Soluble fms-like tyrosine kinase 1 promotes angiotensin II sensitivity in preeclampsia

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Preeclampsia is a hypertensive disorder of pregnancy in which patients develop profound sensitivity to vasopressors, such as angiotensin II, and is associated with substantial morbidity for the mother and fetus. Enhanced vasoconstrictor sensitivity and elevations in soluble fms-like tyrosine kinase 1 (sFLT1), a circulating antiangiogenic protein, precede clinical signs and symptoms of preeclampsia. Here, we report that overexpression of sFLT1 in pregnant mice induced angiotensin II sensitivity and hypertension by impairing endothelial nitric oxide synthase (eNOS) phosphorylation and promoting oxidative stress in the vasculature. Administration of the NOS inhibitor l-NAME to pregnant mice recapitulated the angiotensin sensitivity and oxidative stress observed with sFLT1 overexpression. Sildenafil, an FDA-approved phosphodiesterase 5 inhibitor that enhances NO signaling, reversed sFLT1-induced hypertension and angiotensin II sensitivity in the preeclampsia mouse model. Sildenafil treatment also improved uterine blood flow, decreased uterine vascular resistance, and improved fetal weights in comparison with untreated sFLT1-expressing mice. Finally, sFLT1 protein expression inversely correlated with reductions in eNOS phosphorylation in placental tissue of human preeclampsia patients. These data support the concept that endothelial dysfunction due to high circulating sFLT1 may be the primary event leading to enhanced vasoconstrictor sensitivity that is characteristic of preeclampsia and suggest that targeting sFLT1-induced pathways may be an avenue for treating preeclampsia and improving fetal outcomes.

Introduction

Patients with preeclampsia, a devastating hypertensive complication that occurs after the 20th week of gestation, exhibit a significantly altered angiogenic and hemodynamic profile compared with normal pregnancy (1, 2). In a seminal publication by Gant and colleagues, pregnancy was determined to be a state of relative insensitivity to direct infusion of the pressor hormone angiotensin II (Ang II), a physiological adaptation that contributes to low systemic vascular resistance (3). Conversely, pregnant women who later developed preeclampsia remained sensitive to the pressor effects of Ang II, requiring increasingly smaller doses to achieve the same rise in blood pressure. However, this phenomenon is not specific to Ang II, as sensitivity to other pressors, including catecholamines, has also been reported in preeclampsia (4, 5). Ang II sensitivity has since been well described to occur prior to the clinical signs and symptoms of preeclampsia (3, 6), yet the mechanism remains unknown, thereby preventing therapeutic intervention. Several hypotheses have been proposed, including agonistic autoantibodies to the Ang II type 1 receptor (AT1R) (7, 8), AT1R and bradykinin B1 heterodimerization (9, 10), regulation of G protein signaling (11), and activation of uteroplacental renin-angiotensin system (RAS) (12, 13). These postulates have yielded critical pathophysiological data; however, as yet no mechanism for the generalized sensitivity to vasopressors in preeclampsia has been elucidated.

Epidemiological and experimental studies support a pathophysiological role for excess circulating soluble fms-like tyrosine kinase 1 (sFLT1) in preeclampsia (1). sFLT1 is a soluble variant of VEGF receptor 1, which acts as an antiangiogenic trap for VEGF and placent al growth factor in tissues and in circulation (14). Circulating levels of sFLT1 are elevated not only during clinical disease, but also 4–6 weeks before the onset of clinical signs and symptoms of preeclampsia (15–17). The observation of a temporal relationship between high circulating sFLT1 and Ang II sensitivity led us to hypothesize that sFLT1 may directly influence the increased sensitivity to Ang II in preeclampsia. We therefore sought to determine, in normal and sFLT1-overexpressing mice, the mechanism of Ang II sensitivity during pregnancy.

Results

Ang II response in normal pregnant and preeclamptic mice mimics human physiology. To confirm that mice exhibit Ang II insensitiv-
arity during gestation as observed in women, we used in vivo and ex vivo approaches. Chronic infusion of a pressor dose of Ang II (0.43 mg/kg/d via s.c. osmotic minipump) to nonpregnant mice induced a predictable rise in blood pressure of more than 40 mmHg (Figure 1, A and B; P < 0.001). The same dose of Ang II administered to pregnant mice at gestational day (gd) 12 elicited a maximum increase in blood pressure of 22 mmHg. This increase was significant compared with mid-gestation nadir (the normal decline in blood pressure observed at gd8-10; Figure 1, A and B; P < 0.05), but not with early or nonpregnant values. The change (∆) in blood pressure after chronic Ang II infusion was significantly less in pregnant compared with nonpregnant mice (Figure 1B; P < 0.001), confirming resistance to Ang II in normal pregnancy. We used the well-characterized sFlt1 overexpression model to induce a preeclampsia-like condition in pregnant mice that reproduces the human disorder (18-20). Hemodynamic parameters were similar between sFlt1-treated and CMV-null-treated pregnant mice until gd12 (4 days after injection), when mean arterial pressure increased significantly in sFlt1-overexpressing mice (Figure 1C and D; P < 0.01). We also confirmed preeclamptic phenotype by postmortem examination of kidneys, which revealed characteristic glomerular endotheliosis-like lesions in sFlt1-overexpressing mice (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83918DS1).

The blood pressure response in the sFlt1-induced preeclampsia model was next examined in response to acute Ang II bolus injection in conscious, unrestrained mice while they were nonpregnant and then at predetermined gestational dates. Acute i.v. injection of Ang II into nonpregnant mice caused a reproducible increase in blood pressure (30 ± 5.9 mmHg) within 5 minutes, which normalized to preinjection levels within 25 minutes (n = 8; Supplemental Figure 1B). The pressor response to Ang II in normal pregnant mice became blunted at gd10, and then was refractory at gd14 (-3.6 ± 1.8 mmHg averaged over 15 minutes after injection; Figure 1E; P < 0.05). Comparable reductions were observed in heart rate at gd14 (-73.6 ± 13.1 bpm compared with nonpregnant; Supplemental Figure 1C and D; P < 0.05). Late-gestation response to Ang II remained blunted with return to normal response postpartum. In pregnant mice treated with sFlt1, response to acute bolus injection of Ang II induced a hemodynamic response similar to that observed in the nonpregnant state (Figure 1E). These data demonstrate that sensitivity to acute infusions of Ang II is blunted in normal mid- to late murine pregnancy, while preeclamptic mice remain sensitive to acute Ang II exposure throughout pregnancy.

Consistent with the acute data, chronic infusion of Ang II caused a more rapid increase in blood pressure in sFlt1-treated preeclamptic mice (Figure 1F). Chronic Ang II increased blood pressure in both CMV-null (control pregnant) and sFlt1 pregnant mice, although the magnitude of change was significant in only the sFlt1 mice (Figure 1G; P < 0.01). We confirmed that plasma sFlt1 levels at gd17 were significantly elevated in sFlt1-injected mice compared with CMV-null (P < 0.001), and that chronic Ang II infusion did not alter plasma sFlt1 levels (Supplemental Figure 1B). These data indicate that increasing circulating sFlt1 may contribute to the maintenance of responsiveness to vasoconstrictors observed in preeclamptic women. To further explore this idea, we isolated second-order mesenteric resistance vessels from gd17 CMV-null or sFlt1-treated mice for ex vivo contraction studies (Figure 1H). Contractile responses to Ang II and to the adrenergic agonist phenylephrine were significantly increased in vessels from sFlt1-treated pregnant mice compared with those with normal pregnancy (P < 0.05). These data confirm that exposure to sFlt1 is sufficient to induce the heightened vasoconstrictor and pressor response observed in preeclampsia. Removal of the vessel from contact with circulating sFlt1 during the ex vivo study suggests a persistent alteration in vascular function.

sFlt1 impairs vascular endothelial function independently of alterations in renin-angiotensin pathway. To evaluate mechanisms for Ang II sensitivity, we sought first to examine whether the renin-angiotensin pathway was upregulated. Plasma renin and Ang II were assayed at gd17 in CMV-null pregnant mice compared with sFlt1-treated preeclamptic mice with higher blood pressure. sFlt1 mice had suppressed renin compared with CMV-null mice (Figure 2A; P < 0.05), while no significant differences were found for plasma Ang II (Figure 2B). Ang II receptor (at1ra, at1tb, and at2r) mRNA expression was quantified in several candidate tissues over time in sFlt1-overexpressing compared with CMV-null mice. Ang II receptors were assessed in kidney (Figure 2C), mesenteric vessels (Figure 2D), uterine vessels (Supplemental Figure 2A), and uterus (Supplemental Figure 2B). We further assessed gene expression alterations of bradykinin receptor B2 (Bdkrb2) and endothelin-1 (Edn1) in both uterine and mesenteric vasculature (Supplemental Figure 2, C and D). Expression was dynamic over gestation; however, no differences were observed between CMV-null and sFlt1-overexpressing mice, indicating that alterations in Ang II receptors may not be responsible for the sensitivity noted in preeclampsia. Taken together with our murine studies (Figure 1H) and prior human studies suggesting a general sensitivity to vasocostricitors (4), these data do not support a direct role for alterations in circulating RAS to explain heightened sensitivity to Ang II in preeclampsia.

Impaired endothelial NO synthase and enhanced oxidative stress in preeclampsia-related Ang II sensitivity. Preeclampsia is characterized by decreased NO synthesis (21), and NO metabolites are inversely related to circulating sFlt1 concentrations (22). In the vasculature, endothelial NO synthase (eNOS) activity is the major regulator of NO production (23). VEGF and other growth factors are known to increase eNOS phosphorylation at serine 1177 (Ser1177), which is associated with increased enzyme activity (24-26). We therefore hypothesized that high sFlt1, an endogenous VEGF inhibitor, may block eNOS function and this could underlie Ang II sensitivity in preeclampsia. We examined protein expression of eNOS and its active form, phosphorylated eNOS (phospho-eNOS) (Ser1177), in cultured endothelial cells. As predicted, human endothelial cells exposed to VEGF demonstrated increased phosphorylation of Ser1177 residue on the eNOS protein in a dose-dependent manner that was inhibited in the presence of excess sFlt1 (Figure 3, A and B). We then examined both localization and expression of phospho-eNOS in mesenteric resistance vessels in pregnant sFlt1 mice or CMV-null mice. Positive phospho-eNOS signal was detected in arterial vascular endothelium. Robust signal intensity was observed in vessels from CMV-null mice, while phospho-eNOS expression was attenuated in sFlt1 mice (Figure 3C and Supplemental Figure 3A; P < 0.01). Pregnant mice given l-NAME, a NOS inhibitor, developed Ang II sensitiv-
monal factors, as well as Ang II, work in concert with diminished NO to induce hypertension during pregnancy. We next examined indicators of vascular damage in aortic sections from pregnant CMV-null mice, pregnant sFlt1-expressing mice, or l-NAME–treated pregnant mice (Figure 3F). sFlt1- or l-NAME–treated mice demonstrated increased vascular oxidative stress as measured by positivity for MitoSOX Red and nitrotyrosine immunoreactivity in
Inhibition of phosphodiesterase type 5 rescues sFlt1-induced hypertension and Ang II hypersensitivity. NO promotes vasodilation by activating smooth muscle guanylate cyclase to produce cGMP, which is then degraded by phosphodiesterases (PDEs). To determine whether sFlt1 overexpression directly impairs endothelial function via NO depletion, we explored whether enhancing cGMP with the PDE5 inhibitor sildenafil citrate (FDA-approved for treatment of pulmonary hypertension and erectile dysfunction) would prevent the adverse vascular effects of sFLT1 in pregnant mice. Preeclampsia was induced in pregnant mice by sFlt1 adenovirus (as above), and mice were randomized to sildenafil in drinking water or control beginning on gd8. Dams treated with sFlt1 and sildenafil remained normotensive throughout gestation and postpartum (Figure 4, A and B), relative to sFlt1-only dams. Plasma sFLT1 was not different between groups at gd17 (Figure 4B), indicating that sildenafil does not alter sFLT1 production. Despite similar sFLT1 levels, treatment with sildenafil significantly reduced contractile responsiveness of ex vivo mesenteric vessels from gd17 pregnant mice to Ang II (Figure 4C; *P < 0.05). Sildenafil also decreased aortic superoxide and nitrotyrosine immunoreactivity in mice exposed to sFLT1 during pregnancy (Figure 4, D and E). Urinary cGMP was elevated in animals receiving sildenafil (Figure 2).
Figure 3. sFlt1 alters the vascular endothelium by decreasing eNOS function. (A) VEGF induces dose-dependent activation of eNOS in HUVECs, as measured by eNOS phosphorylation at Ser1177 (phospho-eNOS) relative to total eNOS by Western blotting. This is inhibited by excess sFlt1 (1 μg/ml). Representative of 4 individual experiments. (B) Densitometry of phospho-eNOS/total eNOS bands plotted as a percentage of control. VEGF-induced eNOS phosphorylation in HUVECs was approximately 2.5-fold above control at 10 ng/ml of VEGF (P < 0.001 vs. all other groups). The increase in VEGF-induced phosphorylation was blocked when cells were preincubated with excess of sFlt1; *P < 0.001. Analysis by 2-way repeated-measures ANOVA, n = 4 individual experiments. (C) IHC for phospho-eNOS expression in mesenteric resistance vessels from gd17 pregnant mice given CMV-null (left) or sFlt1 (right). Images are representative of 4–5 mice per group. Scale bar: 100 μm. (D) Administration of L-NAME in drinking water during pregnancy phenocopies sFlt1-induced Ang II hypersensitivity. Pregnant mice were given 1 g/L L-NAME in drinking water at gd8 and then implanted with s.c. osmotic minipumps infusing Ang II or saline control at gd12. Blood pressure became significantly elevated only in mice given L-NAME and Ang II (n = 4 per group; daily mean ± SEM). Two-way repeated-measures ANOVA, *P < 0.01. (E) Comparison of MAP and plasma sFlt1 between L-NAME + saline and L-NAME + Ang II pregnant mice (n = 4 per group). ΔMAP was obtained by subtraction of individual pressure at gd17 from that at gd7. Gd7 was selected as the latest time point when all animals had no pressure differences (prior to intervention, permitting comparison). *P < 0.01. Analysis by 1-way ANOVA with Bonferroni’s post-hoc test. (F) Representative images of aorta isolated from CMV-null (left), sFlt1 (middle), and L-NAME (right) mice at gd17 staining for MitoSOX Red (top; scale bar: 50 μm) and nitrotyrosine (bottom; scale bar: 20 μm). All data represent the mean ± SEM.
Sildenafil improves fetal outcomes and improves placental perfusion. Due to enhanced vasoconstriction, preeclampsia is a condition of impaired placental blood flow that results in fetal growth restriction and risk of fetal loss (30). Thus, we next evaluated the safety and potential uteroplacental benefits of sildenafil in the sFlt1 overexpression model. Pregnant mice were injected with sFlt1 at gd8, plasma sFLT1 was measured after 72 hours, and mice with similar sFlt1 expression were paired and randomized to treatment (sildenafil) or control (water) at gd12. We first permitted the mice to deliver spontaneously to examine both premature delivery and live birth weight. Similarly to humans with preeclampsia, sFlt1-overexpressing mice are prone to fetal losses or premature delivery (Supplemental Table 1). Live birth weights were measured the morning of delivery in mice treated with sFlt1 or sFlt1 plus sildenafil (sFlt1 + SILD). Overall, birth weights were higher in sFlt1 + SILD pups (Figure 5A). When separated into groups based on delivery date, sFlt1 + SILD pups were heavier, even if born prematurely (Figure 5B).

In order to examine placental weight and morphometry, a second study was conducted, and all dams were euthanized at gd17 (prior to spontaneous delivery). No significant changes were observed in fetal weights, litter size, or resorption rate (Supplemental Figure 4, A–E); however, placental weight increased in sFlt1 mice treated with sildenafil (Supplemental Figure 4B; *P < 0.01). As a proxy indicator of placental insufficiency, the ratio of the placental weight to the fetal weight was compared. This ratio was increased in sFlt1 + SILD mice, implying that the heavier placenta...
Figure 5. Sildenafil treatment in sFlt1-expressing dams improves fetal outcomes by enhancing uterine blood flow. (A) Live birth weights from mice injected at gd8 with sFlt1 adenovirus and randomized to sildenafil (sFlt1 + SILD, n = 7 dams) or water (sFlt1, n = 8 dams) at gd12. Plasma sFLT1 was similar prior to randomization. *P < 0.001. (B) Live birth weights showing distribution by birth date (gd). Pups born to sFlt1 + SILD dams (n = 7) had higher birth weights compared with those born to sFlt1 dams (n = 8), independent of date of birth. *P < 0.001. Data presented as boxplot (median, interquartile ranges, minimum and maximum), analyzed by 1-way ANOVA with Tukey’s post-hoc test. (C) Placental histology from gd17 mice given sFlt1 or sFlt1 + SILD. Scale bar: 50 μm. (D and E) Representative Doppler waveform of the uterine artery at gd16 in sFlt1-treated (D) and sFlt1 + sildenafil–treated mice (sFlt1 + SILD) (E), highlighting differences in total flow (peak amplitude) and resistance (height of end diastolic velocity). (F) Uterine artery peak systolic velocity (PSV) and end diastolic velocity (EDV) obtained at gd16 via Doppler ultrasound (n = 5 per group). Sildenafil treatment increases EDV in comparison with sFlt1-only mice. *P < 0.05. (G) Uterine artery resistance index was reduced in the sFlt1 + SILD group compared with sFlt1 alone (n = 5 per group). Data are mean ± SEM, analyzed by unpaired 2-tailed t test, except where noted. *P < 0.05.
To explore safety and fetal effects in normal pregnancy, CD-1 mice were randomized to sildenafil or control at gd12. Plasma sFLT1 was not different between untreated (CON) and sildenafil-treated (SILD) dams (Figure 6A). As expected, sildenafil increases urinary excretion of cGMP (Figure 6B; \( P < 0.05 \)). Doppler ultrasound of the uterine arteries was assessed at gd16 and found that sildenafil treatment in normal pregnant mice does not impair blood flow or resistance index (Figure 6, C and D). At gd17, fetal (Figure 6E; \( P < 0.001 \)) and placental weights (Figure 6F; \( P < 0.05 \)) were increased with sildenafil treatment, while litter sizes were unaffected (Figure 6G). Interestingly, mothers treated with sildenafil had lower resorption rates than control mice (Figure 6H; \( P < 0.05 \)). Importantly, no maternal or fetal morbidities or mortalities were observed in the sildenafil-treated group. Together, these studies provide preclinical data supporting that PDE5 inhibition may be an effective strategy to circumvent sFLT1-induced endothelial dysfunction in preeclampsia, and that this therapy is safe in pregnancy and may improve both maternal and fetal outcomes in preeclampsia.

Impaired eNOS phosphorylation in placentas from preeclamptic women. We next examined the link between eNOS phosphorylation
and sFLT1 secretion in humans to determine the relevance of the mechanism identified in mice to the human condition. We examined placental histology from a cohort of preeclamptic subjects and uncomplicated gestations. IHC staining of phospho-eNOS was localized to the syncytiotrophoblast in placental samples from uncomplicated and preeclamptic subjects (Figure 7A). Adjacent sections were stained for sFLT1 expression (Figure 7B). Mean phospho-eNOS staining intensity was robust in normal pregnant samples and attenuated in preeclamptic samples (Figure 7C; \( P < 0.001 \)). As previously reported, sFLT1 staining was weak and sparse in normal placenta with over 3-fold upregulation in the syncytiotrophoblast layer of the preeclamptic placenta (\( P < 0.001 \)). Correlation of mean staining intensities revealed a negative relationship between phospho-eNOS and sFLT1 expression in the placenta (Figure 7D; \( R^2 = 0.7; P < 0.001 \)). These data in human placentas are consistent with the model determined from our in vitro and in vivo observations in which elevated sFLT1 inhibits phosphorylation of eNOS in women with preeclampsia.

**Discussion**

In this article, we explored the mechanism and potential treatment of sensitivity to vasopressors in preeclampsia and report the following: First, overexpression of sFLT1, a soluble protein that is elevated in preeclampsia, is sufficient to induce vascular sensitivity to vasopressors (including Ang II) in pregnant mice. Second, sFLT1 acts on the maternal endothelium to inhibit eNOS phosphorylation, and this is associated with increased blood pressure, vessel oxidative stress, and sensitivity to vasopressors. Third, sildenafil, a PDE5 inhibitor that raises cGMP levels, is sufficient to restore Ang II resistance during pregnancy and inhibit sFLT1-mediated hypertension. Sildenafil also increased blood flow to the fetal-placental unit and improved fetal outcomes. This finding is unique and contrary to the placental effects of current antihypertensive therapies used in pregnancy, which are often associated with decreased uterine blood flow to the fetus, leading to further risk of fetal growth restriction (30). Finally, sFLT1 expression correlates inversely with eNOS phosphorylation in human placental tissue. These data support a model in which endothelial damage due to high circulating sFLT1 leads to dysfunctional eNOS signaling, which, in turn, promotes vascular oxidative stress and enhances sensitivity to vasoconstrictors (Figure 8) that results in maternal hypertension and fetal growth restriction. Taken together with prior clinical data that sFLT1 alterations antedate hypertension of preeclampsia (35), we suggest that ameliorating the toxic effects of sFLT1 acting downstream of eNOS may alleviate preeclamptic morbidity in the mother without adverse consequences to the fetus.

The modified vascular responses that occur during normal pregnancy that are prevented during hypertensive pregnancy complications have intrigued clinicians and researchers for decades. It was postulated that discerning the physiology observed in normal pregnancy could provide insight for improved treatment of many hypertensive disorders. While resistance to Ang II during normal pregnancy has been most studied, literature supports generalized insensitivity to vasoactive substances in normal pregnancy (5). Conversely, Ang II sensitivity during preeclampsia has become so carefully documented that it is a diagnostic method to predict future development of hypertension during pregnancy (3, 6). Studies in pregnant rodents have been instrumental in determining contributions to hemodynamic and vascular alterations (36, 37). Importantly, Ang II resistance observed during pregnancy has been found to be specific to vascular smooth muscle cells, and not to actions on the renal tubules (38). Together with data show-
Figure 8. Summary model of findings. Our data are consistent with a model in which the preeclamptic placenta produces excess sFLT1, which acts on endothelial cells to impair NO production. This results in elevated plasma sFLT1 as biomarkers) would be far more beneficial to the mother and the fetus than during overt maternal hypertension. It is therefore not surprising that in a small clinical trial, sildenafil given to women with established preeclampsia was not associated with improvement in pregnancy outcomes (42). Furthermore, since preeclampsia is a very heterogeneous disease, it may be critical to focus therapeutic studies with sildenafil specifically in subgroups of patients who have augmented circulating sFLT1 and increased pressor response to Ang II.

In normal pregnancy, despite enhanced Ang II and volume expansion, NO production is amplified (21, 43–47), likely by growth factors such as VEGF and placental growth factor that are required for normal placental angiogenesis (24, 25, 48). The actions of NO to reduce blood pressure during pregnancy are pleiotropic, primarily through endothelial-dependent dilation and resulting smooth muscle relaxation, which decreases vascular resistance. NO also negatively regulates the activity of many vasoconstrictors, including endothelin-1 and Ang II (49–51), and is anti-thrombotic (52). Our study and others, including in vivo studies of pregnant eNOS mutant mice and rodents given L-NAME during pregnancy, provide valuable experimental evidence that inhibition of NO during pregnancy induces hypertension, similar to pre-eclampsia (34, 36, 50, 53, 54). Decreased NO bioavailability can also lead to hypertension by enhancing vascular oxidative stress, as NO is the principal scavenger of oxidants in the vasculature (55). Vascular oxidative stress exacerbates vasoconstriction, and there is ample evidence that angiotensin-induced hypertension requires oxidants produced in the vasculature (56). Our finding that interference with NO signaling by sFLT1 leads to enhanced vascular sensitivity to pressors that can be reversed by sildenafil also has relevance outside of pregnancy. Vascular endothelial dysfunction is characterized by impaired NO production and is an early manifestation of numerous systemic pathologies, including diabetes and atherosclerosis, and is a major contributor to cardiovascular and cerebrovascular disease (57). Genetic polymorphisms in VEGF and its receptors have been associated with susceptibility to coronary heart disease (58). Also, cancer patients treated with novel anti-VEGF pathway inhibitors almost universally develop hypertension and a preeclampsia-like syndrome (59, 60). It will be important to determine whether similar mechanisms of enhanced vascular sensitivity and Ang II sensitivity are involved and whether sildenafil or other drugs downstream of NO may be novel therapeutic strategies for endothelial dysfunction and hypertension in these other clinical scenarios.

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Our article leaves many unanswered questions and paths for future work. We do not know whether other pathways that are downstream of VEGF signaling such as endothelial prostacyclins and hydrogen sulfide may also contribute to the sFLT1-mediated Ang II sensitivity and hypertension (61). We also do not know whether other molecules known to be upregulated in human preeclampsia enhance the effects of sFLT1 on vascular function. For example, soluble endoglin, which is also elevated in pre-eclampsia, may contribute to vascular sensitivity to vasoconstrictors by affecting eNOS enzyme at other activation sites (62) or by other mechanisms. It would also be important to evaluate the effects of angiotensin receptor autoantibody (AT1-AA) in the context of high sFLT1. While suppression of circulating RAS is typical (63), there is ample evidence that AT1-AA is present in patients with preeclampsia (7, 64–66). We also do not know the mechanisms of...
Ang II resistance during normal pregnancy that is maximal during mid-gestation, but we speculate that vasodilatory factors such as relaxin and other growth factors that induce NO may be responsible. Finally, we do not know whether high circulating sFLT1 and the associated impairment of eNOS function and increased oxidative stress during pregnancy contribute to vascular changes that persist into the postpartum period, including epigenetic changes and persistent Ang II sensitivity that may predispose to long-term cardiovascular risk in women exposed to preeclampsia (67, 68).

In summary, our data demonstrate that pharmacological inhibition of PDE5 with sildenafil in the setting of sFLT1-induced hypertension and vascular dysfunction in pregnancy restores the actions of endothelial NO: this appears to be a final common pathway in the pathogenic mechanism of preeclampsia and associated Ang II sensitivity. With NO production restored, there is decreased vascular oxidative stress, improved vascular function, and normalization of blood pressure in the mother. This, in turn, increased uteroplacental blood flow, permitting optimal nutrient availability to the fetus (69) with improved fetal growth and survival. These findings may also have important implications in the pathogenesis of VEGF pathway inhibitor-induced hypertension in cancer patients and of Ang II sensitivity in nonpregnant states such as in women with a history of prior preeclampsia (70-72), major risk factors for cardiovascular disease. Prospective clinical trials are needed to evaluate the beneficial effects of sildenafil started in women with early biomarker evidence of preeclampsia in the prevention of preeclampsia-related morbidity.

Methods

Additional methods can be found in the Supplemental Methods online.

Mice. Adult male and female CD-1 [Crl:CD1(ICR)] mice were purchased from Charles River Laboratories. Animals were housed in a temperature- and humidity-regulated environment with a 12-hour light cycle. For all timed pregnancy experiments, virgin female mice (8 to 12 weeks old) were placed with a stud male overnight. Observation of a copulation plug the following morning was denoted gestational day 0 (gd0). Plugged females were weighed and removed from the stud cage. Pregnant females were injected on gd8 with either murine sFlt1 adenovirus or CMV-null adenovirus (control pregnant) diluted in sterile saline via tail vein under isoflurane anesthesia, as described previously (18). In CD-1 pregnant mice, live birth typically occurs at gd18-19, compared with gd19-20 for inbred strains (Supplemental Table 1). sFlt1 overexpression can result in embryo/fetal resorption, miscarriage, or premature delivery depending on the dose and duration of exposure of sFLT1 protein (19). sFlt1 adenovirus dose was titrated on the basis of batch efficacy to provide plasma sFLT1 values 100-750 ng/ml, which result in hypertension phenotype without significant fetal resorption (73).

Alternatively, pregnant mice were given N-nitro-L-arginine methyl ester hydrochloride (l-NNAME, a NOS inhibitor; Sigma-Aldrich) in drinking water at gd8. Dose of l-NAME was calculated as 100 mg/kg/d (1 g/l). Mice were sacrificed as nonpregnant or at gd14, gd17, or 72 hours postpartum via cardiac puncture under deep isoflurane anesthesia, and organs were collected for vascular reactivity studies, histology, protein, and RNA.

Blood pressure measurement. Direct blood pressure monitoring was done using radiotelemetry (Data Sciences International). Surgical implantation of TA11PA-C10 transmitters was performed as previously described (74). Briefly, 10-week-old female CD-1 mice were anesthetized with inhaled isoflurane in O2, with meloxicam (5 mg/kg, s.c.) as analgesic. Mice were permitted 1 week to recover from surgical implantation, followed by data acquisition for 3-5 days to assess data quality and establish normal blood pressure (nonpregnant) values. Mice were then mated (as above) and recorded throughout pregnancy (74). Blood pressure data were averaged daily per animal.

Acute or chronic Ang II infusion studies. In the acute studies, Ang II (30 ng in 30 μl; Sigma-Aldrich) was injected i.v. to awake, unrestrained, telemetry-implanted mice during recording. Bolus injections were administered up to 3 times to nonpregnant mice and once on gd10, gd14, and gd17, and 72 hours postpartum. Analysis of acute Ang II response used blood pressure/heart rate normalized before injection (t = 0) every 5 minutes for 20 minutes, presented as moving average of the change relative to t = 0.

In chronic studies, telemetry mice that had received either sFlt1 or null adenovirus at gd8 were surgically implanted with s.c. osmotic minipumps at gd12 (model 1002; Alzet) containing Ang II (0.43 mg/kg/d) diluted in sterile saline. Pumps were filled and implanted according to the manufacturer’s directions. Chronic Ang II response was also assessed in nonpregnant mice to compare with normal pregnancy. Nonpregnant telemetry mice were recorded for minimally 14 days before implantation of s.c. minipumps (as above) filled with Ang II. During l-NNAME experiments, telemetry mice were given l-NNAME in drinking water at gd8 (in lieu of sFlt1 adenovirus), and then implanted at gd12 with s.c. minipumps containing Ang II or sterile saline alone.

Sildenafil citrate studies. Experiments using sildenafil citrate (Cayman Chemical) for prevention of preeclamptic-like signs were conducted in telemetry-implanted mice, as above. In these animals, 100 mg/kg/d sildenafil was provided in the drinking water immediately following adenovirus injection (gd8), and animals were sacrificed at gd17 to evaluate preeclampsia phenotypes. In other studies, pregnant mice were injected with sFlt1 adenovirus at gd8. Seventy-two hours later (gd11), plasma was obtained and assayed for circulating sFlt1 protein. Mice were paired and randomized to treatment (sFlt1 + SILD) or normal water (sFlt1) on gd12 based on similar elevations in circulating sFLT1 protein. At gd17, mice were euthanized for vascular reactivity studies or tissue collection. In a separate study, paired mice were permitted to deliver spontaneously to permit assessment of preterm delivery and live birth outcomes.

An additional set of experiments was conducted to more thoroughly examine the impact of sildenafil on maternal and fetal outcomes. Pregnant mice were assigned to no treatment (CON) or sildenafil treatment (SILD) from gd12 to determine the impact of treatment on normal pregnancy. All groups had Doppler ultrasound of the uterine arteries at gd16 and were euthanized at gd17 for measurement of fetal and placental weights.

Western blot analysis. Eighty to ninety percent confluent HUVECs (ATCC) were incubated in 0.25% serum/EGM-2 medium without additional growth factors overnight. Cells were stimulated with increasing doses of recombinant VEGF-165 (R&D Systems) in the presence and absence of 1 μg/ml recombinant human sFlt1 (R&D Systems) for 15 minutes in EGM-2 medium without serum. Cells were washed with cold PBS and lysed with 1× cold cell lysis buffer supplemented with 1 mM Na3VO4, 10 mM NaPO4.2H2O, 25 mM NaF, and protease inhibitors (Cell Signaling Technology). Cell lysates were quantified for...
protein concentrations, and 50 μg protein was separated in 8% SDS-PAGE and immunoblotted with antibodies against phospho-eNOS (Ser1177) (07-428; EMD Millipore) or antibody that recognizes total eNOS (07-520; EMD Millipore). Densitometry was performed using UN-SCAN-IT gel software (Silk Scientific).

**Human subjects.** Details regarding preeclampsia diagnosis and placental collection have been published (75). All subjects presented to Beth Israel Deaconess Medical Center for delivery. Placental biopsies were obtained within 30 minutes of delivery and placed in 10% neutral buffered formalin for routine tissue processing.

**Histology and IHC.** Mouse tissues collected for histology and IHC were immediately placed in 10% neutral buffered formalin for routine processing and staining. Human placental tissue was obtained uncompli cated or pre eclampsia pregnancies for IHC of phospho-eNOS. Histopathology of mouse kidneys was conducted to assess “glomerular endotheliosis” as described previously (18). For nitrotyrosine IHC of the abdominal aorta, 5-μm paraffin sections were cut and deparaffinized. Slides were rinsed in PBS followed by a 15-minute quenching of endogenous peroxidase with 3% hydrogen peroxide in distilled water. To block nonspecific binding, slides were incubated at 37°C with 2.5% normal horse serum in PBS for 40 minutes. Rabbit polyclonal nitrotyrosine antibody (A-21285 IgG; Thermo Fisher) was used at 1:500 overnight at 4°C. Slides were then rinsed in PBS and developed using ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit, according to directions (Vector Laboratories). IHC staining for phospho-eNOS was completed on mesenteric resistance vessels from mouse and term human placenta. Slide preparation and blocking methods were similar to those for nitrotyrosine, with an antigen retrieval step in citrate buffer, according to the manufacturer’s recommendations (Vector Laboratories). Rabbit polyclonal phospho-eNOS (Ser1177) from Cell Signaling Technology (1:100; 9570) was used for mouse samples and from Abcam (1:1,000; ab87750) for human samples. Development for visualization used VECTASTAIN Elite ABC Kit (Rabbit IgG; PK-6101; Vector Laboratories), according to the manufacturer’s recommendations. Serial sections of human placenta were stained for human sFLT1 (1:200; AF231; R&D Systems), as previously described (75). Slides were counterstained with hematoxylin, dehydrated, and mounted. Alternatively, tissues were embedded in OCT compound (Thermo Fisher) and snap-frozen in liquid nitrogen for immunofluorescence. Five-micrometer cryosections of thoracic aorta were cut and equilibrated in PBS for 10 minutes at 37°C, followed by incubation with 5 nM MitoSOX Red (Invitrogen) for 10 minutes at 37°C. Slides were then rinsed with PBS, fixed, and mounted.

**Statistics.** Longitudinal data (hemodynamic data, serial sFLT1 measurements) were compared using 2-way repeated-measures ANOVA, followed by Šidák’s or Bonferroni’s post test, as applicable. Data with multiple measurements per n were compared using 2-way ANOVA. Data comparing 3 or more groups were analyzed using 1-way ANOVA with Tukey’s multiple comparison test or Kruskal-Wallis with Dunn’s post-test. Two-group comparisons were made using paired or unpaired t tests with or without Welch’s correction, as applicable. Pearson r test was used to assess correlation. GraphPad Prism version 6 was used for all analyses. A P value of less than 0.05 was considered statistically significant.

**Study approval.** All animal procedures and surgical protocols were reviewed and approved by the IACUCs at Beth Israel Deaconess Medical Center and Tufts Medical Center. Beth Israel Deaconess Medical Center and Tufts Medical Center are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The IRB at Beth Israel Deaconess Medical Center approved collection and use of discarded human placentas.

**Author contributions**

SDB and SAK designed the study. SDB characterized the mouse model of preeclampsia including telemetry studies and performed rescue/safety experiments with sildenafil. ZKZ and IES performed all histology and immunohistochemistry studies. EVK and SDB performed ultrasound flow studies. ASL and AR conducted phosphorylation experiments. DZ measured renin and angiotensin. CDC, AW, DZ, and SDB performed PCR and immunooassays. JJD, AM, and MEM performed vascular reactivity studies. SDB, ZKZ, EVK, IES, IZJ, and SAK analyzed data. SDB and SAK wrote the manuscript with major contributions from EWS, PMK, IES, and IZJ. All authors reviewed the manuscript prior to submission.

**Acknowledgments**

S.D. Burke, C.D. Clark, A. Rajakumar, and S.A. Karumanchi were supported by the Howard Hughes Medical Institute. E.V. Khankin was supported by KO8 DK101568 from the NIH. I.Z. Jaffe was supported by ROI grants HL095590 and HL119290 from the NIH.

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60. Vigneau C, et al. All anti-vascular endothelial growth factor drugs can induce pre-eclampsia-