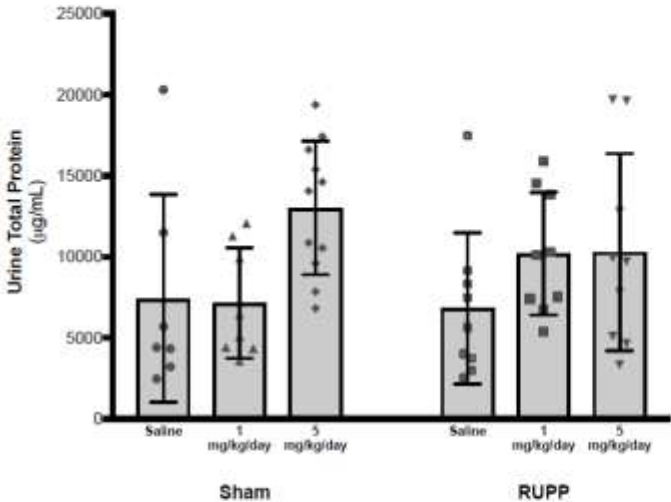


Figure S3. Fetal resorption rate was calculated for each dam in each treatment group. * Significant difference between sham and RUPP surgery groups.

