Negative Regulation of Soluble Flt-1 and Soluble Endoglin Release by Heme Oxygenase-1

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Background—Preeclampsia is characterized clinically by hypertension and proteinuria. Soluble Flt-1 (sFlt-1; also known as soluble vascular endothelial growth factor receptor-1 [VEGFR-1]) and soluble endoglin (sEng) are elevated in preeclampsia, and their administration to pregnant rats elicits preeclampsia-like symptoms. Heme oxygenase-1 (HO-1) and its metabolite carbon monoxide (CO) exert protective effects against oxidative stimuli. Thus, we hypothesized that HO-1 upregulation may offer protection against preeclampsia by inhibiting sFlt-1 and sEng release.

Methods and Results—Preeclamptic villous explants secreted high levels of sFlt-1 and sEng. Adenoviral overexpression of HO-1 in endothelial cells inhibited VEGF-mediated sFlt-1 release and interferon-γ- and tumor necrosis factor-α–induced sEng release, whereas HO-1 inhibition potentiated sFlt-1 and sEng production from endothelial cells and placental villous explants. Consistent with these findings, mice lacking HO-1 produced higher levels of sFlt-1 and sEng compared with wild-type mice. Using selective ligands (VEGF-E and placental growth factor) and a receptor-specific inhibitor (SU-1498), we demonstrated that VEGF-induced sFlt-1 release was VEGFR-2 dependent. Furthermore, CO–releasing molecule-2 (CORM-2) or CO decreased sFlt-1 release and inhibited VEGFR-2 phosphorylation. Treatment of endothelial cells with statins upregulated HO-1 and inhibited the release of sFlt-1, whereas vitamins C and E had no effect.

Conclusions—The present study demonstrates that the HO-1/CO pathway inhibits sFlt-1 and sEng release, providing compelling evidence for a protective role of HO-1 in pregnancy, and identifies HO-1 as a novel target for the treatment of preeclampsia. (Circulation. 2007;115:1789-1797.)

Key Words: endothelium ■ endothelium-derived factors ■ heme oxygenase-1 ■ preeclampsia ■ pregnancy ■ statins ■ angiogenesis

Cardiovascular disease and preeclampsia share some common risk factors, such as insulin resistance, obesity, diabetes mellitus, and inflammation.1,2 The disruption of endothelial homeostasis and inflammation are fundamental to the initiation and progression of atherosclerosis3 and preeclampsia.4 Preeclampsia is a maternal systemic endothelial disease defined clinically as hypertension and proteinuria after 20 weeks’ gestation that affects 3% to 8% of all pregnancies and women. Women with a history of preeclampsia and their offspring are at greater risk of developing cardiovascular disease later in life.6,7

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Preeclampsia involves dysregulated placental angiogenesis,8 resulting in the release of soluble antiangiogenic factors that induce systemic endothelial dysfunction.9 Two key antiangiogenic circulating factors that give the highest strength of association with preeclamptic outcome are soluble Flt-1 (sFlt-1) and soluble endoglin (sEng).10–12 Maternal serum levels of sFlt-1 are elevated 5 weeks before the clinical onset of preeclampsia.10,13–16 sEng, a placenta-derived 65-kDa cleaved form of endoglin (also known as CD105), a coreceptor for transforming growth factor-β, is elevated in the serum of preeclamptic women 8 to 12 weeks before the clinical onset of the disease.12 In rats, administration of recombinant sFlt-1 or a vascular endothelial growth factor (VEGF)–neutralizing antibody results in glomerular endothelial cell damage and proteinuria,17 and adenoviral delivery of sFlt-1 to pregnant rats mimics the clinical manifestations of preeclampsia,18 suggesting that excess circulating sFlt-1 may
play a role in the pathogenesis of preeclampsia. Indeed, we demonstrated that the conditioned medium from preeclamptic placenta exhibited reduced capillary tube–forming activity compared with that of normal placenta and that the removal of sFlt-1 from preeclamptic conditioned medium eliminates this suppressive activity. Like sFlt-1, sEng also inhibited capillary morphogenesis. Furthermore, sEng acts synergistically with sFlt-1 to induce endothelial dysfunction; simultaneous adenoviral administration of sFlt-1 and sEng induced severe preeclampsia-like symptoms in pregnant rats. However, the molecular mechanism(s) regulating the release of these antiangiogenic factors are unknown. Any intervention that would reduce the prevalence of these circulating factors not only may prolong the pregnancy but also protect the mother from permanent vascular damage.

It was proposed that the resolution of oxidative stress and inflammation associated with pregnancy may be controlled by vascular protective factors and that the lack of such compensatory systems leads to preeclampsia. Heme oxygenase-1 (HO-1) is an inducible, endoplasmic reticulum–bound enzyme that catalyzes the nicotinamide adenine dinucleotide phosphate–cytochrome P450 reductase–dependent oxidation of heme to biliverdin in a 3-step process that liberates carbon monoxide (CO) and Fe²⁺. HO-1 is antiinflammatory and provides a defense against oxidant injury. Exogenous HO-1 is widely acknowledged to be protective against ischemia-reperfusion injury, and HO-1 is upregulated after reperfusion. HO-1 is also critical for the successful outcome of pregnancy. Administration of a HO-1 antagonist to pregnant rats resulted in complete fetal resorption, and adenoviral overexpression of HO-1 rescues pregnancy in abortion-prone mice. In women, HO-1 maintains uterine quiescence, which is further supported by lower end-tidal CO levels in women with uterine contractions, indicating reduced HO activity. Women with preeclampsia have significantly decreased CO concentrations in their exhaled breath compared with healthy pregnant women, indicating a decreased expression or activity of HO and protein levels of HO-1 and HO-2 are decreased in preeclamptic placenta. In the present study, we report that adenoviral overexpression of HO-1 in endothelial cells results in a decreased production of sFlt-1 and sEng, whereas HO-1 small interfering RNA (siRNA) knockdown potentiated sFlt-1 and sEng release from endothelial cells, and pharmacological inhibition of HO activity in placental villous explants stimulated the release of sFlt-1 and sEng. The loss of HO activity may be central to the pathogenesis of preeclampsia.

Methods

Reagents
Recombinant growth factors were purchased from RELIATEch (Braunschweig, Germany). Tin protoporphyrin-IX (SnPP) was obtained from Alexis Biochemicals (Nottingham, UK). The VEGF receptor-2 (VEGFR-2) tyrosine kinase inhibitor SU-1498 was purchased from Calbiochem (Nottingham, UK). Tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule [CORM-2]), ruthenium (III) chloride hydrate (CORM-2 control), simvastatin, mevastatin, flavusatin, farnesyl pyrophosphate, vitamin C, vitamin E, tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and all other cell culture reagents and chemicals were obtained from Sigma Aldrich (Poole, UK).

Cell Culture and Stimmulations
Human umbilical vein endothelial cells (HUVECs) and porcine aortic endothelial cells expressing human VEGFR-2 (PAEVEGFR-2) were used as described previously. HUVECs were stimulated with VEGF-A (20 ng/mL), placental growth factor-1 (20 ng/mL), VEGF-E (20 ng/mL), TNF-α (10 ng/mL), or IFN-γ (10 ng/mL), and conditioned media was collected and assayed for sFlt-1 or sEng by ELISA. For inhibitor studies, HUVECs were incubated with SU-1498 (10 μmol/L), SnPP (20 μmol/L), CORM-2 (50 μmol/L), inactivated CORM-2 (CORM-2; 50 μmol/L; reconstituted CORM-2 exposed to air for 24 hours), statins (10 μmol/L), vitamin C (1 mmol/L), or vitamin E (1 mmol/L) for 30 minutes before the addition of VEGF-E.

Adenoviral Gene Transfer
The recombinant, replication-deficient adenovirus-encoding rat HO-1 (AdHO-1) was used as described previously. Optimal multiplicity of infection for AdHO-1 was determined to be 50 infectious units/cell by Western blotting using a rabbit anti-HO-1 antibody (StressGen Biotechnologies Corp [now Nventa], Victoria, BC, Canada).

Quantitative Real-Time Polymerase Chain Reaction
Sample preparation and real-time polymerase chain reaction were performed as described previously. Briefly, mRNA was prepared with TRIzol and DNase-1 digestion/purification on RNAeasy columns (Qiagen, West Sussex, UK) and reverse transcribed with the cDNA Synthesis Kit (Promega, Madison, Wis). Triplicate cDNA samples and standards were amplified in SensMix containing SYBR green (Quintace, London, UK) with primers specific for HO-1 (sense, 5'-GGG TGA TAG AAG AGG CCA AGA CT-3'; antisense, 5'-GCA GAA TCT TGC ACT TTG TTG CT-3') or β-actin. The mean threshold cycle for each HO-1 was normalized to β-actin and expressed relative to control.

sRNA Knockdown of HO-1
HUVEC were trypsinized, and ~1×10⁶ cells were electroporated with ~3 μg HO-1 (sense, 5'-GGGAGAGGGUGUAAGAAGAUU-3'; antisense, 5'-UCUUCUAACCCUCUGCUU-3') or control sRNA using the HUVEC kit II and Nucleofector (Amaxa GmbH, Cologne, Germany) as described previously.

HO-1–Null Mice
The generation of the HO-1⁻/⁻ mice has been previously described.

Placental Tissue Collection and Preparation
Human placental tissue was obtained from normal pregnancies and gestationally matched pregnancies complicated by preeclampsia. Preeclampsia was defined as blood pressure >140/90 mm Hg on at least 2 consecutive measurements and proteinuria of at least 300 mg/24 h. Informed consent was obtained from the patients, and the study had the approval of the South Birmingham Ethical Committee (Birmingham, UK). Villous explants were prepared and exposed to hypoxia as described previously in the presence or absence of SnPP (20 μmol/L), VEGF (20 ng/mL), TNF-α (50 ng/mL), or simvastatin (10 μmol/L) for 24 hours, and conditioned media was assayed for sFlt-1 or sEng.

sFlt-1 and sEng ELISA
sFlt-1 levels were measured as previously described, and sEng was measured by ELISA according to the manufacturer’s instructions (R&D Systems, Abingdon, UK).
Immunoprecipitation and Western Blotting

After 48 hours of serum starvation, PAEV-VEGFR-2 were preincubated for 30 minutes with CORM-2 (50 μmol/L), iCORM-2 (50 μmol/L), CORM-2 control compound (100 μmol/L), or medium containing CO gas. Cells were stimulated with VEGF-E (20 ng/mL) for 10 minutes and lysed in radio immunoprecipitation assay buffer, and the lysate was subjected to overnight immunoprecipitation with rabbit anti-VEGFR-2 (C-1158) (Autogen Bioclear, Wiltshire, UK). Protein-A-agarose (Amersham-Pharmacia, Chalfont St. Giles, UK)–captured immunoprecipitates were separated on 6% SDS-PAGE, and phosphotyrosines were detected with mouse anti-PY99 (Autogen Bioclear, Wiltshire, UK). HUVECs were incubated for 24 hours with simvastatin (10 μmol/L), vitamin C (1 mmol/L), or vitamin E (1 mmol/L), and radio immunoprecipitation assay lysates were subjected to 15% SDS-PAGE and Western blotted with rabbit anti-HO-1 antibody (StressGen Biotechnologies Corp).

Statistical Analysis

All data are expressed as mean±SEM. Statistical comparisons were performed with 1-way ANOVA, followed by the Student-Newman-Keuls test as appropriate. Statistical significance was set at P<0.05. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results and Discussion

HO-1 Is a Negative Regulator of VEGFR-2–Mediated sFlt-1 Release

Previous studies have shown that HO-1 protein is decreased and sFlt-1 release is increased in preeclamptic placenta. Circulating total VEGF is increased in preeclampsia, and VEGF is known to stimulate the release of sFlt-1 from endothelial cells and placental explants. Using selective ligands (VEGF-E and placental growth factor-1) and a receptor-specific inhibitor (SU-1498), we demonstrate that VEGF-induced sFlt-1 release is VEGF receptor-2 (VEGFR-2) dependent (see Figure I in the Data Supplement). To determine whether HO-1 affects sFlt-1 release, endothelial cells were infected with an AdHO-1. Overexpression of HO-1 inhibited VEGF-E–mediated release of sFlt-1 and reduced IFN-γ–stimulated sFlt-1 production (Figure 1A). Consistent with these findings, the HO inhibitor SnPP (Figure 1B) and siRNA knockdown of HO-1 (Figure 1C) potentiated VEGF-E–induced sFlt-1 secretion, suggesting that HO-1 is a negative regulator of VEGFR-2–mediated sFlt-1 release in endothelial cells.
thelial cells. In line with this, significantly higher levels of sFlt-1 were secreted from HO-1–null murine lung biopsy explants compared with wild-type litter mates (Figure 1D). The ability of HO-1 to suppress cytokine-induced damage\textsuperscript{20} and to inhibit sFlt-1 release strongly supports the concept that loss of HO activity may be central to the pathogenesis of preeclampsia.

**CO Inhibits VEGFR-2–Mediated sFlt-1 Release**

To determine whether CO, the gaseous product of HO activity, was involved in the inhibitory effect on sFlt-1 release observed after HO-1 overexpression, HUVECs were treated with CORM-2.\textsuperscript{51} VEGF-E–stimulated sFlt-1 release was inhibited by CORM-2, a CO-releasing molecule, whereas inactivated CORM-2 (iCORM-2) had no effect (Figure 2A). This demonstrates that the CO produced by HO-1 is responsible, at least in part, for this inhibition. Women with preeclampsia have significantly decreased CO concentrations in their exhaled breath compared with healthy pregnant women, indicating decreased expression or activity of HO.\textsuperscript{34,35} Furthermore, women who smoke throughout their pregnancies are 33% less likely to develop preeclampsia\textsuperscript{30} and have reduced serum levels of sFlt-1.\textsuperscript{53} In contrast, women who use snuff, a form of smokeless tobacco, are at an increased risk of developing preeclampsia.\textsuperscript{54} It is likely that exposure to elevated concentrations of exogenous CO, one of the combustion products of cigarette smoke, offers protection against preeclampsia.\textsuperscript{55}

Because sFlt-1 release was VEGFR-2 mediated (Figure I in the online Data Supplement), experiments were undertaken to ascertain whether CORM-2 could inhibit VEGFR-2 phosphorylation induced by VEGF-E. PAE\textsubscript{VEGFR-2} cells were serum deprived for 48 hours and then preincubated with 50 μmol/L CORM-2, iCORM-2, or 100 μmol/L CORM-2 control for 30 minutes (B) or CO gas–saturated medium for 45 minutes and stimulated with VEGF-E (20 ng/mL) for 10 minutes (C). Cell lysates were immunoprecipitated with rabbit anti–VEGFR-2 and Western blotted with an anti-phosphotyrosine antibody (PY99). Western blots are representative of 4 different experiments.

**HO Inhibition Stimulates Release of sFlt-1 From Placental Explants**

To determine whether inhibition of placental HO activity potentiates sFlt-1 release, normal placental explants were incubated with SnPP for 24 hours under tissue normoxia (5% O\textsubscript{2}; P\textsubscript{O\textsubscript{2}}=60 mm Hg), and sFlt-1 was assayed in the conditioned medium. This resulted in a 2-fold increase in sFlt-1 secretion (Figure 3A). HO activity depends on the availability of oxygen,\textsuperscript{21} and the activity of HO isolated from chorionic villi is reported to decrease under hypoxic conditions.\textsuperscript{56} Exposure to hypoxia (1% O\textsubscript{2}; P\textsubscript{O\textsubscript{2}}=16 mm Hg) resulted in elevated sFlt-1 release from normal placental villous explants (Figure 3A) as reported previously.\textsuperscript{47} Although not significant, the addition of SnPP resulted in a slight increase in sFlt-1 release under hypoxia (Figure 3A). Exposure of syncytiotrophoblast cultures to hypoxia resulted in a significant reduction in HO-1 expression.\textsuperscript{57} Under hypoxia, a decrease in but not a complete inhibition of HO activity occurs, which may account for the elevated sFlt-1 levels seen in preeclampsia. Interestingly, when preeclamptic placental explants were placed under atmospheric conditions, inhibition of HO led to an upregulation of sFlt-1 (Figure 3B), suggesting that HO is biologically active in the preeclamptic placenta but at a reduced level compared with normal. Pre-eclampsia is a heterogeneous disease, and it is likely that hypoxia is experienced only in certain areas, with the distribution and degree depending on the severity of the disease. Histological studies showed that preeclamptic placenta appears to be normal in certain areas but infarcted or damaged at other sites where decreased HO expression exists.\textsuperscript{37} If HO expression and activity were to be enhanced therapeutically, the enzyme would be active in areas that are sufficiently oxygenated and may offer protection.
HO-1 Is a Negative Regulator of sEng Release
Maternal serum levels of sEng are elevated in preeclampsia 8 to 12 weeks before the clinical onset of the disease, and preeclamptic placental tissues express elevated endoglin. Here, we show that like sFlt-1, preeclamptic placental villous explants release significant amounts of sEng into the culture medium compared with normal gestationally matched explants (Figure 4A). Ischemia/reperfusion injury in certain areas of the placenta is associated with the progression of preeclampsia. Hepatic ischemia/reperfusion injury in HO-1−/− mice results in increased inflammatory cell recruitment and induction of proinflammatory cytokines such as TNF-α and IFN-γ compared with wild-type littermates. Exacerbated inflammation and elevated TNF-α and IFN-γ levels

Figure 3. Inhibition of HO induces sFlt-1 release from placental explants. A, Placental villous explants incubated with SnPP (20 μmol/L) under hypoxia (1% O2) or tissue normoxia (5% O2) for 24 hours. B, Normal or preeclamptic placental villous explants were rested overnight and then incubated in the presence of SnPP (20 μmol/L) under Birmingham, UK, atmospheric conditions for 24 hours. Conditioned media was collected and assayed for sFlt-1 by ELISA. Data are mean (±SEM) of 3 separate experiments (n=9).

Figure 4. HO-1 negatively regulates sEng release. A, Levels of sEng released by normal or preeclamptic placental villous explants after 24 hours. *P<0.001 vs preeclamptic. Data are mean (±SEM) of 3 separate experiments (n=9). B, HUVECs infected with 50 infective units/cell of AdHO-1 were stimulated with 10 ng/mL TNF-α or IFN-γ for 24 hours, and sEng was measured in the supernatants. Data are mean (±SEM) of 3 separate experiments (n=9). C, Placental villous explants were incubated in the presence of TNF-α (10 ng/mL) and/or SnPP (20 μmol/L) for 24 hours, and conditioned media was assayed for sEng by ELISA. *P<0.01 vs vehicle; **P<0.001 vs TNF-α (n=6). D, Circulating sEng levels measured in the plasma of 8-week-old HO-1-null (KO) and wild-type (WT) mice. *P<0.001 vs WT.
also occur in preeclampsia. Interestingly, hypoxia induces IFN-γ release, which has been shown to suppress HO-1 expression, whereas CO antagonizes this effect in macrophages. Similar to the inhibition of sFlt-1, adenoviral HO-1 overexpression inhibited basal and TNF-α– and IFN-γ–mediated sEng release (Figure 4B). In addition, TNF-α–induced release of sEng from normal placental villous explants was potentiated by inhibition of HO activity with SnPP (Figure 4C). The hypothesis that HO-1 is a global protective factor is supported by our data showing that circulating sEng is elevated in serum from HO-1 mice compared with wild-type litter mates (Figure 4D).

Vitamins C and E Have No Effect on Release of sFlt-1 or sEng
Increased oxidative stress is a causative factor of preeclampsia. This finding has led to several clinical trials of vitamin supplements in pregnant women at risk of developing preeclampsia. Disappointingly, the results of 2 recently published studies show that vitamins C and E do not prevent or reduce the severity of disease. In fact, the Vitamins in Preeclampsia study showed that vitamin supplementation has a detrimental effect on infant outcome. On the basis of our hypothesis, we assessed the effects of vitamins C and E on HO-1 expression and sFlt-1 and sEng release. These vitamins had no effect on HO-1 expression (Figure 5A) or on the release of sFlt-1 or sEng under basal or stimulated conditions (Figure 5B and 5C). It is possible that the lack of successful outcome of these trials may be due in part to the failure of these vitamins to modify the expression and/or secretion of these 2 key antiangiogenic proteins. Our data suggest that upregulation of endogenous protective factors like the HO pathway or other antioxidant systems such as the thioresdoxin family of proteins may alleviate the symptoms of preeclampsia.

Statins Inhibit sFlt-1 Release
Agents that increase HO expression and reduce the release of antiangiogenic factors may be beneficial as therapeutic agents in preeclampsia. The antiinflammatory and antiproliferative effects of simvastatin occur in part through HO-1. Simvastatin was reported to upregulate HO-1 mRNA in HUVECs, and here we show that simvastatin upregulates HO-1 protein (Figure 6A). Although long-term treatment with atorvastatin after acute myocardial infarct increased circulating sFlt-1 levels, addition of simvastatin, fluvastatin, or mevastatin (Figure 6B) significantly decreased the basal production and VEGF-E–induced release of sFlt-1 from endothelial cells. This inhibition was specific to the cholesterol biosynthesis pathway because it could be rescued by supplementation with farnesyl pyrophosphate to bypass hydroxy-methyl-glutaryl-Coenzyme A reductase (Figure 6C). Furthermore, simvastatin decreased the VEGF-induced sFlt-1 release from normal-term placental villous explants (Figure 6D). Additionally, treatment with simvastatin led to a reduction in sEng release, although this was not statistically significant. Currently, statins are contraindicated in pregnancy because skeletal malformations in rat fetuses were reported with high-dose lovastatin (800 mg · kg⁻¹ · d⁻¹, 700 times the maximum recommended dose in humans) or active metabolites of statins. However, no increase in congenital abnormalities

Figure 5. Vitamins C and E do not affect HO-1 expression or release of sFlt-1 or sEng. A, HUVECs were incubated with 1 mmol/L vitamin C and/or vitamin E for 24 hours, and cell lysates were Western blotted for HO-1. B, HUVECs were incubated with 1 mmol/L vitamin C and/or vitamin E in the presence or absence of VEGF-E (20 ng/mL) for 24 hours, and the conditioned media was assayed for sFlt-1 (B) or sEng (C) by ELISA. Data are mean (±SEM) of ≥3 separate experiments performed in duplicate.
above that of the normal population has been reported when statin treatment was inadvertently continued throughout pregnancy in women with familial hypercholesterolemia.\textsuperscript{74–77} Once a predictive and accurate biomarker has been established for early-onset preeclampsia, pregnant women at risk of developing preeclampsia could be managed with statins. We postulate that statins may alleviate the symptoms of preeclampsia by upregulating HO-1 and inhibiting the 2 key antiangiogenic factors, which have been shown to underpin the clinical syndrome.

The present report demonstrates that HO-1 inhibits sFlt-1 and sEng release from endothelial cells and placental explants and that the pathophysiology of preeclampsia may involve the loss of HO activity. We propose that the HO/CO pathway acts as a gatekeeper, preventing the onset of preeclampsia by inhibiting the 2 key antiangiogenic factors, which have been shown to underpin the clinical syndrome.

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**CLINICAL PERSPECTIVE**

Preeclampsia is a maternal systemic disorder with a clinical presentation of hypertension and proteinuria after 20 weeks’ gestation that affects 3% to 8% of all pregnancies. Women with a history of preeclampsia are at an increased risk of developing cardiovascular disease later in life. Long-term risk for cardiovascular disease also is augmented in the offspring of preeclamptic women. Despite the many theories for preeclampsia, the delineation of the mechanism of action for potential therapeutic agents has been lacking. Because the only effective treatment for preeclampsia resolution is the delivery of the placenta, it is generally accepted that placental factors that inhibit the normal function of the endothelium are involved. Recent retrospective cross-sectional studies demonstrated that 2 anti-angiogenic factors, soluble Flt-1 and soluble endoglin, are elevated in the sera of preeclamptic women weeks before the clinical onset of the disease. In the present study, we have identified heme oxygenase-1, a cytoprotective enzyme, as the key component in suppressing the release of soluble Flt-1 and soluble endoglin from the placenta. We also show that treatment of endothelial cells with statins upregulated HO-1 and inhibited the release of soluble Flt-1, whereas vitamins C and E had no effect, providing a possible explanation for the failure of the recent antioxidant vitamin supplementation trials with vitamins C and E to prevent preeclampsia in women at risk. Our study opens a new avenue of investigation for increasing our understanding of the origin of preeclampsia and provides a novel therapeutic target in placental-based pregnancy complications.
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