Autoantibody From Women With Preeclampsia Induces Soluble Fms-Like Tyrosine Kinase-1 Production via Angiotensin Type 1 Receptor and Calcineurin/Nuclear Factor of Activated T-Cells Signaling

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Abstract

Preeclampsia is a pregnancy-specific hypertensive syndrome that causes substantial maternal and fetal morbidity and mortality. Recent evidence indicates that maternal endothelial dysfunction in preeclampsia results from increased soluble Fms-like tyrosine kinase-1 (sFlt-1), a circulating antiangiogenic protein. Factors responsible for excessive production of sFlt-1 in preeclampsia have not been identified. We tested the hypothesis that angiotensin II type 1 (AT_1) receptor activating autoantibodies, which occur in women with preeclampsia, contribute to increased production of sFlt-1. IgG from women with preeclampsia stimulates the synthesis and secretion of sFlt-1 via AT_1 receptor activation in pregnant mice, human placental villous explants, and human trophoblast cells. Using FK506 or short-interfering RNA targeted to the calcineurin catalytic subunit mRNA, we determined that calcineurin/nuclear factor of activated T-cells signaling functions downstream of the AT_1 receptor to induce sFlt-1 synthesis and secretion by AT_1-receptor activating autoantibodies. AT_1-receptor activating autoantibody–induced sFlt-1 secretion resulted in inhibition of endothelial cell migration and capillary tube formation in vitro. Overall, our studies demonstrate that an autoantibody from women with preeclampsia induces sFlt-1 production via angiotensin receptor activation and downstream calcineurin/nuclear factor of activated T-cells signaling. These autoantibodies represent potentially important targets for diagnosis and therapeutic intervention.

Keywords

preeclampsia; renin-angiotensin system; angiotensin receptor; autoantibody; angiogenesis; cell signaling

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Preeclampsia is a pregnancy-specific syndrome of hypertension and proteinuria, resulting in substantial maternal and neonatal morbidity and mortality.[1] Although the underlying pathogenic mechanisms of the disorder are not well understood, preeclampsia is largely

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Disclosures
None.

believed to be associated with uteroplacental ischemia and maternal endothelial cell dysfunction.[2–5] It is a widely held view that "toxic factors" secreted by the placenta into the maternal circulation are responsible for systemic endothelial dysfunction, hypertension, and multiorgan damage.[6–11] Recent research has shown that soluble Fms-like tyrosine kinase-1 (sFlt-1) is 1 of the key "toxic factors" released by the placenta into the maternal circulation and that it contributes to the hypertension, proteinuria, and endothelial cell dysfunction associated with this disorder.[12–15] sFlt-1 is a soluble form of the vascular endothelial growth factor receptor that lacks the cytoplasmic tail and transmembrane domain but retains the extracellular ligand-binding domain (sFlt-1 is also named sVEGFR1). Therefore, sFlt-1 prevents circulating vascular endothelial growth factor and placental growth factor interactions with their proangiogenic receptors and functions as an antiangiogenic factor. The level of sFlt-1 in the plasma of women with preeclampsia is elevated in comparison with that in women with uncomplicated pregnancies,[16–18] a feature consistent with microarray data showing that sFlt-1 mRNA levels are elevated in placentas from women with preeclampsia compared with placentas of normotensive pregnant women.[12] The physiological consequences of elevated sFlt-1 in preeclampsia have been examined recently in animal studies. Administration of sFlt-1 to rats resulted in elevated blood pressure, proteinuria, and renal changes, indicating that excessive placental-derived sFlt-1 may contribute to these classic features preeclampsia. In view of the critical contributions of sFlt1 to disease pathogenesis, it is important to identify factors responsible for increased synthesis and secretion of sFlt-1 by the placentas of women with preeclampsia.

A local renin-angiotensin system plays an important role in regulating placental development.[19–24] Decidual tissue is enriched in the components needed for angiotensin II (Ang II) production (ie, renin, angiotensinogen, and angiotensin-converting enzyme), and placental tissue is enriched in the Ang II type 1 (AT_1) receptor.[23] We have shown recently that Ang II stimulates sFlt-1 production by human trophoblast cells, placental villous explants, and pregnant mice.[25] These findings suggest that Ang II regulates sFlt-1 synthesis and secretion by trophoblast cells of the placenta during pregnancy. Because Ang II levels are not elevated in preeclampsia beyond that occurring in uncomplicated pregnancies,[22,26] we must look for other factors to account for the additional synthesis and secretion of sFlt-1 associated with preeclampsia. Likely candidates are the angiotensin receptor–activating autoantibodies present in women with preeclampsia.[27] These autoantibodies interact with a common epitope on the second extracellular loop of the AT_1 angiotensin receptors. Autoantibody-mediated activation of AT_1 receptors on human trophoblast cells results in increased production of reduced nicotinamide-adenine dinucleotide phosphate oxidase, tissue factor, and plasminogen activator inhibitor-1. Activation of AT_1 receptors by these autoantibodies on various cell types provokes biological responses relevant to the pathophysiology of preeclampsia.[27–32] We report here that IgG from preeclamptic but not normotensive pregnant individuals stimulates sFlt-1 synthesis and secretion by pregnant mice, by human placental villous explants, and by cultured human trophoblasts. We show that these autoantibodies are capable of inducing endothelial dysfunction via sFlt-1 induction. Notably we show that losartan and a 7-amino acid epitope peptide can prevent AT_1 receptor-activating autoantibody (AT1-AA)–induced sFlt-1 secretion and endothelial dysfunction in vitro. Finally, we demonstrate that nuclear factor of activated T cells (NFAT), a well known transcription factor activated by calcineurin signaling functioning downstream of the AT_1 receptor, may regulate sFlt-1 induction by AT1-AA at the transcriptional level. Our findings suggest that angiotensin receptor agonistic autoantibodies represent potentially important targets for diagnosis and therapeutic intervention in the management of preeclampsia.

Methods

Reagents

One-step RT-PCR kit, cell culture medium, antibiotics, and fetal bovine serum (FBS) were purchased from Invitrogen. Ang II was from Sigma. Losartan was a generous gift from Merck and Co, Inc. FK506 was purchased from Fujisawa Pharmaceuticals. Endothelial cell culture medium EGM-2 was from Cambrex.

Patients

Patients who were admitted to Memorial Hermann Hospital were identified by the obstetric faculty of the University of Texas Medical School at Houston. Patients were diagnosed with severe preeclampsia based on the definition set by the National High Blood Pressure Education Program Working Group report. The criteria include the presence of high blood pressure of ≥160/110 mm Hg and the presence of protein in urine of ≥0.3 g in a 24-hour period. These women had no previous history of hypertension. Other criteria included the presence of persistent headache, visual disturbances, epigastric pain, or the HELLP syndrome in women with blood pressure of ≥140/90 mm Hg. Normotensive pregnant individuals were characterized by uncomplicated pregnancies with normal, term deliveries. Characteristics of the patient population used for this study are provided in the Table. Blood samples were centrifuged at 18 000g for 10 minutes, and the serum samples were stored at −80°C. The research protocol, including the consent form, was approved by the institutional committee for the protection of human subjects.

Preparation of the Immunoglobulin Fraction

The IgG fraction was isolated, as described previously,[28] using GammaBind G Sepharose (Amersham Biosciences). Typically, 200 μL of patient sera were applied to the column matrix and eluted in 1.8 mL of buffer according to the manufacturer-recommended protocol. This approach resulted in an ≈9-fold dilution of IgG from that originally present in patient sera. Various dilutions of these samples, typically 1:10 or 1:20 dilution, were used for in vitro assay using cultured cells or placental explants.

Synthetic Peptides

For the neutralization experiments, a synthetic peptide corresponding to a sequence present on the second extracellular loop of the human AT_1 receptor, AFHYESQ, was used. This peptide was purchased from Baylor College of Medicine.

Introduction of Antibody Into Mice

C57BL/6J pregnant or nonpregnant mice (18 to 22 g; Harlan) were used in our study. All of the animal studies were reviewed and approved by the animal welfare committee of the University of Texas Houston Health Science Center. Mice were anesthetized with sodium pentobarbital (50 mg/kg IP), and concentrated IgG purified from 200 μL of patient's serum was introduced into 13-day pregnant mice or nonpregnant mice by orbital sinus injection. Some animals also received losartan (0.24 mg) or a 7-amino acid peptide corresponding with an epitope on the second extracellular loop of the AT_1 receptor (1.5 mg). We collected serum on gestation day 19 for measurement of the sFlt-1 concentration.

Human Placental Villous Explants Cultured With IgG

Human placental tissue was obtained from uncomplicated term pregnancies delivered by elective cesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies, as described previously.[33,34] Informed consent was obtained from the patients, and the study had the approval of the South Birmingham Ethical

Committee. Five small fragments (15 to 20 mg wet weight) of placental villi were dissected from the placenta, teased apart, and placed in 24-well plates. Placental villous explants were cultured in phenol red-free DMEM (Sigma Ltd) supplemented with 100 μg/mL of streptomycin, 100 U/mL of penicillin, and 0.2% BSA in 5% CO_2 at 37°C. IgG purified from normotensive and preeclamptic patients was added to the villous fragments at the indicated concentrations and incubated for ≤72 hours at 37°C. For the inhibitor studies, losartan at 100 nM or a 7-amino acid peptide (AFHYESQ; 0.1 μmol/L) corresponding with an epitope on the second extracellular loop of the AT_1 receptor was added for 30 minutes before stimulation with IgG. After 72 hours, the conditioned medium (CM) was collected and stored at –20° for further analysis.

IgG Treatment and Collection of Conditioned Medium

For sFlt-1 induction, 1×10^5 human trophoblast cells HTR-8/SVneo[35] were plated in 12-well plates overnight. The next day, cells were changed to serum-free medium, treated with a 1:10 dilution of purified IgG, and incubated for an additional 3 or 4 days. For some experiments, losartan (100 nM), FK506 (1 μmol/L), or the 7-amino acid epitope peptide (AFHYESQ; 0.1 μmol/L) was added to culture medium 30 minutes before the addition of IgG. After 3 or 4 days, cells and culture medium were collected and centrifuged at room temperature for 15 minutes at 100g. Cell pellets were used for Western blot analysis. Supernatants were termed conditioned medium (CM) and used for a variety of experiments. The concentration of sFlt-1 in CM was determined by ELISA (see below). CM was also used in the in vitro endothelial cell migration and capillary tube formation assays, as we have described previously.[25] For some experiments, sFlt-1 was depleted by incubation with monoclonal anti-Flt antibody for 2 hours at 4°C before adding protein A overnight. The following day, supernatants were collected after centrifugation to remove immunoprecipitates and were used in endothelial cell tube formation and migration assays.

ELISA for sFlt-1

sFlt-1 levels in CM and in mouse serum were measured using a commercial kit (R and D Systems).

Transfection of Small-Interfering RNA

HTR cells (1.0×10^5) were plated on 6-well plates overnight, and transfected with 3 μg of small-interfering RNA (siRNA) specific for calcineurin catalytic subunit-α (CN) or with nonspecific siRNA (Dharmacon) using RNAiFect transfection reagent (Qiagen). Non-specific siRNA was used as a negative control. At 48 hours after transfection, cells were cultured in serum-free medium and treated with IgG (1:10 dilution) from normotensive women or from women with preeclampsia for 72 hours.[25] Secreted sFlt-1 in cell culture medium was measured by ELISA.

RNA Isolation and Semiquantitative RT-PCR

TRIzol reagent was used for the isolation of total RNA. RT-PCR was performed according to the manufacturer's recommended protocol (Invitrogen). One microgram of RNA was used per reaction, and the annealing temperature for PCR was 55°C. sFlt-1 primer sequences and PCR conditions were as described[14]: sense primer, 5′-TTTGCATAGCTTCCAATAAAGTTG, and antisense primer, 5′-CATGACAGTCTAAAGTGGTGGAAC. β-Actin was used as an internal control, and primer sequences were as described.[25] RT-PCR products were revealed on 2% agarose gels and quantified using Bio-Rad quantity-1 software. sFlt-1 mRNA expression was represented by the ratio of sFlt-1 mRNA:β-actin mRNA.

Luciferase Activity

Chinese Hamster Ovary cells (1×10^5 cells) containing stably integrated copies of a minigene encoding the rat AT_1 receptor and a $4 \times NFAT$-driven luciferase construct were plated on 24-well plates overnight. The next day cells were changed to serum-free medium and treated with IgG (1:10 dilution) for 24 hours. Luciferase activity in cell lysates was measured using a luciferase assay kit (Promega).

In Vitro Endothelial Cell Migration and Tube Formation

The in vitro cell migration assays were preformed as described.[36] Briefly, human umbilical vein endothelial cells (HUVECs; 2.5×10^4 per well) were prepared with 500 μL of EGM-2 basal medium and added to the inserts of the 24-well plates (BD Biosciences). CM was used as a chemoattractant and added to the outside of the insert chamber. After 24 hours of incubation at 37°C, migrated cells were stained with Quick-diff and counted. From 4 to 10 fields of cells were counted and the average recorded. This procedure was carried out in triplicate for each sample of CM.

The endothelial cell in vitro tube formation assays were performed as described[12] using 96-well plates coated with growth factor–reduced Matrigel (BD Biosciences) and incubated at 37°C for 30 minutes. HUVECs, at a density of 2×10^4 cells per well, were incubated with CM, plated on precoated 96-well plates, and incubated at 37°C in a CO_2 incubator. Images were taken after 4 hours of incubation, and tube lengths were measured using MetaMorph software (Molecular Devices). Five to 10 fields were used to average the tube length.

Statistical Analysis

All of the values are expressed as the mean±SEM. Data were analyzed for statistical significance using GraphPad Prism software (GraphPad Software). A value of $P < 0.05$ was considered significant.

Results

IgG From Women With Preeclampsia Stimulates sFlt-1 Secretion in Pregnant Mice

To evaluate the potential in vivo role of AT1-AA on sFlt-1 secretion in preeclampsia, we introduced IgG from either preeclamptic or normotensive patients into pregnant and nonpregnant mice. Because sFlt-1 secretion is normally induced late in pregnancy, we chose to introduce IgG at gestation day 13. Mice were euthanized 5 days after injection, and serum levels of sFlt-1 were determined by ELISA. The results show that circulating levels of sFlt-1 were significantly increased in the pregnant mice 5 days after injection of IgG from preeclamptic women over that observed in the pregnant mice after injection of IgG from normotensive pregnant women (Figure 1A). The concentration of sFlt-1 was very low in nonpregnant mice and was not stimulated by injection with IgG from either normotensive or preeclamptic women (Figure 1B), suggesting that placenta is the major source for excessive secretion of sFlt-1 in antibody-injected mice. The antibody-mediated sFlt-1 induction in the pregnant mice was prevented by the coinjection of losartan, an AT_1 receptor antagonist, or by a 7-amino acid peptide that corresponds with a site on the second extracellular loop of the AT_1 receptor that is recognized by autoantibodies from women with preeclampsia (Figure 1A). These findings indicate that IgG from preeclamptic patients induces sFlt-1 secretion in pregnant mice through AT_1 receptor activation.

IgG From Preeclamptic Women Stimulates Excess sFlt-1 Secretion by Human Placental Villous Explants via AT$_1$ Receptor Activation

To determine whether AT1-AA stimulates sFlt-1 production by the placenta, human placental villous explants were incubated with IgG purified from preeclamptic or normotensive pregnant individuals. After 72 hours, the CM was collected, and the concentration of sFlt-1 was determined. IgG purified from women with preeclampsia induced an excessive secretion of sFlt-1 (Figure 2A). Similar to in vivo studies from pregnant mice, the autoantibody-induced sFlt-1 secretion in pregnant mice was blocked by losartan or the 7-amino acid epitope peptide. The autoantibody-induced stimulation of sFlt-1 secretion was prevented by the presence of losartan, an AT$_1$ receptor antagonist, and by a 7-amino acid peptide that corresponds to a site on the second extracellular loop of the AT$_1$ receptor that is recognized by autoantibodies from women with preeclampsia. These findings indicate that IgG from women with preeclampsia is capable of inducing sFlt-1 secretion via AT$_1$ receptor activation in human placentas.

To determine whether Ang II and AT1-AA can function additively to stimulate sFlt-1 secretion, we incubated human placental villous explants with limiting concentrations of Ang II or AT1-AA individually or in combination. After 72 hours, the concentration of sFlt-1 in the medium was determined. sFlt-1 secretion was significantly induced by Ang II or AT1-AA alone (Figure 2B). However, the combination of Ang II and AT1-AA resulted in a significantly higher stimulation than either alone (Figure 2B). These findings imply that the excessive production of sFlt-1 in women with preeclampsia may be because of the additional autoantibody-mediated activation of AT$_1$ receptors in their placentas.

IgG From Women With Preeclampsia Stimulates sFlt-1 Secretion by Human Trophoblast Cells via AT$_1$ Receptor Activation

We have reported recently that Ang II stimulates sFt-1 synthesis and secretion from human trophoblast cells.[25] Thus, it is likely that trophoblast cells are the source of the autoantibody-induced sFlt-1 production from placental villous explants.[14,37,38] To test this possibility, we incubated immortalized human trophoblasts cells HTR-8/SVneo with IgG purified from preeclamptic or normotensive pregnant individuals and determined the concentration of sFlt-1 in the CM after 4 days. IgG from preeclamptic individuals induced a significant increase in sFlt-1 secretion, whereas IgG from normotensive pregnant women did not (Figure 3A). The autoantibody-mediated induction of sFlt-1 secretion in the human trophoblast cells was inhibited by the presence of losartan or the 7-amino acid epitope peptide (Figure 3A). These results provide in vitro evidence that autoantibodies associated with women with preeclampsia are capable of activating AT$_1$ receptors on human trophoblast cells, resulting in increased sFlt-1 secretion.

As with human placental villous explants, we found that Ang II and AT1-AA can function additively to stimulate sFlt-1 secretion by cultured human trophoblast cells (Figure 3B). Specifically, we found that sFlt-1 secretion was significantly induced by Ang II or AT1-AA alone (Figure 3B). The combination of Ang II and AT1-AA resulted in a significantly higher stimulation than either alone (Figure 3B). These findings imply that the excessive secretion of sFlt-1 in preeclampsia may be because of additional AT$_1$ receptor activation by AT1-AA present in these women.

Calcineurin/NFAT Signaling Functions Downstream of the AT$_1$ Receptor and Is Required for Autoantibody-Induced sFlt-1 Production by Human Trophoblast Cells

Calcineurin signaling pathways play important roles in multiple pathological conditions, including hypertension and cardiac hypertrophy.[39–41] Previous studies have shown that calcium calmodulin–dependent calcineurin signaling functions downstream of the AT$_1$

receptor in response to AT_1-AA–mediated plasminogen activator inhibitor-1 induction in human trophoblast cells.[35] To determine whether calcineurin signaling is associated with autoantibody-mediated stimulation of sFlt-1 secretion by human trophoblast cells, we initially used FK506, a well-known specific inhibitor of calcineurin activity. The results (Figure 4A) show that the increased sFlt-1 secretion mediated by IgG from women with preeclampsia was blocked by the presence of FK506. FK506 had no effect on sFlt-1 secretion in the cells treated with IgG from normotensive pregnant women (Figure 4A). The involvement of calcineurin signaling was confirmed using siRNA specific to mRNA encoding the calcineurin catalytic subunit $α$.[25] As with FK506, we found that autoantibody-induced stimulation of sFlt-1 secretion was significantly inhibited by the presence of calcineurin-specific siRNA (CN siRNA) but not by the presence of nonspecific siRNA (Figure 4B). Taken together, these findings show that calcineurin signaling functions downstream of autoantibody-induced AT_1 receptor activation to stimulate sFlt-1 secretion by human trophoblast cells.

Realizing that the abundance of sFlt-1 mRNA is elevated in placentas from women with preeclampsia,[12] we wanted to know whether AT1-AA-induced sFlt-1 secretion in human trophoblast cells was accompanied by an increase in sFlt-1 mRNA. To address this issue, trophoblast cells were treated in a variety of ways, and the abundance of sFlt-1 mRNA was assessed using RT-PCR. The results (Figure 5A and 5B) show that sFlt-1 mRNA abundance was significantly induced by IgG from women with preeclampsia but not IgG from normotensive pregnant women and that the induction was inhibited by losartan or the 7-amino acid epitope peptide. In addition, preincubation with FK506 blocked the antibody-mediated induction of sFlt-1 mRNA (Figure 5A and 5B), suggesting that sFlt-1 mRNA upregulation by AT1-AA requires calcineurin signaling.

Calcineurin plays a critical role in a number of signal transduction pathways by catalyzing the dephosphorylation of the transcription factor, NFAT, thereby allowing the latter to enter the nucleus and participate in the transcriptional activation of target genes. Thus, we hypothesize that autoantibody-mediated stimulation of sFlt-1 mRNA levels through AT_1 receptor activation is a transcriptional response associated with the calcineurin-mediated activation of NFAT. To assess this possibility, we measured the activity of an NFAT-luciferase reporter construct in Chinese hamster ovary cells treated with IgG from normotensive pregnant women or from women with preeclampsia in the presence or absence of losartan, the 7-amino acid epitope peptide, or FK506. IgG from preeclamptic patients stimulated the activity of the NFAT-luciferase reporter gene, whereas IgG from normotensive pregnant women did not (Figure 5C). This induction was blocked by the presence of losartan, the 7-amino acid epitope peptide or FK506 (Figure 5C). These findings suggest that the AT1-AA–mediated induction of sFlt-1 mRNA results from an NFAT-mediated transcriptional enhancement of the *Flt-1* gene.

Autoantibody-Induced sFlt-1 Inhibits Endothelial Cell Function

Placental-derived sFlt-1 is believed to contribute to endothelial dysfunction in preeclampsia.[12,42] To determine whether the autoantibody-induced sFlt-1 produced by cultured human trophoblast cells is biologically active, we incubated human trophoblast cells with IgG from preeclamptic or normotensive pregnant individuals and tested CM for its impact on endothelial cell migration and tube formation. CM from human trophoblast cells treated with IgG from women with preeclampsia showed a 50% reduction in endothelial cell (HUVEC) migration (Figure 6A) and tube formation (Figure 6B and 6C) when compared with CM from trophoblast cells treated with IgG from normotensive pregnant women. Importantly, removal of sFlt-1 using anti–sFlt-1 antibody from preeclamptic IgG-treated trophoblast CM eliminated the antimigratory properties (Figure 6A) and reduced the in vitro antiangiogenic activity (Figure 6B and 6C). These results indicate that sFlt-1 accounted for

the reduction in angiogenic activity seen in CM from trophoblast cells treated with IgG from preeclamptic individuals. The inhibitory properties of the CM from preeclamptic IgG-treated trophoblast cells were prevented by the presence of losartan or FK506, indicating that blockade of AT_1 receptor activation or downstream calcineurin signaling prevented the autoantibody-mediated induction of sFlt-1 (Figure 6). The presence of the 7-amino acid epitope peptide also prevented the appearance of autoantibody-induced antiendothelial properties of CM. Taken together, these studies show that autoantibody-mediated AT_1 receptor activation results in the increased production of trophoblast-derived sFlt-1 that can inhibit endothelial cell migration and tube formation resulting in autoantibody-induced endothelial dysfunction.

Discussion

We report here that IgG from women with preeclampsia stimulates sFlt-1 synthesis and secretion by pregnant mice, human placental villous explants, and human trophoblast cells through AT_1 receptor activation and that the increased production of sFlt-1 results in the inhibition of endothelial cell migration and capillary tube formation. Notably, losartan or an antibody-blocking epitope peptide prevented the excessive sFlt-1 secretion, thereby highlighting therapeutic possibilities for the treatment of preeclampsia. In addition, we have shown that NFAT, a transcription factor and a well-known calcineurin substrate, is required for antibody-mediated transcriptional enhancement of the *FLT-1* gene. In summary, our studies indicate that autoantibodies functioning as Ang II are capable of activating AT_1 receptors and contribute to excess sFlt-1 secretion in preeclampsia.

The level of sFlt-1 in the maternal circulation increases significantly during the third trimester.[16,43,44] We have shown recently that Ang II stimulates increased production of sFlt-1 by human placental villous explants, human trophoblast cells, and in pregnant mice.[25] We speculate that the increase in circulating sFlt-1 that occurs during the third trimester of a normal pregnancy is because of the increase in circulating Ang II that occurs at this time.[20–22,45] Preeclampsia is characterized by an earlier and more extensive increase in the concentration of circulating sFlt-1.[16–18] Factors accounting for the precocious and excessive accumulation of sFlt-1 associated with preeclampsia have not been identified previously. Ang II levels are not elevated in preeclampsia over that occurring in a normal pregnancy,[20,22,26,45,46] and, therefore, other factors may contribute to the elevated levels of sFlt-1 observed in women with preeclampsia. We hypothesize that AT1-AA present in women with preeclampsia contributes to the increased production of sFlt-1 observed in these women. These autoantibodies are detected at as early as 18 to 20 weeks of gestation in women with impaired placental development,[47] thereby placing their appearance within a time-frame congruent with subsequent increases in circulating sFlt-1.[16] Here, we showed that IgG from women with preeclampsia activates AT_1 receptors and thereby stimulates increased sFlt-1 secretion from human placental explants and cultured human trophoblast cells. We also showed that Ang II and AT1-AA can function additively to induce increased sFlt-1 secretion through additional AT_1 receptor activation. We suggest that Ang II is a key regulator of sFlt-1 synthesis and secretion during normal pregnancy[25] and that the excessive accumulation of sFlt-1 observed in women with preeclampsia is because of additional activation of AT_1 receptors mediated by AT1-AA.

The following lines of evidence indicate that autoantibody-induced sFlt-1 secretion results from transcriptional activation of the *FLT-1* gene. First, we have shown here that autoantibody-induced sFlt-1 secretion is accompanied by a corresponding increase in sFlt-1 mRNA abundance. Second, a transcriptional control mechanism is suggested by our finding that antibody-mediated AT_1 receptor activation is accompanied by downstream activation of the calcineurin/NFAT signaling pathway. Calcineurin is a calcium/calmodulin-dependent

phosphatase that dephosphorylates multiple residues within the regulatory domain of the transcription factor NFAT, leading to its nuclear translocation and the activation of target genes. Additional evidence for the importance of autoantibody-induced NFAT activation comes from experiments using Chinese hamster ovary cells stably transformed with an NFAT-luciferase reporter gene. We found that the NFAT-luciferase reporter gene was activated by IgG from women with preeclampsia and that the activation was blocked by the calcineurin inhibitor FK506. Finally, a role for NFAT in the transcriptional regulation of the *FLT-1* gene is consistent with the presence of 4 consensus NFAT binding sites in the 5′ flanking region of the gene within 550 bp of the transcription start site (GenBank accession No. D64016). Overall, our findings suggest that NFAT, functioning downstream of calcineurin, may directly regulate *FLT-1* transcriptional activation after autoantibody-mediated AT_1 receptor activation.

In addition to AT1-AA, other factors may contribute to the increased sFlt-1 secretion associated with preeclampsia. Preeclampsia is often associated with poorly developed placentas characterized by shallow trophoblast invasion, deficient spiral artery remodeling, and reduced perfusion. These placental abnormalities result in a hypoxic environment for the placenta that is believed to contribute to increased sFlt-1 production in preeclampsia.[14,48–50] It has been suggested that hypoxia-induced upregulation of sFlt-1 expression is through the presence of hypoxia-inducible factor-1α response elements in the *sFLT-1* promoter region.[51] Reduced oxygen tension is also associated with increased production of placental cytokines, including tumor necrosis factor-α, which is elevated in the maternal circulation of women with preeclampsia.[42,52] Recent studies show that tumor necrosis factor-α, in a concentration-dependent manner, stimulates the release of sFlt-1 from placental villous explants.[42] Complement activation, especially C5a, is associated with increased sFlt-1 production.[53] Negative regulators of sFlt-1 production have also been identified. The carbon monoxide produced by the heme oxygenase reaction serves as a negative regulator of sFlt-1 production.[54] Thus, multiple factors influence sFlt-1 production by the placenta. Stepan et al[55] have recently examined the relationship between AT1-AA and elevated sFlt-1 in pregnant women. They found that most of the preeclamptic patients were characterized by high sFlt-1 levels and the presence of AT1-AA. However, discordance between AT1-AA and sFlt-1 was observed in a population of patients characterized by reduced uterine perfusion and no other pregnancy complications. In these cases, AT1-AA was frequently present, whereas sFlt-1 was not usually elevated. They also identified an occasional preeclamptic patient with elevated sFlt-1 who lacked AT1-AA. Thus, in addition to AT1-AA, other factors influence sFlt-1 production and may account for those cases identified by Stepan et al[55] in which there is discordance between sFlt-1 and AT1-AA in pregnant women.

Important recent work by Walther et al[47] shows that AT-AA can be detected before 20 weeks in women with impaired uterine perfusion detected by Doppler sonography. The fact that AT1-AA can be detected many weeks before the symptoms of preeclampsia has significant implications regarding early diagnosis and therapeutic intervention. If maternal circulating AT1-AA contributes to the pathophysiology of preeclampsia, the removal of these autoantibodies from women with preeclampsia may provide therapeutic benefit. Another possible therapeutic approach is to block the action of the autoantibody. AT1-AAs typically recognize a 7-amino acid sequence present on the second extracellular loop of the AT_1 receptor. Available evidence indicates that a 7-amino acid peptide, corresponding with this epitope, blocks antibody-induced stimulation of the AT_1 receptor.[27,28] Our current studies show that this 7-amino acid peptide can neutralize AT1-AA and thereby prevent autoantibody-induced sFlt-1 production in placental explants and cultured trophoblast cells. Thus, the use of epitope peptide therapy to block the action of angiotensin receptor–

activating autoantibodies has the potential of being a safe and effective treatment of preeclampsia.

Perspectives

In 1999, Wallukat et al[27] reported their remarkable findings that sera from women with preeclampsia contain autoantibodies that react with the AT_1 receptor in a stimulatory fashion. In recent years, these important findings have been extended in numerous ways showing that these autoantibodies activate AT_1 receptors on cardiac myocytes, trophoblast cells, endothelial cells, mesangial cells, vascular smooth muscle cells, and Chinese hamster ovary cells.[56,57] Here we reported that these autoantibodies stimulate increased production of sFlt-1 by human trophoblast cells, human villous explants, and in pregnant mice and in this way may contribute to the production of a major soluble factor released from the placenta that contributes to the maternal syndrome. Altogether, the studies show that the AT1-AAs activate AT_1 receptors on a variety of cell types and provoke biological responses that are relevant to the pathophysiology of preeclampsia. These findings provide a new way of thinking about the abnormalities associated with preeclampsia and raise the possibility that preeclampsia may be an autoimmune disease. Thus, removing AT1-AA or inhibiting AT_1 receptor activation by 7-amino acid antibody-blocking epitope peptide may be a potential safe and effective therapeutic treatment for preeclampsia.

Acknowledgments

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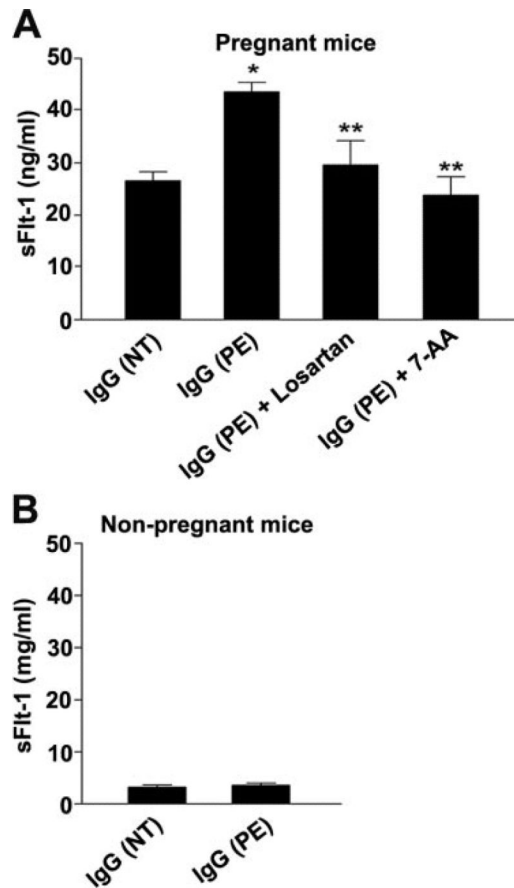
Figure 1.
IgG from women with preeclampsia stimulates sFlt-1 secretion in pregnant mice. IgG from normotensive or preeclamptic pregnant women was introduced by retro-orbital injection into pregnant (day 13) (A) or nonpregnant mice (B). A, Pregnant mice were injected with IgG from women with preeclampsia in the presence or absence of losartan or the 7-amino acid epitope peptide (7-AA). Mice were euthanized 5 days after injection, and the serum concentrations of sFlt-1 were determined by ELISA. *$P<0.05$ vs pregnant mice injected with normotensive IgG. **$P<0.05$ vs pregnant mice injected with preeclampsia IgG alone. n=5 to 8.

Figure 2.
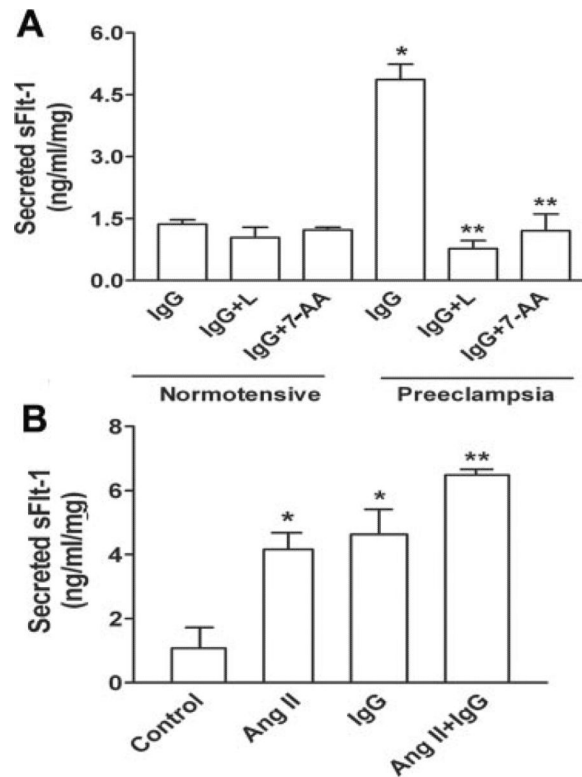IgG from women with preeclampsia stimulates sFlt-1 secretion in human placental villous explants through AT_1 receptor activation. A, Placental villous explants were incubated in serum-free medium containing 0.2% BSA and treated with IgG (1:10 dilution) for 72 hours with or without losartan (L) or 7-amino acid epitope peptide (7-AA) corresponding to a sequence present on the second extracellular loop of the AT_1 receptor that is recognized by AT1-AA (0.1 μmol/L). The CM was collected, and the amount of secreted sFlt-1 was determined by ELISA. B, Additive effects of Ang II and AT1-AA resulted in higher induction of sFlt-1. Human placental villous explants were treated with IgG from preeclamptic patients (1:20) or Ang II (10 nM), alone or in combination, for 72 hours. The concentration of sFlt-1 present in the CM was determined by ELISA specific for human sFlt-1. Data are expressed as mean±SEM. *P<0.05 relative to control; **P<0.05 relative to Ang II or IgG-treated samples.
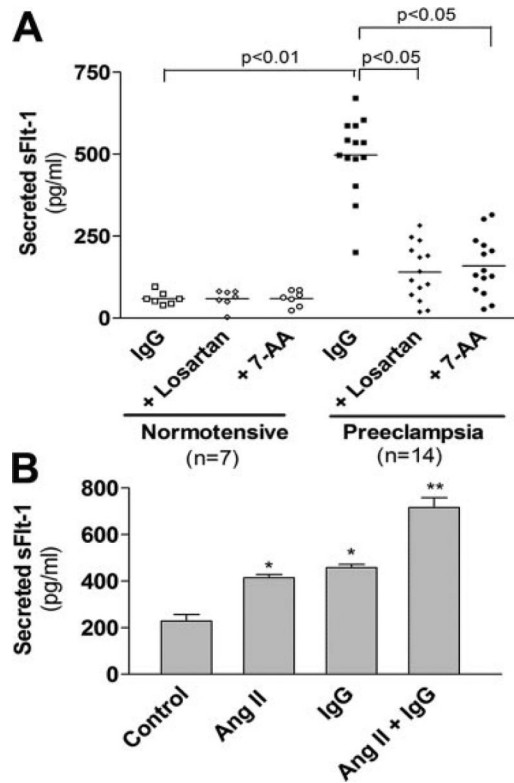
Figure 3.
IgG from women with preeclampsia stimulates secretion of sFlt-1 via AT_1 receptor activation in human trophoblast cells. A, Immortalized human trophoblast cells (HTR-8/SVneo) were treated with IgG (1:10 dilution) for 4 days in the presence or absence of losartan or a 7-amino acid peptide (7-AA; 0.1 μmol/L). The concentration of sFlt-1 in CM was determined by ELISA. Each sample was analyzed in triplicate, and the resulting value for each patient sample is shown. The horizontal line represents the mean for the data points in each group. B, HTR-8/SVneo cells were treated with IgG from preeclamptic patients (1:20) or Ang II (10 nmol/L), alone or in combination, for 72 hours. The concentration of sFlt-1 present in the CM was determined by ELISA specific for human sFlt-1. Data are expressed as mean±SEM. *P<0.05 relative to control; **P<0.05 relative to Ang II or IgG-treated samples.
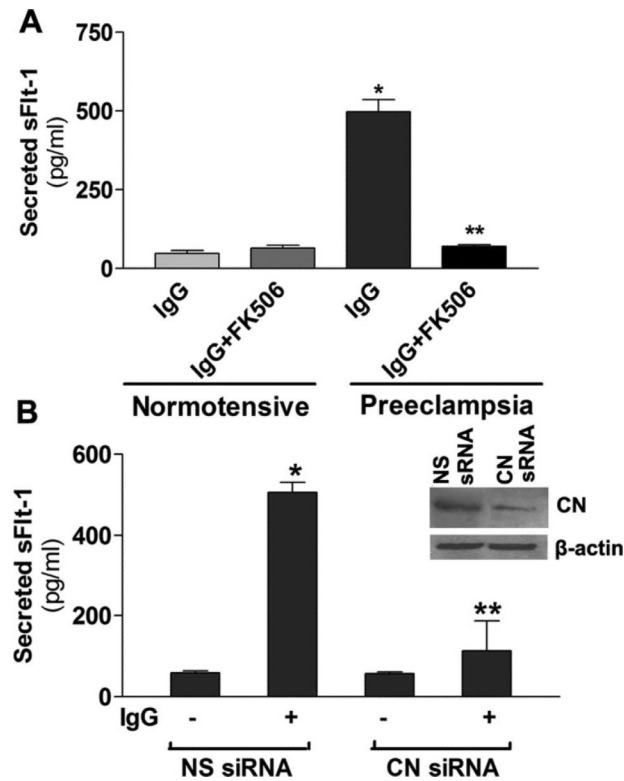
Figure 4.
Calcineurin signaling is required for AT1-AA–mediated sFlt-1 induction. A, HTR-8/SVneo cells were incubated with IgG from normotensive pregnant women (n=7) or from women with preeclampsia (n=14) for 4 days in the presence or absence of FK506 (1 μmol/L). The concentration of sFlt-1 present in the culture medium was determined by ELISA specific for human sFlt-1. Each sample was analyzed in triplicate. Data are expressed as mean±SEM. *P<0.01 vs normotensive IgG treatment; **P<0.05 vs preeclampsia IgG treatment. B, HTR-8/SV-neo cells were transfected with nonspecific siRNA (NS siRNA) and specific siRNA for calcineurin catalytic subunit α mRNA (CN siRNA). Forty-eight hours after transfection, the cellular level of calcineurin catalytic subunit α (CN) was evaluated by Western blot analysis. A typical result is shown in the inset. The transfected cells (48 hours posttransfection) were treated with IgG for 4 days, and the concentration of sFlt-1 in the CM was determined by ELISA. Each sample was analyzed in triplicate. Data are expressed as mean±SEM. *P<0.01 vs control; **P<0.05 vs preeclampsia IgG treatment.
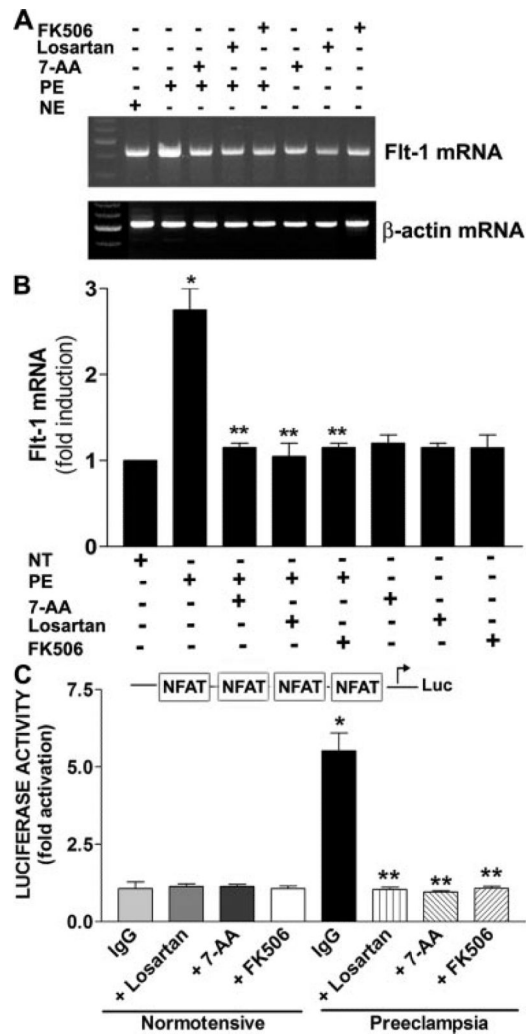
Figure 5.
NFAT functions downstream of calcineurin to regulate AT1-AA–mediated sFlt-1 induction at transcriptional level. A, Total RNA was isolated from HTR-8/SVneo cells treated with IgG from normotensive pregnant women (NT) and women with preeclampsia (PE). Semiquantitative RT-PCR was used to quantify sFlt-1 mRNA from the treated cells. RT-PCR products were visualized by electrophoresis on 2% agarose gels, and *β*-actin was used to normalize the sFlt-1 mRNA abundance. B, The ratio of sFlt-1 mRNA:*β*-actin was obtained by using densitometric analysis of multiple agarose gels. Data are expressed as mean±SEM. *$P<0.05$ vs control; **$P<0.05$ vs preeclamptic IgG treatment. C, Cells stably transformed with an NFAT (4 copy)-driven luciferase (Luc) reporter gene were cultured in serum-free medium and treated with IgG from normotensive pregnant women or IgG from women with preeclampsia for 4 days in the presence or absence of losartan, 7-amino acid epitope peptide (7-AA; 0.1 *μ*mol/L), or FK506 (1 *μ*mol/L). Luciferase activity was determined in cell lysates. Each sample was analyzed in triplicate. Data are expressed as mean±SEM. *$P<0.01$ vs normotensive IgG treatment; **$P<0.05$ vs preeclampsia IgG treatment.
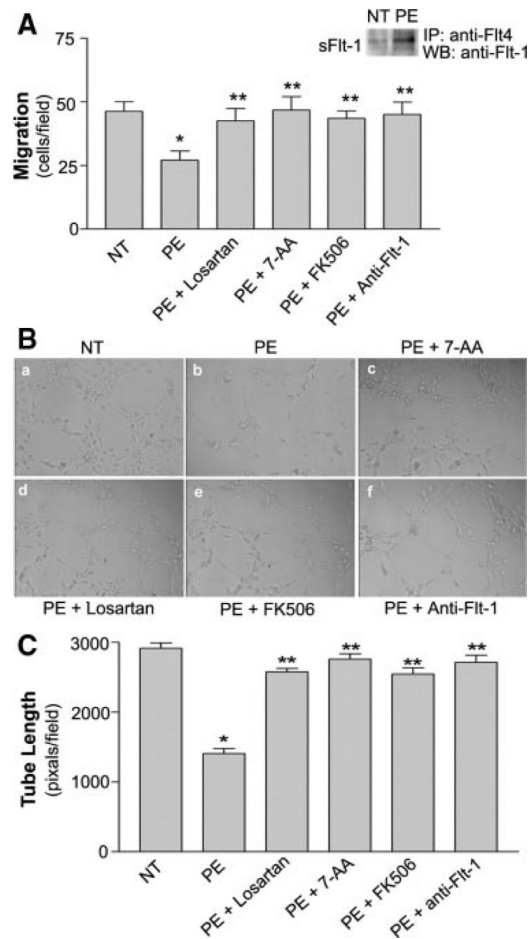
Figure 6.
Autoantibody-induced sFlt-1 inhibits endothelial cell functions. CM from HTR-8/SVneo cells treated with IgG from normotensive or preeclamptic pregnant women was added to cultured endothelial cells, and the effect on cell migration (A) and tube formation (B and C) were determined. In some cases, HTR-8/SVneo cells were treated with the 7-amino acid epitope peptide (7-AA; 0.1 μmol/L) or losartan (1 μmol/L) in addition to IgG. A, For cell migration, HUVECs were grown in serum-free medium in a cell migration chamber, and CM was used as chemoattractant. Migrated cells were counted after 24 hours of incubation. Each sample was counted in triplicate and repeated 3 times. Data are expressed as mean ±SEM. *P<0.05 preeclampsia vs normotensive IgG; **P<0.05 vs preeclampsia IgG treatment alone. Inset, CM from IgG treated HTR-8 SV40/neo cells was incubated with anti–Flt-1 antibody, and the immunoprecipitated (IP) protein was analyzed by Western blot analysis (WB) using specific Flt-1 antibody. B, For tube formation, HUVEC cells were plated on growth factor–reduced Matrigel with different CM and incubated for 4 hours at 37°C. Images were taken by light microscopy and are representative photomicrographs of HUVECs plated on growth factor–reduced Matrigel and stimulated with different CMs. C, Tube circumferences were measured using MetaMorph software. Four to 10 fields were used to obtain average tube length. Data are expressed as mean±SEM. * P<0.05 preeclampsia vs normotensive IgG; **P<0.05 vs preeclampsia IgG treatment.

Table

Clinical Characteristics of the Patients Involved in This Study

Features of Patients	Normal (n=7)	Severe Preeclampsia
Maternal age, mean±SEM, y	32.6±0.8	31.5±0.9
Gestational age, mean±SEM, wk	38.7±0.5	32.7±0.9
Primiparous, %	26	85
Systolic blood pressure, mm Hg	<140	164±7.5
Diastolic blood pressure, mm Hg	<90	105±4.5
sFlt-1, ng/mL	<0.3	6.7±1.5
AT1-AA induced NFAT-luc activity	1.1±0.5	6.4±1.4 [*]

sFlt-1- and AT1-AA–induced NFAT-luciferase activity were measured as described in the Methods section. Data are expressed as mean±SEM.

[*] $P<0.05$ for preeclampsia vs normotensive IgG.