Dysregulation of Hydrogen Sulfide (H$_2$S) Producing Enzyme Cystathionine γ-lyase (CSE) Contributes to Maternal Hypertension and Placental Abnormalities in Preeclampsia

Running title: Wang et al.; Low hydrogen sulfide in preeclampsia

Keqing Wang, MD, PhD$^{1,2}$; Shakil Ahmad, PhD$^{1,2}$; Meng Cai, PhD$^{1,2}$; Jillian Rennie, BSc$^2$; Takeshi Fujisawa, PhD$^2$; Fatima Crispi, MD, PhD$^3$; James Baily, MD$^2$; Mark R. Miller, PhD$^2$; Melissa Cudmore, PhD$^2$; Patrick W. F. Hadoke, PhD$^2$; Rui Wang, MD$^4$; Eduard Gratacós, MD$^3$; Irina A. Buhimschi, MD$^5$; Catalin S. Buhimschi, MD$^5$; Asif Ahmed, PhD$^{1,2}$

$^1$Vascular Medicine Unit, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, England; $^2$Gustav Born Centre for Vascular Biology, BHF Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom; $^3$Dept of Maternal-Fetal Medicine, Hospital Clinic-IDIBAPS, University of Barcelona, Spain; $^4$Lakehead University, Thunder Bay, Ontario, Canada; $^5$Dept of Obstetrics, Gynecology and Reproductive Science, Yale School of Medicine, Yale University, New Haven, CT

Address for Correspondence:
Asif Ahmed, PhD
Aston University
Birmingham B4 7ET
England, United Kingdom
Tel: +44 204 4967
Fax: +44 121 204 3696
E-mail: asif.ahmed@aston.ac.uk.

Abstract:

**Background**—The exact etiology of preeclampsia is unknown, but there is a growing evidence of an imbalance in angiogenic growth factors and abnormal placentation. Hydrogen sulphide (H$_2$S), a gaseous messenger produced mainly by cystathionine γ-lyase (CSE, also known as CTH), is a pro-angiogenic vasodilator. We hypothesised that a reduction in CSE activity may alter the angiogenic balance in pregnancy and induce abnormal placentation and maternal hypertension.

**Methods and Results**—Plasma levels of H$_2$S were significantly decreased in women with preeclampsia (p<0.01), which was associated with reduced placental CSE expression as determined by real-time PCR and immunohistochemistry. Inhibition of CSE activity by DL-propargylglycine (PAG) reduced placenta growth factor (PIGF) 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 (HUVEC) by siRNA increased the release of soluble fms-Like tyrosine kinase-1 (sFlt-1) and soluble endoglin, (sEng) as assessed by ELISA while adenoviral-mediated CSE overexpression in HUVEC inhibited their release. Administration of PAG to pregnant mice induced hypertension, liver damage, and promoted abnormal labyrinth vascularisation in the placenta and decreased fetal growth. Finally, a slow releasing H$_2$S-generating compound, GYY4137 inhibited circulating sFlt-1 and sEng levels and restored fetal growth in mice that was compromised by PAG treatment demonstrating that the effect of CSE inhibitor was due to inhibition of H$_2$S production.

**Conclusions**—These results imply that endogenous H$_2$S is required for healthy placental vasculature and a decrease in CSE/H$_2$S activity may contribute to the pathogenesis of preeclampsia.

**Key words:** Hydrogen sulfide; preeclampsia, angiogenesis; fetal growth restriction; soluble Flt-1; vascular endothelial growth factor.
Introduction

Hydrogen sulfide (H$_2$S), a gaseous signaling molecule, promotes vasodilatation$^1$ and stimulates angiogenesis in the vasculature.$^2$ H$_2$S has anti-inflammatory properties$^3$ and is also cytoprotective against cellular damage induced by lethal hypoxia or reperfusion injury.$^4,5$

Cystathionine $\gamma$-lyase (CSE) is the principal enzyme responsible for the endogenous production of H$_2$S.$^6$ Chronic administration of the CSE inhibitor DL-propargylglycine (PAG) leads to elevated blood pressure and vascular remodelling in the rat$^7$ and both CSE and H$_2$S levels are reduced in pulmonary hypertensive rats.$^8$ Mice genetically deficient in CSE develop age-dependent hypertension, severe hyperhomocysteinaemia, and endothelial dysfunction.$^9$ Clearly H$_2$S has multiple roles in health and disease,$^{10,11}$ however, its role in pregnancy-induced hypertension is unknown.

Preeclampsia is a hypertensive syndrome that affects 4-7% of all pregnancies and is a major contributor to maternal and fetal morbidity and mortality worldwide.$^{12}$ The exact aetiology of preeclampsia is unknown, abnormal placentation$^{13,14}$ and imbalance in angiogenic factors$^{15,16}$ have been implicated in preeclampsia pathogenesis. Importantly, circulating levels of soluble Flt-1 (sFlt-1), the endogenous inhibitor of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) as well as soluble endoglin (sEng, the cleaved product of the accessory transforming growth factor-$\beta$1 receptor endoglin) are elevated several weeks prior to the onset of the clinical manifestations of preeclampsia,$^{17,18}$ while PIGF is reduced in the first trimester of pregnant women who subsequently developed the syndrome.$^{19-25}$ Together with endothelial dysfunction these have become the biochemical hallmarks of severe preeclampsia. Few studies have investigated the functions of CSE/H$_2$S in pregnancy. Recently, Patel et al.$^{2}$ demonstrated that both cystathionine $\beta$-synthase and CSE are present in human intrauterine tissues and
placenta.\textsuperscript{26, 27} Given that the placenta is a highly vascular organ we hypothesize that the
dysregulation of CSE/H\textsubscript{2}S pathway may contribute to placental abnormalities and a
preeclampsia-like condition.

In the current study, we demonstrated that plasma H\textsubscript{2}S levels in the mother and CSE
expression in the placenta are reduced in pregnancies complicated by preeclampsia as compared
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 marked decrease in placenta growth
factor (PIGF) production and trophoblast invasion \textit{in vitro} is inhibited. Inhibition of CSE activity
induces hypertension, increases sFlt-1 and sEng levels and causes placental abnormalities in
time-pregnant mice due to inhibition of H\textsubscript{2}S production. A slow releasing, H\textsubscript{2}S 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/ H\textsubscript{2}S
pathway may contribute to the pathogenesis of preeclampsia and provide the first direct evidence
for H\textsubscript{2}S therapy in this condition.

\textbf{Materials and Methods}

\textbf{Placental Tissue Collection and Preparation}

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 Labour and Delivery Unit. All women were
followed prospectively from enrolment until delivery. Human plasma and placental tissues were
collected from pregnancies complicated by preeclampsia and from normotensive pregnant
women. Samples of placental tissue were processed for RNA extraction and maternal plasma
from the same patients (n=14 preeclampsia and n=14 control) were used for analysis. From another set of patients placenta (n=5 preeclampsia and n=5 control) was collected for the immunohistochemical study. Preeclampsia was defined as blood pressure >140/90 mm Hg on at least two consecutive measurements and maternal proteinuria of at least 300 mg/24 h. First trimester placental tissues (6–9 weeks gestational age) were retrieved from normal pregnancies that had undergone elective termination. Villus explants were prepared as described previously. Briefly, human placental villus explants were incubated with or without PAG for 24 hours, and conditioned media collected and assayed for sFlt-1 or sEng and PlGF. Data was analyzed after normalization for total tissue protein.

**Animal Experimental Protocol**

Eight to ten week old C57BL/6 mice were mated. The first day of pregnancy (E0.5) was defined by the presence of a vaginal plug the following morning. Pregnant mice were randomly assigned into four groups: (i) saline (vehicle control), (ii) 25 mg/kg DL-propargylglycine (PAG; Sigma, Poole, U.K.), (iii) 50 mg/kg group PAG and (iv) 50 mg/kg PAG with 0.25 mg/kg of slow releasing H$_2$S donor GYY4137 (Sigma). Mice were injected intraperitoneally with saline or increasing concentrations of PAG from E8.5 and blood pressure measured by tail cuff plethysmography. In addition, arterial blood pressure was also measured pregnant mice at E17.5 as described previously for GYY4137 studies. Briefly, mice were anesthetized using a Ketamine/Xylazine cocktail and the carotid artery was isolated and cannulated with a Millar 1-French Mikro-Tip pressure catheter connected to a pressure transducer (ADInstruments Ltd. Oxford, UK). After 30 minutes of blood pressure stabilisation, arterial pressure recorded and averaged over a further 10 minutes period. Following measurements, blood sampling was undertaken, the animals sacrificed and their kidneys, livers, and placentas were collected. The
live fetuses and placentas were counted and weighed. All experimentation was conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 using procedures approved by the University Ethical Review Committee.

**Cell Culture**

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

**RNA Interference**

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

**Adenoviral Gene Transfer**

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

**Enzyme-Linked Immunosorbent Assay**

Enzyme-linked immunosorbent assay (ELISA) 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-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.

**Real-time Polymerase Chain Reaction**

Sample preparation and real-time quantitative was performed as described previously.\textsuperscript{31}

**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). HUVEC (1×10\textsuperscript{4} cells/well) were seeded in 96-well Matrigel-coated plates for 24 hours. Next day 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 six hours, cells were observed with a Nikon inverted microscope and images recorded and analysed using the Image Pro-Plus image analysis software (Media Cybernetics).

**Measurement of H\textsubscript{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 termination of pregnancy. H\textsubscript{2}S levels were measured as described previously.\textsuperscript{33} Briefly, 75 μl plasma was mixed with 250 μl of 1% (w/v) zinc acetate and 425 μl water, followed by 250 ml 50% trichloroacetic acid to remove proteins. To the mixture was added 133 μl 20 mM N-dimethyl-p-phenylenediamine sulphate in 7.2 mM HCl and 133 μl 30 μM FeCl\textsubscript{3} in 1.2 mM HCl. After 10 min incubation at room temperature, reaction mixture was centrifuged at 10 000g for two minutes. The absorbance of the resulting solution was measured at 670 nm with a spectrophotometer in a 96 well plate. The concentration of H\textsubscript{2}S in the solution was calculated against a calibration curve of NaHS.

**Statistical Analysis**
Data sets were tested for normality of distribution using the Shapiro-Wilk’s method and presented as either mean and SEM or median and range as appropriate. Comparison between two groups was performed using Student t-test or paired t-test (parametric) or Mann-Whitney U-test (non-parametric). Comparisons among three or more groups were performed using one-way ANOVA or repeated measures ANOVA followed by Student-Neuman Keuls post-hoc tests. Differences in proportions were tested by Fisher’s exact test. An observer blinded to treatment performed the analyses. Statistical significance was set at p<0.05.

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 controls group (Fig. 1A). Quantitative real-time PCR revealed that the CSE mRNA expression was significantly reduced in preeclamptic placenta (Fig. 1B) and decreased in fetal growth restricted pregnancy (Supplemental Fig. S1). Immunohistochemical staining confirmed that CSE immunoreactivity was dramatically reduced in preeclamptic placentas (Fig. 1C iv) 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 (Fig. 1C iii). Clinical characteristics of the study patients are described in Table 1.

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 anti-angiogenic factors generated by the placenta may account for the widespread maternal endothelial dysfunction in preeclampsia. While exposure of human first trimester placental explants to the CSE inhibitor PAG had no significant effect in sFlt-1 (p=0.254, Fig 2A) and sEng (p=0.361, Fig 2B) release, and PAG dramatically reduced PlGF production (p=0.003, Fig 2C). The inability to detect differences in sFlt-1 and sEng may be due to a Type II error as this dataset is finite. However, given that in the same explants we were able to detect differences in PlGF at a level of <0.01 we can confidently conclude that PAG affects to a larger extent the release of PlGF rather than 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 μM) compared with the vehicle control (Supplemental Fig. S2A and S2B, p=0.004) suggesting that a diminished CSE activity may compromise normal pregnancy.

CSE modulates sFlt-1 and sEng release in endothelial cells

Although placenta has been considered to be the main source of sFlt-1 and sEng release in preeclampsia patients, some studies have shown that levels of sFlt-1 remained higher in women with a history of preeclampsia compared with those without preeclampsia an average of 18 months postpartum. To investigate whether CSE affects sFlt-1 and sEng release in endothelial cells, CSE expression was modulated by siRNA or adenovirus in HUVECs. Downregulation of CSE (Fig. 3A) increased both sFlt-1 (Fig. 3B) and sEng (Fig. 3C) release, while overexpression of CSE (Fig. 3C) inhibited sFlt-1 (Fig. 3D) and sEng (Fig. 3E) release by HUVECs. These data further support the concept that 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 removal of sFlt-1 from preeclampsia samples restores angiogenesis. To assess whether H₂S can reverse the anti-angiogenic effects of preeclampsia, plasma from normotensive or preeclamptic women was added to HUVEC grown on growth factor-reduced Matrigel in the presence of a H₂S donor (100 mM NaHS), and *in vitro* tube formation assay performed. Consistent with earlier findings, preeclamptic plasma inhibited capillary tube network formation compared with normal control sera (Fig. 4). More importantly, NaHS partially restored the ability of HUVECs to form tube-like structure (Fig. 4A, 4B).

**Blocking endogenous H₂S causes hypertension and abnormal placental vascularisation 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 eight 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 approximately 50% (Fig. 5A). Consistent with these data we found significantly elevated mean blood pressure in a PAG concentration-dependent fashion in the treated group compared with vehicle-injected controls (Fig. 5B). Although proteinuria was not detected in the PAG-treated animals (Supplemental Table) but elevated liver enzyme aspartate transaminase (AST) indicated liver damage in these animals (Supplemental Table).

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.
(Fig. 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.\textsuperscript{38,39} Using isolectin B\textsubscript{4} to highlight the fetal endothelial cells,\textsuperscript{40} we compared the anatomical features of the labyrinth zone in vehicle and 50 mg/kg PAG-treated mice. In control mice the labyrinth appeared as organised fetal vessels with well-developed branching morphogenesis. In contrast the fetal vasculature of the placenta in PAG-treated animals was observed as irregular branching (Fig. 5D).

**H\textsubscript{2}S rescues PAG-induced hypertension, abnormal placental vascularisation 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 co-administration of 0.25 mg/kg GYY4137 (Fig. 6A). Plasma levels of sFlt-1 (Fig. 6B) and sEng (Fig. 6C) increased in PAG-treated pregnant mice (Fig. 6B and 6C) and were attenuated by 0.25 mg/kg of GYY4137 (Fig. 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 H\textsubscript{2}S can help to restore normal angiogenic status in vivo. Fetal weight was significantly decreased in mice that received the higher dose of PAG (Fig. 6D). Most importantly, GYY4137 treatment restored fetal growth (Fig. 6D) and the placental vasculature compromised by the CSE inhibitor (Fig. 6E).

**Discussion**

Chronic administration of a CSE inhibitor leads to reduced H\textsubscript{2}S and increased blood pressure in rats.\textsuperscript{7} Thus it is plausible that a reduction in the circulating H\textsubscript{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 due to inhibition of H$_2$S production as 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 shown to play a role in uterine contractility.$^{27}$ Recent placental studies provided contradictory findings probably due to the small sample size of these studies.$^{26,42,43}$ Holwerda and colleagues observed no changes in CSE expression in placenta from severe preeclampsia samples, while Cindrova-Davies et al reported that placental CSE level was reduced from pregnancies complicated with severe IUGR and preeclampsia.$^{43}$ In the present study, placental CSE levels were dramatically reduced in preeclamptic patients compared with normotensive controls and there was also a reduction in circulating maternal H$_2$S level. 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.$^{44,45}$ In this study, CSE was found to be a negative regulator of anti-angiogenic 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 VEGF and PlGF
activity in preeclampsia is believed to be the result of excess sFlt-1.\textsuperscript{15,17} As sFlt-1 levels are comparable to healthy controls during the first trimester of pregnancy, this theory does not explain why the circulating levels of PI GF are low in early pregnancy in women who subsequently develop preeclampsia.\textsuperscript{46} Our findings that inhibition of endogenous placental H\textsubscript{2}S generation by CSE inhibitor attenuates the production of PI GF in first trimester placental explants provides a possible explanation and a new hypothesis for testing: namely, the decrease in PI GF expression in early pregnancy is due to loss or reduction in the enzymes producing H\textsubscript{2}S. Furthermore, inhibition of CSE activity abolished the invasion of first-trimester extravillus trophoblast cells suggesting that dysregulation of CSE/H\textsubscript{2}S pathway may not only change the balance of placental pro and anti-angiogenesis factors, but also dysregulate maternal spiral artery remodeling and placental development.

In pregnant mice CSE inhibition reduced endogenous H\textsubscript{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 non-proteinuric (atypical) preeclampsia. Preeclampsia is also strongly associated with placental abnormalities including compromised villus volume and surface area, as well as reduced placental vascularisation.\textsuperscript{14,47} In the PAG-treated mice, the fetal labyrinth showed impaired branching morphogenesis, indicating endogenous H\textsubscript{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 vascularisation with altered patterning, branching and dilation.\textsuperscript{48} Blood pressure, liver function and fetal weight compromised by PAG-treatment were rescued by the slow releasing H\textsubscript{2}S-generating compound GYY4137 demonstrating that the effects of CSE inhibitor were due to inhibition of H\textsubscript{2}S production. These results imply that endogenous H\textsubscript{2}S is
required for healthy placental vasculature to support fetal and maternal wellbeing.

**Funding Sources:** This work was supported by grants from the British Heart Foundation (PG/06/114) and Medical Research Council (G0601295 and G0700288). We gratefully acknowledge the generous financial support provided by Dr Tim Watts of Pertemps.

**Conflict of Interest Disclosures:** None.

**References:**


23. Savvidou MD, Noori M, Anderson JM, Hingorani AD, Nicolaides KH. Maternal endothelial


fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest.* 2003;111:649-658.


**Table 1.** 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, years #</td>
<td>29.1 [19-40]</td>
<td>28.9 [18-44]</td>
<td>0.911</td>
</tr>
<tr>
<td>Gestational age, weeks #</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>24h-protein excretion, grams/24h#</td>
<td>N.A</td>
<td>4.5 [0.3-7.4]</td>
<td>N.A</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>

# Data presented as median [interquartile range] and analyzed by Mann–Whitney U test.
† Data shown as number of cases and percentage and analyzed tested by Fisher’s exact test.

**Figure Legends:**

**Figure 1.** CSE expression and H₂S levels in preeclampsia. (A) Plasma level of H₂S were measured spectrophotometrically in patients with preeclampsia (n=14) and normotensive controls (n=14). Data is shown as median (thick line) with interquartile range (box limits) and largest and smallest values (whisker limits) and analyzed using Mann-Whitney test; (B) Placental CSE mRNA expression was determined by real-time PCR in placentas from preeclampsia (n=14) and normotensive controls (n=14). Data is shown as mean+SEM and analyzed by Student t-test;
(C) Representative immunohistochemical staining of CSE (iii and iv) and isotype (i and ii) control in placenta from (i and iii) normotensive (n=5) and (ii and iv) preeclamptic pregnancies (n=5) MB: maternal blood; ST: syncytiotrophoblast; FV: fetal vessel; MC: mesenchymal cells.

**Figure 2.** Effects of CSE inhibition on angiogenesis factor release from human first trimester placenta. Villous explants were prepared from first trimester placental tissues (6–9 weeks gestational age) retrieved from normal pregnancies that had been terminated (n=7), and were incubated with or without a series of concentrations (25, 50, 100, 250 μM) of the CSE inhibitor PAG for 24 hours. (A) sFlt-1, (B) sEng, and (C) PlGF levels in conditioned media (CM) were measured by ELISA. Results of are expressed as mean+SEM and analyzed by one-way repeated measures ANOVA followed by Student-Newman-Keuls post-hoc tests. Means marked with asterisk are significantly different (p<0.01) from the PlGF level in untreated wells (zero dose).

**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). (B) sFlt-1 and (C) sEng 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); (E) sFlt-1 and (F) sEng were measured in culture medium of in HUVECs. The data is shown as mean+SEM of 3 (A and B) or 4 (C and D) experiments and analyzed by paired t-tests.

**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 normal (n=5) or preeclamptic women (n=5) in the absence or presence of
H₂S donor NaHS (100mM). (B) In vitro tube formation was measured as total tube length per field. Quantification of tube length was performed using Image Pro Plus image analysis software and expressed in μm/field. The data is shown as mean+SEM and analyzed by one-way repeated measures ANOVA.

Figure 5. Inhibition of CSE reduces endogenous circulating H₂S and promotes hypertension and abnormal placental vascularisation in pregnant mice. (A) Plasma was obtained from three groups of mice (n=4-5/group). H₂S levels were measured in pooled plasma from each group of animals. (B) Mean blood pressure was measured in these mice by tail cuff method. The results expressed as mean+SEM (n=6 mice/group) and analyzed by one-way ANOVA followed by Student-Newman-Keuls tests. (C) Hematoxylin and eosin staining of a representative mouse placenta treated either with (i) vehicle or (ii) 50 mg/kg PAG injection. (D) Isolectin B₄ staining of haemotrichorial labyrinth zone from mice received either (i) vehicle or (ii) 50 mg/kg PAG injection. Black solid arrow: maternal blood sinus; black thin arrow: fetal vessel; blue arrow: sinusoidal trophoblast giant cell.

Figure 6. H₂S 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 Millar system through carotid artery through microtip catheter. (B) Soluble Flt-1 (sFlt-1) and (C) soluble endoglin (sEng) in mouse plasma measured by ELISA. (D) Mean fetal weight determined at E17.5. (E) Isolectin B₄ staining of haemotrichorial labyrinth zone from mice received either (i) 50 mg/kg PAG alone or (ii) in combination with GYY4137. The results are expressed as mean+SEM (n=5 animals/group) and analyzed by one-way ANOVA followed by Student-Newman-Keuls tests.
Figure 1

A

Hydrogen Sulfide (ȝM)

Control | Preeclampsia

B

CSE mRNA

Control | Preeclampsia

C

i

ii

iii

iv

by guest on April 13, 2017 http://circ.ahajournals.org/ Downloaded from
Figure 2

A

Human sFlt-1 (pg/mg protein)

PAG (μM) [log]

B

Human sEng (pg/mg protein)

PAG (μM) [log]

C

Human PlGF (pg/mg protein)

PAG (μM) [log]
Figure 3

A

siControl  siCSE
CSE
β-actin

B

Human sFlt-1 (ng/ml)

siControl  siCSE

C

Human sEng (ng/ml)

siControl  siCSE

p = 0.013

p = 0.026

D

AdEV  AdCSE
CSE
β-actin

E

Human sFlt-1 (ng/ml)

AdEV  AdCSE

p = 0.007

p = 0.023

F

Human sEng (ng/ml)

AdEV  AdCSE

p = 0.023
Figure 4

A

B

Control  PE  PE+H₂S

Tube length (μm)

Control  PE  PE+NaHS

p=0.004

p=0.001

p=0.002
Figure 5

A

Hydrogen sulfide (µM)

PAG (mg/kg)

B

Mean Blood Pressure (mmHg)

PAG (mg/kg)

C

Control

PAG 50

D

Control

PAG 50
Figure 6

A  
Mean Blood Pressure (mmHg)  
- Control  
- PAG50  
- PAG50 + GYY  
- p = 0.004  
- p < 0.001

B  
Mouse sFlt-1 (ng/mL)  
- Control  
- PAG25  
- PAG50  
- PAG50 + GYY  
- p = 0.045  
- p = 0.036

C  
Mouse sEng (ng/mL)  
- Control  
- PAG25  
- PAG50  
- PAG50 + GYY  
- p = 0.005  
- p = 0.034

D  
Fetal weight (grams)  
- Control  
- PAG25  
- PAG50  
- PAG50 + GYY  
- p = 0.008  
- p = 0.025

E  
PAG50  
PAG50 + GYY437
Dysregulation of Hydrogen Sulfide (H$_2$S) Producing Enzyme Cystathionine $\gamma$-lyase (CSE) Contributes to Maternal Hypertension and Placental Abnormalities in Preeclampsia

Keqing Wang, Shakil Ahmad, Meng Cai, Jillian Rennie, Takeshi Fujisawa, Fatima Crispi, James Baily, Mark R. Miller, Melissa Cudmore, Patrick W. F. Hadoke, Rui Wang, Eduard Gratacós, Irina A. Buhimschi, Catalin S. Buhimschi and Asif Ahmed

*Circulation.* published online May 23, 2013;
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2013/05/23/CIRCULATIONAHA.113.001631

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/05/23/CIRCULATIONAHA.113.001631.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

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

Keqing Wang$^{1,2}$, Shakil Ahmad$^{1,2}$, Meng Cai$^{1,2}$, Jillian Rennie$^2$, Takeshi Fujisawa$^2$, Fatima Crispi$^3$, James Baily$^2$, Mark Miller$^2$, Melissa J. Cudmore$^2$, Patrick W. F. Hadoke$^2$, Rui Wang$^4$, Eduard Gratacós$^3$, Irina A. Buhimschi$^5$, Catalin S. Buhimschi$^5$, and Asif Ahmed$^{1,2,5}$

$^1$Vascular Medicine Unit, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, England, U.K.; $^2$Gustav Born Centre for Vascular Biology, University/BHF Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom; $^3$Department of Maternal-Fetal Medicine (Institut Clínic de Ginecologia, Obstetrícia i Neonatologia), Hospital Clinic-IDIBAPS, University of Barcelona, Spain; $^4$Lakehead University, Thunder Bay, Ontario, Canada; $^5$Department of Obstetrics, Gynecology and Reproductive Science, Yale School of Medicine, Yale University, New Haven, CT 06520, USA.

**Running title:** Low hydrogen sulfide in preeclampsia

$^5$**Corresponding author:** Prof. Asif Ahmed, Aston University, Birmingham B4 7ET, England, U.K. Email: asif.ahmed@aston.ac.uk. Tel: +44 204 4967.
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.\(^1\) Biotin-labelled isolectin-B\(_4\), 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.\(^2\) 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 extravillous 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>
</tr>
<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 graph showing CSE mRNA expression in Control and IUGR groups with a p-value of 0.070.](image-url)
Figure S2

A

Control

PAG

B

Cell Invasion (% of control)

p=0.004

control

PAG