Chapter 3

Hydrogen sulfide producing enzymes in pregnancy and preeclampsia


Abstract

Preeclampsia, a human pregnancy specific disorder is characterized by an anti-angiogenic state. As hydrogen sulfide (H₂S) has pro-angiogenic and anti-oxidative characteristics, we hypothesized that H₂S levels could play a role in the pathogenesis of preeclampsia and studied the placental expression of the H₂S-producing enzymes cystathionine-γ-lyase (CSE) and cysthationine-β-synthase (CBS). CBS and CSE protein are expressed in the fetoplacental endothelium and CBS only in Hofbauer cells. CBS mRNA expression is decreased (p = 0.002) in early-onset preeclampsia, while CSE mRNA is unchanged. Thus, down regulation of CBS during early onset preeclampsia may result in less H₂S production and may aid in the anti-angiogenic state.
Introduction

Preeclampsia (PE), a human pregnancy specific disorder, is characterized by placental ischemia and maternal endothelial dysfunction. The poorly perfused and ischemic placenta releases excess amounts of anti-angiogenic factors causing generalized endothelial damage.

Hydrogen sulfide (H$_2$S) is produced from the amino acid L-cysteine by two pyridoxal 5’ phosphate-dependent enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS). H$_2$S induces vasorelaxation by opening ATP-sensitive K-channels in smooth muscle cells and up regulates vascular endothelial growth factor. Furthermore, H$_2$S also has antioxidant capacity by direct scavenging of nitrogen or reactive oxygen species. CBS and CSE are down regulated in several cardiovascular and pulmonary diseases. Exogenous H$_2$S (NaHS) administration is proposed as a novel therapy in animal models of cardiovascular and ischemic diseases. CBS is also an important enzyme in the homocysteine pathway, since homocysteine is converted to cystathionine by CBS. Pregnant CBS transgenic mice show a moderate increase of homocysteine which associated with blunted endothelial-dependent relaxation in arteries.

We hypothesized that H$_2$S, because of its pro-angiogenic and anti-oxidative characteristics and the involvement of CBS in homocysteine degradation, might play a role in the pathogenesis of PE. The aim of the present study was to identify and compare the expression and localization of CBS and CSE in placental tissue from both normotensive and early- and late-onset PE.

Methods

Sample collection

Placental biopsies were obtained from patients (n = 36) with early-onset PE, late-onset PE and mode of delivery matched healthy pregnant controls after informed consent. The local UMCG Medical Ethical Committee approved the study. PE was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy: diastolic blood pressure of > 90 mm Hg and proteinuria ≥ 300 mg/24 hours. PE present before 34 weeks of gestation was defined as early-onset, these patients delivered by Cesarean section. Intrauterine growth restriction (IUGR) was defined as birth weight under the tenth percentile.
Clinical characteristics of pregnant women with early- and late-onset preeclampsia, and mode of delivery matched controls

<table>
<thead>
<tr>
<th></th>
<th>Delivery by Cesarean section</th>
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<th>Spontaneous delivery</th>
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<tbody>
<tr>
<td></td>
<td>Control pregnancy</td>
<td>Early-onset preeclampsia</td>
<td>Control pregnancy</td>
<td>Late-onset preeclampsia</td>
</tr>
<tr>
<td>Number</td>
<td>9</td>
<td>10</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>(26 – 35)</td>
<td>(25 – 26)</td>
<td>(27 – 37)</td>
<td>(26 – 33)</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>38±6 (38.6 – 39.9)</td>
<td>28±5 (27.5 – 30.9)**</td>
<td>39±1 (38.1 – 41.0)</td>
<td>38±1 (37.0 – 39.7) **</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>132 (125 – 147)</td>
<td>178 (166 – 190)*</td>
<td>120 (118 – 120)</td>
<td>155 (150 – 163)* **</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 (80 – 85)</td>
<td>110 (110 – 115)**</td>
<td>76 (70 – 80)</td>
<td>97 (92 – 105)**</td>
</tr>
<tr>
<td>Proteinuria (grams/24 hours)</td>
<td>0 (0.9 – 4.9)**</td>
<td>3.0</td>
<td>0.8 (0.7 – 0.8)**</td>
<td>0.8</td>
</tr>
<tr>
<td>Birth weight (grams)</td>
<td>3880 (3610 – 4085)</td>
<td>933 (713 – 1103)**</td>
<td>3390 (3180 – 3700)</td>
<td>2770 (2569 – 3071)*</td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IUGR</td>
<td>0</td>
<td>2 (20)</td>
<td>0</td>
<td>1 (11)</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile range) and numbers (%). For statistical analysis the Mann Whitney U test and Fisher exact test were used. IUGR, intrauterine growth restriction; HELLP, hemolysis elevated liver enzymes low platelets. *p-value < 0.05, **p-value < 0.001, when compared to healthy pregnancy with delivery by Cesarean section. ¥p-value < 0.05, ¥¥p-value < 0.001, when compared to healthy pregnancy with spontaneously delivery.

Immunohistochemistry
As previously described,11 placental cryosections were stained with mouse monoclonal antibodies against CSE (1:100, donated by dr. N. Nishi, Kagawa Medical School, Japan) and CBS (1:250, Abnova, Tapei, Taiwan). Primary antibody was replaced by PBS in negative controls. For immunofluorescence double staining, CD31 (1:100, Sigma-Aldrich, St. Louis, MO) was used.

Real time RT-PCR
For real time RT-PCR, RNA was isolated from several parts of the placenta, pooled, and purified as previously described.12 We analyzed mRNA expression of CBS and CSE using Assay-on-Demand Gene Expression (Applied Biosystems, USA). PSMD4 (proteasome non-ATPase regulatory subunit 4) was used as a housekeeping gene, the expression of this gene was constant over the four study groups.13
Western Blot

Total proteins from placental biopsies were extracted and western analysis was performed according to published procedures. CBS and CSE monoclonal antibodies (Abnova) were used at 1:500 dilution.

**Results and discussion**

The major finding of this study is that CBS mRNA expression is significantly downregulated in placental villous tissue derived from pregnancies complicated by early-onset PE when comparing to mode of delivery matched controls (figure 1A). Furthermore, we demonstrated that CBS and CSE are mainly localized in the endothelium in the fetal vessels from the chorionic- and stem-villi (figure 2A). The endothelial origin of both enzymes is confirmed by double staining with CD31 (figure 2B). Hofbauer cells express CBS (figure 2A). There were no differences in CBS/CSE in protein expression between PE and delivery matched controls (figure 1B). However, CBS/CSE protein expression was significantly down regulated in all placentae after spontaneous delivery compared to Cesarean delivery (figure 1B).

Although protein expression of CBS and CSE was not affected by PE, we found a down regulation of mRNA of CBS in early-onset PE. This discrepancy between mRNA and protein expression is remarkable, but has been reported previously. In ischemic brain tissue, decrease in mRNA corresponded to decreased CBS-activity and H$_2$S production, while protein levels did not. Although protein levels do not imply protein-activities, we did not evaluate CSE- and CBS-activity or H$_2$S production. However, Patel et al. showed that H$_2$S is endogenously produced in the placenta, production rate was increased under low-oxygen levels. Another study confirmed placental catalytic CBS-activity by converting homocysteine. So far, in PE no CBS- and CSE-activity or H$_2$S production is reported. Therefore, the exact role of endogenous H$_2$S in PE needs to be elucidated.

Differential CBS mRNA expression may be gestational age related, as has been previously documented for other genes, however no differences in CBS mRNA expression measured in first-trimester and term human placentae were reported. For late-onset PE, placental CBS mRNA expression is not altered compared to healthy pregnancies (figure 1B). This is in line with the growing evidence that there are differences between the pathophysiology of early- and late-onset PE. In our total study group, 3 patients with a
pregnancy complicated by IUGR were present. The data of these patients with respect to mRNA and protein expression fitted well within their study groups.

Figure 1 - Placental expression of CBS and CSE mRNA and protein after normotensive pregnancy and pregnancy complicated by preeclampsia

(A) CBS mRNA expression is down regulated in the early onset preeclampsia group, compared to the mode for delivery matched group (delivery by cesarean section). No differences were observed in CBS mRNA expression in the late-onset PE group or in CSE mRNA expression in all four groups. Median and interquartile range is given. For statistical analysis, Mann-Whitney U test was used. (B) Quantitative western blot analysis showed a significant down regulation of both CBS and CSE in all spontaneous delivery placentae, compared to the cesarean groups. No differences were observed in expression of both enzymes between both PE groups and mode of delivery matched controls. Median and interquartile range is given. For statistical analysis, Mann-Whitney U test was used, *p-value < 0.05.
Figure 2 - Placental expression of CBS and CSE in healthy pregnancy

IHC and IF staining was performed on all samples of the 4 groups. No difference in localization were observed between groups, therefore only representative cryostat sections of control placental villous tissue stained for CSE (A, E) and CBS (B, C, F) are shown. Positive staining is shown in fetal endothelial cells in chorionic villi (long arrow), fetal endothelial cells in stem villi (closed short arrow) and in hofbauer cells (arrow-head). Syncytio- and cytotrophoblasts are negative (open short arrow). No positive staining is observed in the negative control, in which the primary antibody was replaced by PBS (D).

For the IF staining (E, F), nuclear staining (DAPI) is shown in blue, CD31 protein is shown in red, CSE and CBS protein in green, and colocalization of CD31 with CBS or CSE in white. IF staining confirmed the endothelial expression of both CSE and CBS protein. All images were taken at the same exposure and with a magnification of x20 (A, B, D, E, F) or x40 (C).
CBS and CSE protein, but not mRNA expression was significantly down regulated in control and PE placentae after spontaneous delivery compared to Cesarean delivery. This is in line with a recent report of down regulation of the enzymes and reduced H$_2$S production in the myometrium during labor.$^{21}$ Increased turnover of the enzymes could be involved in transition of the labor process.

The fetal endothelial expression of CBS and CSE is in accord with the expressions in other organs.$^{22}$ Moreover, we showed expression of CBS by Hofbauer cells. Hofbauer cells or fetal tissue macrophages are placental immune cells; several studies suggest that Hofbauer cells play a direct role in placental vasculogenesis.$^{23}$ Although CSE has been shown to be expressed by macrophages,$^{24}$ the expression of CBS by macrophages has not been reported. The expression of CBS by Hofbauer cells may be in line with the role of these cells in vasculogenesis.

In conclusion, the present study provides novel insights into the expression of H$_2$S-producing enzymes during normal and PE. Future studies will compare the placental CBS/CSE-activity and H$_2$S production in pregnancies and explore the possible therapeutic role of H$_2$S during PE.

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References
