Dysregulation of Hydrogen Sulfide Producing Enzyme Cystathionine γ-lyase Contributes to Maternal Hypertension and Placental Abnormalities in Preeclampsia

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Background—The exact etiology of preeclampsia is unknown, but there is growing evidence of an imbalance in angiogenic growth factors and abnormal placenta. Hydrogen sulfide (H₂S), a gaseous messenger produced mainly by cystathionine γ-lyase (CSE), is a proangiogenic vasodilator. We hypothesized that a reduction in CSE activity may alter the angiogenic balance in pregnancy and induce abnormal placenta and maternal hypertension.

Methods and Results—Plasma levels of H₂S were significantly decreased in women with preeclampsia (P<0.01), which was associated with reduced placental CSE expression as determined by real-time polymerase chain reaction and immunohistochemistry. Inhibition of CSE activity by DL-propargylglycine reduced placental growth factor production from first-trimester (8–12 weeks gestation) human placental explants and inhibited trophoblast invasion in vitro. Knockdown of CSE in human umbilical vein endothelial cells by small-interfering RNA increased the release of soluble fms-like tyrosine kinase-1 and soluble endoglin, as assessed by enzyme-linked immunosorbent assay, whereas adenoviral-mediated CSE overexpression in human umbilical vein endothelial cells inhibited their release. Administration of DL-propargylglycine to pregnant mice induced hypertension and liver damage, promoted abnormal labyrinth vascularization in the placenta, and decreased fetal growth. Finally, a slow-releasing H₂S-generating compound, GYY4137, inhibited circulating soluble fms-like tyrosine kinase-1 and soluble endoglin levels and restored fetal growth in mice that was compromised by DL-propargylglycine treatment, demonstrating that the effect of CSE inhibitor was attributable to inhibition of H₂S production.

Conclusions—These results imply that endogenous H₂S is required for healthy placental vasculature and that a decrease in CSE/H₂S activity may contribute to the pathogenesis of preeclampsia. (Circulation. 2013;127:2514-2522.)

Key Words: angiogenesis ■ fetal development ■ fms-like tyrosine kinase-1 ■ hydrogen sulfide ■ preeclampsia ■ placental growth factor ■ vascular endothelial growth factor

Hydrogen sulfide (H₂S), a gaseous signaling molecule, promotes vasodilatation1 and stimulates angiogenesis in the vasculature.2 H₂S has anti-inflammatory properties3 and is also cytoprotective against cellular damage induced by lethal hypoxia or reperfusion injury.4,5 Cystathionine γ-lyase (CSE) is the principal enzyme responsible for the endogenous production of H₂S.6 Chronic administration of the CSE inhibitor DL-propargylglycine (PAG) leads to elevated blood pressure and vascular remodeling in the rat,7 and both CSE and H₂S levels are reduced in pulmonary hypertensive rats.8 Mice genetically deficient in CSE develop age-dependent hypertension, severe hyperhomocysteinemia, and endothelial dysfunction.9 Clearly, H₂S has multiple roles in health and disease,10,11 but its role in pregnancy-induced hypertension is unknown.

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Preeclampsia is a hypertensive syndrome that affects 4% to 7% of all pregnancies and is a major contributor to maternal and fetal morbidity and mortality worldwide.12 The exact etiology of preeclampsia is unknown; abnormal placenta13,14 and imbalance in angiogenic factors15,16 have been implicated in preeclampsia pathogenesis. Importantly, circulating levels of soluble fms-like tyrosine kinase-1 (sFlt-1), the endogenous inhibitor of vascular endothelial growth factor and placental...
growth factor (PIGF), and soluble endoglin (sEng, the cleaved product of the accessory transforming growth factor-β1 receptor endoglin), as well, are elevated several weeks before the onset of the clinical manifestations of preeclampsia, whereas PIGF is reduced in the first trimester of pregnant women who subsequently develop the syndrome. Together with endothelial dysfunction, these have become the biochemical hallmarks of severe preeclampsia. Few studies have investigated the functions of CSE/H₂S in pregnancy. Recently, Patel et al demonstrated that both cystathionine β-synthase and CSE are present in human intrauterine tissues and placenta. Given that the placenta is a highly vascular organ, we hypothesize that the dysregulation of the CSE/ H₂S pathway may contribute to placental abnormalities and a preeclampsia-like condition.

In the current study, we demonstrated that plasma H₂S levels in the mother and CSE expression in the placenta are reduced in pregnancies complicated by preeclampsia in comparison with gestational age–matched controls. Inhibition of CSE activity ex vivo in placental explants from the first trimester (8–12 weeks) of pregnancy results in a marked decrease in PIGF production and trophoblast invasion in vitro is inhibited. Inhibition of CSE activity induces hypertension, increases sFlt-1 and sEng levels, and causes placental abnormalities in time-pregnant mice owing to the inhibition of H₂S production. A slow-releasing, H₂S-generating compound, GYY4137, restored fetal growth compromised by CSE inhibition and inhibited the rise in circulating sFlt-1 and sEng levels. These findings indicate that a dysfunctional CSE/H₂S pathway may contribute to the pathogenesis of preeclampsia and provide the first direct evidence for H₂S therapy in this condition.

Materials and Methods

Placental Tissue Collection and Preparation

The Institutional Ethics Committee approved the blood and tissue collection, and written informed consent was obtained. We analyzed blood samples from women with singleton pregnancies recruited in low- and high-risk clinics and labor and delivery units. All women were followed up prospectively from enrollment until delivery. Human placental tissues were collected from pregnancies complicated by preeclampsia and from normotensive pregnancies in the presence or absence of sodium hydrogen sulfide (NaHS). After 6 hours, cells were observed with a Nikon inverted microscope and Image Pro-Plus image analysis software.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described. Experiments were performed on third or fourth passage HUVECs.

RNA Interference

To silence human CSE expression, we performed transfection of small-interfering RNA duplex by using electroporation (Nucleofector, Amaxa). Small-interfering RNAs for control and CSE were synthesized by IDT DNA technologies (Glasgow, UK). Knockdown of CSE in HUVEC was confirmed by using Western blotting.

Adenoviral Gene Transfer

The recombinant replication deficient adenovirus-encoding human CSE and empty vector were purified on CsCl gradients, titered, and stored at −80°C in a viral storage buffer before use as described previously. Optimal multiplicity of infection for adenovirus-encoding human CSE was determined to be 20 infectious units/cell by Western blotting using a rabbit anti-CSE antibody (Abcam). Adenovirus-encoding empty vector–infected HUVECs were used as a negative control.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay kits for human and murine sFlt-1, sEng, and PIGF were obtained from R&D Systems and performed according to the manufacturer’s specifications.

Immunohistochemistry

Human and murine placental tissues were prepared for immunohistochemistry as previously described. Biotin-labeled isocitrat-B₃ anti-CSE (5 mg/mL), and isotype control were used. The staining was analyzed by using a Nikon inverted microscope and Image Pro-Plus image analysis software.

Real-Time Polymerase Chain Reaction

Sample preparation and real-time quantitative polymerase chain reaction was performed as described previously.

In Vitro Angiogenesis Assay

Angiogenic potential was assessed by the spontaneous formation of capillary-like structures by HUVEC on growth factor–reduced Matrigel (Becton Dickinson, Devon, UK). HUVECs (1×10⁵ cells/ well) were seeded in 96-well Matrigel-coated plates for 24 hours. On the next day, the cells were incubated with maternal plasma from pregnancies complicated by preeclampsia or uncomplicated pregnancies in the presence or absence of sodium hydrogen sulfide (NaHS). After 6 hours, cells were observed with a Nikon inverted microscope and images recorded and analyzed by using Image Pro-Plus image analysis software (Media Cybernetics).
Measurement of H$_2$S in Plasma

Citrated blood was obtained from women with uncomplicated pregnancies (n=14) and preeclampsia (n=14) and also from pregnant mice before the termination of pregnancy. H$_2$S levels were measured as described previously. In brief, 75 µL of plasma was mixed with 250 µL of 1% (wt/vol) zinc acetate and 425 µL of water, followed by 250 mL of 50% trichloroacetic acid to remove proteins. To the mixture was added 133 µL of 20 mmol/L N,N-dimethyl-p-phenylenediamine sulfate in 7.2 mmol/L HCl and 133 µL of 30 µmol/L FeCl$_3$ in 1.2 mmol/L HCl. After 10 minutes of incubation at room temperature, the reaction mixture was centrifuged at 10,000 g for 2 minutes. The absorbance of the resulting solution was measured at 670 nm with a spectrophotometer in a 96-well plate. The concentration of H$_2$S in the solution was calculated against a calibration curve of NaHS.

Statistical Analysis

Data sets were tested for normality of distribution by using the Shapiro-Wilks method and presented as either mean and standard error of the mean, or median and range as appropriate. Comparison between the 2 groups was performed by using the Student $t$ test or paired $t$ test (parametric) or Mann-Whitney $U$ test (nonparametric). Comparisons among 3 or more groups were performed by using

Table. Clinical and Outcome Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>Control (n=14)</th>
<th>PE (n=14)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y$^*$</td>
<td>29.1 (19–40)</td>
<td>28.9 (18–44)</td>
<td>0.911</td>
</tr>
<tr>
<td>Gestational age, wk$^*$</td>
<td>32.7 (21–40)</td>
<td>29.7 (25–38)</td>
<td>0.155</td>
</tr>
<tr>
<td>Nulliparity$^+$</td>
<td>7 (50.0)</td>
<td>10 (71.4)</td>
<td>0.110</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg$^*$</td>
<td>114.5 (106–131)</td>
<td>173.6 (156–208)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg$^*$</td>
<td>65.9 (57–80)</td>
<td>101.3 (80–117)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria: urinary dipstick$^*$</td>
<td>0 (0–1)</td>
<td>3 (2–4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>24-h protein excretion, g/24 h$^*$</td>
<td>NA</td>
<td>4.5 (0.3–7.4)</td>
<td>NA</td>
</tr>
<tr>
<td>IUGR$^+$</td>
<td>0 (0)</td>
<td>3 (21.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>HELLP$^+$</td>
<td>0 (0)</td>
<td>4 (28.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HELLP indicates hemolysis, elevated liver enzymes, low platelet count; IUGR, intrauterine growth restriction; NA, not available; and PE, preeclampsia.

$^*$Data presented as median (interquartile range) and analyzed by the Mann–Whitney $U$ test.

$^+$Data shown as number of cases and percentage and analyzed tested by the Fisher exact test.
Results

Placental CSE Expression Is Reduced in Preeclampsia

To investigate whether CSE/H₂S activity is altered in preeclampsia, H₂S was measured in plasma obtained from gestational age–matched control pregnancies and those complicated by preeclampsia. Maternal plasma H₂S levels were significantly reduced in preeclampsia compared with the controls group (Figure 1A). Quantitative real-time PCR revealed that the CSE mRNA expression was significantly reduced in the preeclamptic placenta (Figure 1B) and decreased in fetal growth–restricted pregnancy (Figure II in the online-only Data Supplement). Immunohistochemical staining confirmed that CSE immunoreactivity was dramatically reduced in preeclamptic placentas (Figure 1Civ) suggesting that the changes in placental CSE levels affect maternal circulating H₂S levels. Expression of CSE was located in the trophoblast, the endothelium, and the mesenchymal cells within the core of the chorionic villus. The latter are possibly the Hofbauer cells, which are of mesenchymal origin (Figure 1Ciii). Clinical characteristics of the study patients are described in the Table.

Inhibition of CSE Activity Reduces PI GF Release in Placental Explants

Angiogenic factors produced by placenta are important in regulating placental vascular development. Imbalance of pro- and antiangiogenic factors generated by the placenta may account for the widespread maternal endothelial dysfunction in preeclampsia. Although exposure of human first-trimester placental explants to the CSE inhibitor PAG had no significant effect in sFlt-1 (P = 0.254, Figure 2A) and sEng (P = 0.361, Figure 2B) release, PAG dramatically reduced PI GF production (P = 0.003, Figure 2C). The inability to detect differences in sFlt-1 and sEng may be attributable to a type II error because this data set is finite. However, given that in the same explants we were able to detect differences in PI GF at a level of <0.01, we can confidently conclude that PAG affects the release of PI GF to a larger extent than the release of sFlt-1 and sEng. In addition, a significant decrease in cell invasion was observed when first-trimester trophoblast cells (HTR-8/SVneo) were incubated with PAG (50 μmol/L) in comparison with the vehicle control (Figure IIA and IIB in the online-only Data Supplement; P = 0.004) suggesting that a diminished CSE activity may compromise normal pregnancy.

CSE Modulates sFlt-1 and sEng Release in Endothelial Cells

Although the placenta has been considered to be the main source of sFlt-1 and sEng release in patients with preeclampsia, some studies have shown that the levels of sFlt-1 remained higher in women with a history of preeclampsia than in those without preeclampsia for an average of 18 months postpartum. To investigate whether CSE affects sFlt-1 and sEng release in endothelial cells, CSE expression was modulated by small-interfering RNA or adenovirus in HUVECs. Downregulation of CSE (Figure 3A) increased both sFlt-1 (Figure 3B) and sEng (Figure 3C) release, whereas overexpression of CSE (Figure 3C) inhibited sFlt-1 (Figure 3D) and sEng (Figure 3E) release by HUVECs. This data further supports the concept that the loss of CSE activity may contribute to the pathogenesis of preeclampsia.
H₂S Partially Rescues Preeclamptic Plasma-Induced Inhibition of In Vitro Tube Formation

It has been demonstrated that excess sFlt-1 generated by preeclamptic placenta inhibits in vitro endothelial tube formation, and the removal of sFlt-1 from preeclamptic samples restores angiogenesis. To assess whether H₂S can reverse the antiangiogenic effects of preeclampsia, plasma from normotensive or preeclamptic women was added to HUVECs grown on growth factor-reduced Matrigel and treated with a H₂S donor (100 mmol/L NaHS) and an in vitro tube formation assay was performed. Consistent with earlier findings, preeclamptic plasma inhibited capillary tube network formation in comparison with normal control sera (Figure 4). More importantly, NaHS partially

Figure 3. CSE modulates sFlt-1 and sEng release in endothelial cells. A, CSE protein expression by Western blot in HUVECs electroporated with CSE siRNA (siCSE) or control siRNA (siControl). sFlt-1 (B) and sEng (C) were measured in culture medium. D, CSE protein expression by Western blot in HUVECs infected with adenovirus-encoding human CSE (AdCSE) and empty vector (AdEV). sFlt-1 (E) and sEng (F) were measured in a culture medium of HUVECs. The data are shown as mean+SEM of 3 (A and B) or 4 (C and D) experiments and analyzed by paired t tests. CSE indicates cystathionine γ-lyase; HUVEC, human umbilical vein endothelial cell; sEng, soluble endoglin; SEM, standard error of the mean; sFlt-1, fms-like tyrosine kinase-1; and siRNA, small-interfering RNA.

Figure 4. H₂S rescues preeclamptic sera-induced inhibition of in vitro tube formation. A, Representative photomicrographs of HUVECs plated on growth factor-reduced Matrigel and treated with sera from women who are healthy (n=5) or who have preeclampsia (n=5) in the absence or presence of H₂S donor NaHS (100 mmol/L). B, In vitro tube formation was measured as total tube length per field. Quantification of tube length was performed by using Image Pro Plus image analysis software and expressed in micrometers per field. The data are shown as mean+SEM and analyzed by 1-way repeated-measures ANOVA. ANOVA indicates analysis of variance; CSE, cystathionine γ-lyase; FV, fetal vessel; H₂S, hydrogen sulfide; HUVEC, human umbilical vein endothelial cell; NaHS, sodium hydrogen sulfide; PE, preeclampsia; and SEM, standard error of the mean.
restored the ability of HUVECs to form tubelike structures (Figure 4A and 4B).

**Blocking Endogenous H₂S Causes Hypertension and Abnormal Placental Vascularization in Pregnant Mice**

We predicted that inhibition of CSE in vivo would cause a preeclampsia-like syndrome in pregnant mice. Three groups of pregnant C57Bl6/J mice (5–8/group) were treated daily with vehicle or 25 mg/kg PAG or 50 mg/kg PAG from E8.5 to E17.5. After 8 days of treatment, plasma was pooled from all the animals in each treatment group, and pooled H₂S levels were measured. PAG caused a dose-dependent decrease in circulating H₂S levels. The higher PAG dose reduced plasma H₂S by ≈50% (Figure 5A). Consistent with these data, we found significantly elevated mean blood pressure in a PAG concentration-dependent fashion in the treated group in comparison with vehicle-injected controls (Figure 5B). Although proteinuria was not detected in the PAG-treated animals (Table in the online-only Data Supplement), elevated liver enzyme aspartate transaminase indicated liver damage in these animals (Table in the online-only Data Supplement).

Blinded histological analysis of placental sections showed that the maternal blood space in the labyrinth zone appeared larger in 50 mg/kg PAG-treated animals than in vehicle controls (Figure 5C). The labyrinth zone consists of cells of trophoblast and mesodermal origin that together undergo branching morphogenesis, resulting in a large surface area for nutrient and gas exchange between the mother and fetus. During placental development, the maternal blood space lined by trophoblast becomes progressively more finely divided. Using isolectin B₄ to highlight the fetal endothelial cells, we compared the anatomic features of the labyrinth zone in vehicle and 50 mg/kg PAG-treated mice. In control mice, the labyrinth appeared as organized fetal vessels with well-developed branching morphogenesis. In contrast, the fetal vasculature of the placenta in PAG-treated animals was observed as irregular branching (Figure 5D).

**H₂S Rescues PAG-Induced Hypertension, Abnormal Placental Vascularization, and Rise in sFlt-1 in Pregnant Mice**

The increase in mean blood pressure induced by 50 mg/kg PAG-treated pregnant mice was inhibited by coadministration of 0.25 mg/kg GYY4137 (Figure 6A). Plasma levels of sFlt-1 (Figure 6B) and sEng (Figure 6C) increased in PAG-treated pregnant mice (Figure 6B and 6C) and were attenuated by 0.25 mg/kg GYY4137 (Figure 6B and 6C). Plasma PlGF was below the detection limit of the assay. These data suggest that inhibition of CSE activity alters maternal angiogenic balance and H₂S can help to restore normal angiogenic status.
Fetal weight was significantly decreased in mice that received the higher dose of PAG (Figure 6D). Most importantly, GYY4137 treatment restored fetal growth (Figure 6D) and the placental vasculature compromised by the CSE inhibitor (Figure 6E).

Discussion

Chronic administration of a CSE inhibitor leads to reduced H$_2$S and increased blood pressure in rats. Thus, it is plausible that a reduction in the circulating H$_2$S level may contribute to hypertension in preeclampsia. In this study, we provide evidence that preeclampsia is associated with reduced circulating H$_2$S, which is accompanied by downregulation of placental CSE, the key enzyme responsible for the generation of endogenous H$_2$S. Furthermore, the inhibition of CSE in pregnant mice induces hypertension, increases sFlt-1 and sEng levels, and causes placental abnormalities. This is attributable to the inhibition of H$_2$S production, because a slow-releasing, H$_2$S-generating compound GYY4137 inhibited circulating sFlt-1 and sEng levels and restored fetal growth compromised by CSE inhibition. These findings indicate that a dysfunctional CSE/ H$_2$S pathway may contribute to the pathogenesis of preeclampsia.

H$_2$S is a vasorelaxant factor that acts through K$_{ATP}$ channels causing smooth muscle relaxation$^{1,41}$ and playing a role in uterine contractility.$^{27}$ Recent placental studies provided contradictory findings probably because of the small sample size of these studies.$^{26,42,43}$ Holwerda and colleagues$^{42}$ observed no changes in CSE expression in placenta from severe preeclampsia samples, whereas Cindrova-Davies et al$^{43}$ reported that placental CSE level was reduced from pregnancies complicated with severe intrauterine growth restriction and preeclampsia. In the present study, placental CSE levels were dramatically reduced in preeclamptic patients in comparison in vivo. Fetal weight was significantly decreased in mice that received the higher dose of PAG (Figure 6D). Most importantly, GYY4137 treatment restored fetal growth (Figure 6D) and the placental vasculature compromised by the CSE inhibitor (Figure 6E).

Figure 6. H$_2$S rescues PAG-induced preeclampsia-like phenotype in pregnant mice. A, Mean arterial blood pressure of mice treated with 50 mg/kg PAG alone (PAG50) or in combination with GYY4137 (PAG50+GYY) was measured by the Millar system through carotid artery through a microtip catheter. Soluble Flt-1 (sFlt-1; B) and soluble endoglin (sEng) (C) in mouse plasma was measured by ELISA. D, Mean fetal weight determined at E17.5. E, Isolectin B4 staining of hemotrichorial labyrinth zone from mice received either (left) 50 mg/kg PAG alone or (right) in combination with GYY4137. The results are expressed as mean±SEM (n=5 animals/group) and analyzed by 1-way ANOVA followed by Student-Newman-Keuls tests. ANOVA indicates analysis of variance; ELISA, enzyme-linked immunosorbent assay; Flt-1, fms-like tyrosine kinase-1; H$_2$S, hydrogen sulfide; PAG, dl-propargylglycine; and SEM, standard error of the mean.
with normotensive controls, and there was also a reduction in circulating maternal H$_2$S levels. Studies in genetically deficient CSE mice demonstrated that this enzyme is the major source of H$_2$S in both the vasculature and the peripheral tissues.9

Angiogenic imbalance has been highlighted as the prime culprit in preeclampsia over systemic inflammation.8,45 In this study, CSE was found to be a negative regulator of antiangiogenic factors, sFlt-1 and sEng, in endothelial cells, suggesting that dysregulation of CSE may contribute to the lasting endothelial dysfunction and an elevated risk of cardiovascular disease in women with a history of preeclampsia. In addition, the decrease in vascular endothelial growth factor and PlGF activity in preeclampsia is believed to be the result of excess sFlt-1.15,17 Because sFlt-1 levels are in preeclampsia. In addition, the decrease in vascular endothelial growth factor and PlGF activity in early pregnancy is attributable to loss or reduction in the enzymes producing H$_2$S. Furthermore, inhibition of CSE activity abolished the invasion of first-trimester extravillus trophoblast cells suggesting that dysregulation of the CSE/H$_2$S pathway may not only change the balance of placental pro- and antiangiogenesis factors, but also dysregulate maternal spiral artery remodeling and placental development.

In pregnant mice, CSE inhibition reduced endogenous H$_2$S, and this was accompanied by an increase in blood pressure and liver damage but without visible renal pathologies of proteinuria or glomerular endotheliosis. Thus, murine syndrome was similar to nonproteinuric (atypical) preeclampsia. Preeclampsia is also strongly associated with placental abnormalities including compromised villus volume and surface area, and reduced placental vascularization, as well.13,14 In the PAG-treated mice, the fetal labyrinth showed impaired branching morphogenesis, indicating that endogenous H$_2$S is required for placental development. Impaired placental perfusion and suboptimal oxygen and nutrient diffusion has been reported to occur as a result of inappropriate labyrinth vascularization with altered patterning, branching, and dilation.48 Blood pressure, liver function, and fetal weight compromised by PAG treatment were rescued by the slow-releasing, H$_2$S-generating compound GYY4137 demonstrating that the effects of CSE inhibitor were attributable to inhibition of H$_2$S production. These results imply that endogenous H$_2$S is required for healthy placental vasculature to support fetal and maternal well-being.

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Disclosures
None.

References
Preeclampsia is a de novo maternal hypertensive syndrome that affects 4% to 7% of all pregnancies and is a major contributor to maternal and fetal morbidity and mortality worldwide. Globally, >4 million babies are born with growth restriction and 70 000 women die in pregnancy as a consequence of preeclampsia. Both mothers and their babies are prone to developing chronic diseases including cardiovascular diseases in later life. The exact etiology of preeclampsia is unknown, but soluble fms-like tyrosine kinase-1 and soluble endoglin are key components of maternal hypertension. Hydrogen sulfide is a gaseous signaling molecule that promotes vasodilatation and stimulates angiogenesis. Given that the placenta is a highly vascular organ, we proposed that a decrease in hydrogen sulfide production may promote placental abnormalities and contribute to preeclampsia-like condition by increased expression of soluble fms-like tyrosine kinase-1 and soluble endoglin. The present investigation provides a proof-of-concept study for the role of hydrogen sulfide in preeclampsia, because it shows that dysregulation of this pathway is associated with preeclampsia and that inhibition of cystathionine γ-lyase activity in pregnant mice produces some of the features of preeclampsia, including hypertension and impaired fetal growth. Importantly, it provides the first evidence that a decrease in placenta growth factor in the first trimester, which is associated with poor pregnancy outcome, may stem from a dysregulation of this pathway. These findings support the concept that hydrogen sulfide is an important regulator of the development of placental vasculature, whereas a deficiency in this gaseous second messenger appears to induce preeclampsia-like features. This study has identified hydrogen sulfide as a potential new target for therapeutic intervention against preeclampsia and intrauterine fetal growth restriction.
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SUPPLEMENTAL MATERIAL

Dysregulation of the hydrogen sulfide (H$_2$S)-producing enzyme cystathionine γ-lyase (CSE) contributes to maternal hypertension and placental abnormalities in preeclampsia.

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Running title: Low hydrogen sulfide in preeclampsia

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SUPPLEMENTAL METHODS

Histopathology

Kidney, liver, and placenta were immersion fixed in 4% paraformaldehyde for 24 hours and processed to paraffin. A series of 5 µm sections were cut and processed for hematoxylin & eosin (H&E) staining.

Immunohistochemistry

Serial 3-5-µm sections of formalin fixed, paraffin embedded human and paraformaldehyde-fixed murine placental tissues were prepared for immunohistochemistry as previously described. Biotin-labelled isolectin-B₄, anti-CSE (5mg/ml) and isotype control were used. The staining was analyzed using a Nikon inverted microscope and an Image Pro-Plus image analysis software (Media Cybernetics).

Real-time Polymerase Chain Reaction (PCR)

Sample preparation and real-time quantitative PCR was performed as described previously. Briefly, mRNA from placental tissue was extracted using TRIzol and DNase-1 digestion/purification on RNAeasy columns (Qiagen), and reverse transcribed with the cDNA Synthesis Kit (Promega). Triplicate cDNA samples and standards were amplified in SensiMix containing SYBR green (Quantace) with primers specific for CSE (GCC-CAG-TTC-CGT-GAA-TCT-AA; CAT-GCT-GAA-GAG-
TGC-CCT-TA) or β-actin. The mean threshold cycle (CT) for CSE was normalized to β-actin and expressed relative to control.

**Trophoblast Cell Invasion Assay**

The human extravillus trophoblast (EVT) cell line HTR-8/SVneo was a kind gift from Professor Charles H. Graham, Queen's University, Kingston, Ontario, Canada. The invasion assay was performed as described previously, with modification.30 Briefly, HTR-8/SVneo (50,000) cells treated with or without PAG were placed in the upper chamber of Matrigel-coated (1 mg/ml) transwell inserts (8 µm pore, Falcon, BD, UK) and housed in a 24-well plate. The cells were allowed to invade through the reconstituted extracellular matrix for 24 h in the presence or absence of 50 µM PAG (n=3). Trophoblast cells located on the under-surface of the transwell membrane were fixed with ice-cold methanol and stained with hematoxylin, and brightfield images were obtained with Nikon inverted microscope and Image Pro Plus image analysis software (Media Cybernetics).
SUPPLEMENTAL LEGENDS

Figure S1. CSE mRNA expression in IUGR. CSE mRNA levels were determined by real-time PCR in placentas from IUGR (n=14) and normal controls (n=14).

Figure S2. Trophoblast cell invasion is decreased by CSE inhibition. Transwell migration assays of HTR-8/SVneo cells in the presence of 50 μM of PAG were performed as described in Methods. (A) Migrated HTR-8/SVneo cells were stained with hematoxylin, and brightfield images were captured. (B) Cell numbers were counted, and results are expressed as a percentage of the control (n=3).

References


**Supplemental Table.** PAG increases liver aspartate transaminase AST.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urine albumin/creatinine (mg/mmol)</th>
<th>AST (U/L)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control#</td>
<td>9.92±6.2</td>
<td>111.71±38.4</td>
<td>0.93</td>
</tr>
<tr>
<td>PAG (50mg/kg)*</td>
<td>9.41±2.2</td>
<td>263.14±175.1*</td>
<td>0.03</td>
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<tr>
<td>PAG+GYY4137†</td>
<td>6.60±1.4</td>
<td>102.60±15.9†</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values shown are means ± SEM and analyzed by one-way ANOVA.

#Control vs PAG+GYY *PAG vs control; †PAG vs PAG+GYY
Figure S1

![Bar chart showing CSE mRNA levels in Control and IUGR groups. The p-value is 0.070.](image)
Figure S2

A

Control  PAG

B

Cell Invasion (% of control)

control  PAG

p = 0.004