Hydrogen Sulfide in Preeclampsia
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Chapter 1

General Introduction
Preeclampsia (PE) is a severe pregnancy-induced syndrome that is characterized by the new onset of hypertension and proteinuria in the second half of gestation. Symptoms of PE and eclampsia (the onset of convulsions in PE) were already perceived in ancient times, and the association between the pregnancy-related disorder and a “heavy”, strong and swollen pulse’ was made in the 6th century. However, it was not until 1896, when sphygmomanometry was introduced, that blood pressure measurements were clinically used to diagnose PE. In the 19th century, proteinuria in PE patients was first noted by Lever et al. Despite the great development in the area of PE, the etiology of PE is still partly unclear. Perhaps more importantly, no adequate therapies for PE or methods to predict PE have yet been developed. Consequently, PE is still a leading cause of maternal and fetal mortality and morbidity worldwide. Since the last decades, the role of gasotransmitters such as nitric oxide (NO) and carbon monoxide (CO) in the initiation and progression of PE have been investigated. The gases are thought to be involved in the pathophysiology of PE, and exogenous administration NO, CO, or related compounds have beneficial effects in (animal models for) PE. The most recently discovered and third gasotransmitter hydrogen sulfide (H\textsubscript{2}S) has never been investigated in PE. However, its vasodilatory and pro-angiogenic features make H\textsubscript{2}S an interesting factor to investigate in PE. This prompted us to explore the involvement of H\textsubscript{2}S as a novel factor in the (patho)physiology of pregnancy and PE, and to investigate the possibilities of H\textsubscript{2}S to become a therapeutic agent for this severe pregnancy-related disease.

**Preeclampsia**

Complicating approximately 2-5% of all pregnancies worldwide, PE is a major obstetric problem contributing to both maternal and fetal mortality and morbidity. It is a multi-organ disease that clinically manifests after the 20th week of gestation. The disease is defined by the new onset of hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg), in combination with proteinuria (≥ 300 mg / 24 hours). PE can be subdivided into an early-onset form, with an onset before 34 weeks of gestation, and a late-onset form, developing after 34 weeks of gestation.

Both the maternal and perinatal outcomes of PE worsen with an earlier gestational age at time of onset and with increased severity of the disease. PE can affect several organs, such as the maternal liver, the kidneys, and the brain. Therefore, maternal
complications of PE include the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), renal failure, and eclamptic seizures. In the kidney, PE is also associated with glomerular endotheliosis, which is thought to be a pathognomonic lesion in PE. Glomerular endotheliosis is mainly characterized by the presence of protein droplets and by occlusion of capillary lumens by swollen endothelium. Fetal complications of PE include preterm delivery and fetal growth restriction.

The only effective treatment for PE at the moment is termination of pregnancy (delivery of the placenta), often remote from term. Management options for PE are accurate maternal and fetal monitoring, and avoiding maternal complications by administering anti-hypertensive drugs. Obviously, management decisions are highly dependent on the severity of the disease and the gestational age of presentation. The right balance between the risk for the mother to be subjected to progressive disease and the benefits for the fetus to further develop in utero, is essential in this decision-making. Ideal therapeutic strategies that prolong pregnancy until term and prevent further progression of the maternal disease have not been developed yet.

Pathogenesis of preeclampsia

Although the etiology of PE is still not completely understood, the general hypothesis that the placenta plays a key role in its pathophysiology is leading. However, it is now becoming clear that the extent to which the placenta contributes to the disease is variable. Inadequate placentation is now thought of much greater significance in the development of early-onset PE, rather than in the late-onset form. Late-onset PE, on the other hand, seems to be more associated with a defective pre-gestational maternal endothelium and/or inflammatory function.

The development of early-onset PE is thought to progress in two stages, starting with a preclinical phase, which is associated with abnormal placentation in the early stages of pregnancy (stage I). During the process of placentation in normal pregnancy, fetal cytotrophoblasts invade the maternal myo- and endometrium, and the spiral arteries. The cytotrophoblasts migrate into the wall of the spiral arteries, resulting in the loss of endothelial and vascular smooth muscle cells. Consequently, the spiral arteries become dilated and low-resistant in order to optimize uteroplacental blood flow during gestation. During the development of PE, trophoblast invasion and, consequently, remodeling of the maternal spiral arteries in the maternal myometrium is impaired (stage I). The spiral arteries remain narrow and highly resistant, causing insufficient placental
perfusion (intermittent flow) and subsequent placental oxidative stress. Subsequently, the hypoxic placenta releases various bioactive compounds into the circulation of the mother, such as soluble FMS-like tyrosine kinase 1 (sFlt1), pro-inflammatory cytokines, and syncytiotrophoblast microparticles. Together, these factors contribute to the onset of generalized endothelial cell dysfunction and vascular inflammation, which are consequently thought to initiate the clinical syndrome of PE, i.e. hypertension and proteinuria (the clinical stage; stage II). Early-onset PE is often accompanied by fetal growth restriction, since the oxygen-deprived placenta is not able to meet the increasing fetal demands for nutrients and oxygen along with gestation.

For late-onset PE, maternal factors that predispose to vascular inflammation seem to be relevant. In these women, pregestational endothelial dysfunction is present, due to preexisting conditions such as (early stages of) cardiovascular disease, obesity, or diabetes. It is thought that the preexisting vascular inflammation exaggerates the maternal response to pregnancy and contributes to the clinical signs of PE. However, another hypothesis with regard to the development of late-onset PE was recently proposed. As with the early-onset form, inadequate placental perfusion is observed in late-onset PE. The dysregulation of placental perfusion in late-onset PE, though is not preceded by impaired placentation, but by restricted villous perfusion that is associated with the size of the term placenta. As in early-onset PE, dysregulation of placental perfusion in late-onset PE causes placental stress, release of bioactive compounds, and the subsequent clinical stage (stage II) of PE.

Anti-angiogenic factors belong to the variety of bioactive factors that are released by the affected placenta during PE. These factors contribute to the development of endothelial cell dysfunction, a central feature of PE. The most extensively researched anti-angiogenic factor in PE is sFlt1. The sFlt1 protein is a splice variant of vascular endothelial growth factor (VEGF) receptor 1, lacking the transmembrane and cytoplasmic domains. Circulating in the maternal blood, sFlt1 acts as a powerful antagonist of VEGF and placental growth factor (PIGF). Increased levels of circulating sFlt1 lead to functional VEGF and PIGF deficiency, causing endothelial dysfunction, impaired angiogenesis, impaired capillary repair, and, consequently, hypertension and proteinuria. Moreover, aside from the release of antiangiogenic factors, the diseased placenta sheds pro-inflammatory debris into the maternal circulation containing factors such as cytokines and syncytiotrophoblasts microparticles. These factors activate the maternal endothelium and consequently contribute to the endothelial dysfunction and vascular inflammation.
**Therapeutic strategies for preeclampsia**

Various options have been studied as potential therapies for PE, including anti-oxidants (such as vitamin E), fish oil, and bed rest.\(^{21-23}\) Unfortunately, none of these studies have been convincing. Supplementation of calcium and the use of aspirin started early in gestation have been shown to have small beneficial effects in the prevention of PE in high-risk populations.\(^{24,25}\) Currently, quite a few studies focus on restoring the angiogenic balance during PE. Recent studies have shown that recombinant proteins with functions similar to VEGF have therapeutic effects in animals with experimental PE.\(^{26-28}\) Furthermore, selective removal of plasma sFlt1 in women with early-onset PE shows beneficial effects.\(^{29}\) Finally, current studies also focus on the use of gasotransmitters as a therapy for PE. Because of their regulating function in the vasculature and cytoprotective actions, gasotransmitters are an interesting target in PE.

**Gasotransmitters and preeclampsia**

Gasotransmitters, such as nitric oxide (NO) and carbon monoxide (CO), are small, hydrophilic, gaseous signaling molecules, that diffuse through cell membranes.\(^{30}\) Given that both NO and CO are involved in regulation of vascular tone and that deficiency of the two gases is associated with endothelial dysfunction, their role in PE has been extensively studied.\(^{31-33}\) It has been shown that the bioavailability of NO and CO is impaired in women with PE.\(^{32,34,35}\) Moreover, besides their vasoregulating functions, it is believed that NO and CO might also be involved in influencing the angiogenic balance in PE.\(^{36-38}\) Finally, increased availability of the cytoprotective heme oxygenase-1 (a CO producing enzyme) in PE patients was linked to the increased oxidative and inflammatory stress that is present in these women.\(^{39}\) Supplementation with L-arginine, the precursor of NO, might have a role in the treatment and/or prevention of PE.\(^{40}\) Clinical trials using therapeutic agents that target as well the anti-angiogenic environment as NO deprivation, such as statins and relaxin, are now performed.\(^{41-43}\) Interestingly, statins are also able to increase CO availability.\(^{44}\) Besides NO and CO, a third gasotransmitter was discovered more recently; hydrogen sulfide (H\(_2\)S).
Hydrogen sulfide

For a long time, \( \text{H}_2\text{S} \) has been known as a highly toxic gas with the distinctive smell of rotten eggs. However, during the last few decades, the gas was discovered to be endogenously produced by mammalian cells.\(^\text{30}\) In humans and other mammals, the enzymatic \( \text{H}_2\text{S} \) production is part of the transsulfuration pathway (figure 1).\(^\text{30}\) The two main enzymes in this process are cystathionine-\( \beta \)-synthase (CBS) and cystathionine-\( \gamma \)-lyase (CSE), both using pyridoxal 5’-phosphate (vitamin B6) as a cofactor.\(^\text{45}\) CBS performs the first and rate-limiting step in the transsulfuration pathway by converting homocysteine into cystathionine. This is followed by cleavage of cystathionine by CSE yielding L-cysteine. Both CBS and CSE desulferate L-cysteine into \( \text{H}_2\text{S} \) (figure 1).\(^\text{45}\) \( \text{H}_2\text{S} \) is also produced by 3-mercaptopyruvate sulfurtransferase (3-MST) from L-cysteine and D-cysteine, via cysteine aminotransferase (CAT) and D-amino acid oxidase (DAO), respectively (figure 1).\(^\text{46}\) Oxidation of \( \text{H}_2\text{S} \) yields its metabolites thiosulfate, sulfite, and sulfate, which are excreted in urine (figure 1).\(^\text{47}\)

**Figure 1 - Schematic representation of the \( \text{H}_2\text{S} \) production pathway**

CBS, cystathionine-\( \beta \)-synthase; CSE, cystathionine-\( \gamma \)-lyase; CAT, cysteineaminotransferase; 3-MST, 3-mercaptopyruvate sulfurtransferase; and DAO, D-amino acid oxidase.
Biological functions of hydrogen sulfide

$\text{H}_2\text{S}$ has several biological functions, such as scavenging reactive oxygen species, affecting the immune system by e.g. modulating cytokine production and leukocyte adhesion, and influencing protein activity through sulfhydration.\textsuperscript{48} However, the first and most studied function of $\text{H}_2\text{S}$ is regulation of vascular tone. Genetic deficiency of $\text{CSE}$ causes hypertension, as shown by $\text{CSE}$ knockout mice, which suffer from age-dependent hypertension and loss of endothelium-dependent vasorelaxation.\textsuperscript{49} Mice heterozygous for $\text{CBS}$ also develop increased blood pressure.\textsuperscript{50} Interestingly, individuals with a genetic deficiency of $\text{CBS}$ exhibit hyperhomocysteinemia, which is associated with vascular disease and endothelial dysfunction.\textsuperscript{51,52}

Within the vessel wall, $\text{H}_2\text{S}$ is produced by both endothelial cells and vascular smooth muscle cells and the gas is able to induce vasorelaxation via direct opening of potassium-ATP ($K_{\text{ATP}}$) channels and calcium-dependent potassium channels.\textsuperscript{43,53,54} It has recently been shown that opening of the $K_{\text{ATP}}$ channel by $\text{H}_2\text{S}$ may be initiated by sulfhydration of cysteine residues in this channel.\textsuperscript{55} Furthermore, it has been described that the vasodilatory effects of $\text{H}_2\text{S}$ are partially dependent on nitric oxide (NO),\textsuperscript{56} and that $\text{H}_2\text{S}$ is able to prevent hypertension in rat by affecting the renin-angiotensin aldosterone system (RAAS) through direct inhibition of renin plasma activity.\textsuperscript{57}

$\text{H}_2\text{S}$ is also an efficacious pro-angiogenic agent. The process of angiogenesis is characterized by the sprouting of new blood vessels. An important mediator in angiogenesis is VEGF, which exerts its effects through VEGF receptor 2 (VEGFR2). The pro-angiogenic properties of $\text{H}_2\text{S}$ were first shown by Cai et al. in vitro and confirmed by several other groups in vivo.\textsuperscript{58-60} VEGF expression and activation of its receptor are increased upon $\text{H}_2\text{S}$ treatment.\textsuperscript{60-62} Interestingly, $\text{H}_2\text{S}$ is also able to directly activate VEGFR2 by breaking a disulfide bond in a domain of this receptor.\textsuperscript{63} As with vasorelaxation, the pro-angiogenic actions of $\text{H}_2\text{S}$ seem to be partially dependent on NO.\textsuperscript{56,64}

Hydrogen sulfide as a therapeutic compound

Decreased production of $\text{H}_2\text{S}$ or its producing enzymes is substantially linked to the pathology of cardiovascular disease.\textsuperscript{65} Therefore, compounds able to increase availability and/or functionality of $\text{H}_2\text{S}$ have been shown to have therapeutic potential in these diseases. Such compounds can be divided into several groups. In the vast majority of studies, commercially available sulfide-sodium salts such as $\text{NaHS}$ and $\text{Na}_2\text{S}$ are used. $\text{NaHS}$ and $\text{Na}_2\text{S}$ have shown to have pro-angiogenic, antihypertensive and
antiinflammatory effects. However, a major problem of sulfide-sodium salts is the rapid loss of sulfide from the solution, making it difficult to achieve controllable levels of H$_2$S in vivo and in vitro. For this reason, H$_2$S donors with slow-releasing properties are under development. For example, it was shown that the slow H$_2$S releaser GYY4137 is able to normalize blood pressure in spontaneously hypertensive rats. Another alternative is the administration of natural occurring analogues of cysteine, the precursor of H$_2$S. S-propargyl cysteine for example is able to induce angiogenesis in both in vitro and in vivo settings. Moreover, it was observed that the cysteine analogue diallyl trisulfide (derived from garlic), has protective cardiovascular effects by up regulation of VEGF and NO bioavailability in mice. Finally, the H$_2$S metabolite sodium thiosulfate can also act as an H$_2$S donor. Through the action of thiosulfate reductase, thiosulfate can be converted into H$_2$S. It was recently shown that hypertension, proteinuria, and renal damage induced by angiotensin II infusion was attenuated by sodium thiosulfate.

Hydrogen sulfide in pregnancy and preeclampsia

The placental production of H$_2$S by CSE and CBS from L-cysteine was first described in 2009. The endogenous H$_2$S production by the placenta increases under low oxygen conditions. The catalytic activity of CBS was further analyzed by Mislanova et al., showing that homocysteine increased the accumulation of L-cysteine and CBS in human placental explants. Functionally, it was shown in vitro that H$_2$S mediates vasodilatation within the human placenta via K$_{ATP}$ channels and by interaction with nitric oxide. The presence of CBS was also detected in first trimester human placentae.

Alterations in the transsulfuration pathway have been associated with PE. For example, homocysteine, cystathionine, and cysteine are increased in the plasma of women with PE. Furthermore, hyperhomocysteinemia has been related to a decreased fetal weight in the offspring of both healthy pregnant women and PE patients. Interestingly, pregnant CBS-deficient mice, both homozygotes and heterozygotes, exhibit hyperhomocysteinemia which is associated with blunted endothelial-dependent vessel relaxation. Finally, CBS-deficient mice show impaired decidualization.
Scope of the thesis

Clinically, PE is recognized by proteinuria and hypertension. Pathological processes, such as endothelial dysfunction, impaired angiogenesis, and an exaggerated immune response, are characteristic for PE. As H2S is known for its, immunomodulating, cytoprotective, vasorelaxative, and pro-angiogenic effects, we hypothesize that decreased H2S is involved in the pathophysiology of PE and that H2S could have therapeutic potential in PE. Until now, the expression of the H2S-producing enzymes in pregnancy and PE has never been investigated. Therefore, the main objective of this thesis is to address these issues by investigating alterations in the H2S pathway in association with PE and by testing its therapeutic potential in vivo.

Part I: Human studies – Hydrogen sulfide in the pathophysiology of PE

In the last decade(s), the role of NO and CO in both pregnancy and PE has been investigated extensively. Less is known about the involvement of H2S in pregnancy and in the pathophysiology of PE. As H2S, NO, and CO share several features and functions, a similar involvement of H2S in pregnancy and PE is conceivable. The aim of Chapter 2 was to give an outline of the role of the three gasotransmitters in the physiology of pregnancy. Furthermore, this chapter describes the aberrant production of NO, CO, and H2S in PE. Specific emphasis is put on the therapeutic potential of the three gasotransmitters in PE by overviewing experimental in vivo and in vitro studies.

Chapter 3 aims to evaluate the cellular localization of two H2S-producing enzymes, CBS and CSE, in the human placenta. Moreover, the placental expression of both mRNA and protein of these two enzymes is investigated during healthy pregnancy and PE. Expression of CBS and CSE in placentae from both early-onset and late-onset PE patients is evaluated.

Chapter 4 focuses on the single nucleotide polymorphisms (SNPs) in the CBS gene in association with early- and late-onset PE. In a cohort of 75 healthy pregnant women, 45 patients with early-onset PE and 52 women with the late-onset form, six tagging SNPs in the locus of the CBS gene are assessed. Furthermore, functional effects of the tag-SNPs on plasma and urinary metabolites of the transsulfuration pathway are evaluated in this chapter.
Part II: Animal studies – Hydrogen sulfide as a therapeutic agent in animal models with high sFlt
As H\textsubscript{2}S has pro-angiogenic potential, its therapeutic properties were evaluated in an animal model in which an anti-angiogenic state was induced by sFlt1 overexpression. This animal model was first described by Maynard, et al., who showed that injection of an adenovirus overexpressing the anti-angiogenic protein sFlt1 in pregnant rats induced a PE-like phenotype.\textsuperscript{17} As in the human situation, high levels of circulating sFlt1 antagonize the free circulating levels of the pro-angiogenic protein VEGF. Systemic deprivation of VEGF contributes to the onset hypertension, proteinuria, and glomerular endotheliosis in these rats.\textsuperscript{17} The induction of the phenotype by the sFlt1 adenovirus is not exclusive to pregnant animals. Therefore, the aim of Chapter 5 was to examine the effect of the H\textsubscript{2}S donor NaHS on hypertension, proteinuria and glomerular endotheliosis in a non-pregnant rat model with sFlt1 overexpression. By performing both in vivo and in vitro experiments, the mechanism by which NaHS (partly) influences the anti-angiogenic state is studied in more detail.

Since we are specifically interested in the therapeutic potential of H\textsubscript{2}S in a pregnancy-specific disease, we studied the safety of NaHS treatment in healthy pregnant rats in Chapter 6. Furthermore, the therapeutic potential of NaHS was evaluated in a pregnant rat model with sFlt1 overexpression.

Chapter 7 recapitulates the molecular mechanisms by which H\textsubscript{2}S exerts its vasodilatory and pro-angiogenic functions, while H\textsubscript{2}S-based therapies for PE and other cardiovascular diseases are summarized.

In the final chapter of this thesis, Chapter 8, all findings are summarized and discussed. Furthermore, future research possibilities to increase the knowledge on the role of H\textsubscript{2}S in the pathophysiology and to test its therapeutic potential will be discussed.
References


Part I

Human studies

Hydrogen sulfide in the pathophysiology of preeclampsia