Chapter 7

Hydrogen sulfide: a role in vascular physiology and pathology

K.M. Holwerda, S.A. Karumanchi, A.T. Lely

Abstract

Hydrogen sulfide (H$_2$S), a colorless gas that is endogenously generated in mammals from cysteine, has important biological functions. Within the vasculature it regulates vessel tone and outgrowth of new vessels. This review summarizes recent literature on H$_2$S signaling in the vasculature and its therapeutic potential in vascular disorders.

H$_2$S is able to induce vasorelaxation via ATP-sensitive potassium channels in vascular smooth muscle cells. Large-conductance calcium-dependent K$^+$-channels and K$_v$, voltage-gated K$^+$-channels are also involved in H$_2$S signaling. Vascular endothelial growth factor is the key downstream mediator that is involved in H$_2$S induced angiogenesis. By having both direct effects on its receptor and increasing the bioavailability of vascular endothelial growth factor, H$_2$S is pro-angiogenic. H$_2$S-based therapies in vascular diseases are an expanding area of research. The applications of several compounds, such as natural donors and synthetic slow release compounds, have been extensively studied in vascular diseases such as hypertension, ischemia–reperfusion disorders and preeclampsia.

H$_2$S has a key role in vascular homeostasis during physiology and in pathological states. H$_2$S-based therapies may have a role in several vascular diseases.
Introduction

Hydrogen sulfide (H$_2$S) is a small, water-soluble, freely permeable endogenous gasotransmitter. In humans and other mammals, H$_2$S is produced by two pyridoxal-5-phosphate-dependent enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), from the amino acid L-cysteine. H$_2$S is also produced from L-cysteine in an additional pathway by 3-mercaptopyruvate sulfurtransferase (3-MST) in combination with cysteine aminotransferase.

An important physiological function of H$_2$S in the vasculature is the regulation of vascular tone. CSE knockout mice show age-dependent hypertension and loss of endothelium-dependent vasorelaxation. Within the vessel wall, H$_2$S can be derived from both endothelial cells and vascular smooth muscle cells (VSMCs). By triggering cholinergic activity, and a subsequent increase of intracellular calcium, CSE acutely produces H$_2$S in endothelial cells. In VSMCs, H$_2$S induces vasorelaxation partly by directly opening of potassium-ATP (K$_{ATP}$) channels.

Cai et al. were the first to report the pro-angiogenic effect of H$_2$S in vitro; they demonstrated that H$_2$S stimulated cell proliferation, migration and formation of tube-like structures. Since then, several groups have showed that H$_2$S also acts as a pro-angiogenic factor in vivo. The pro-angiogenic effects of H$_2$S are associated with an increase in vascular endothelial growth factor (VEGF) expression and activation of its receptor.

In the current review, we will summarize more recent studies (2013 – 2014) on the vascular functions of H$_2$S. In addition, we will give an overview of the role of H$_2$S in the activation of ion channels, its pro-angiogenic mechanisms and the interaction between H$_2$S and nitric oxide. Finally, we will discuss the possible therapeutic role of H$_2$S in hypertension, ischemia-reperfusion disorders and preeclampsia.

Activation of ion channels by hydrogen sulfide

H$_2$S is able to activate ion channels in both VSMCs and endothelial cells, as shown in figure 1A. In 2011, the ability of H$_2$S to activate ion channels by S-sulfhydration was discovered. S-sulfhydration is a covalent change of a cysteine residue, yielding a persulfide moiety (-SSH). In the Kir6.1 subunit of K$_{ATP}$ channels, H$_2$S can sulfhydrate cysteine-43 leading to activation of the channel. Furthermore, H$_2$S induces vasorelaxation via intermediate-
conductance and small-conductance calcium-dependent K⁺ channels (IK\textsubscript{Ca} and SK\textsubscript{Ca} channels) in endothelial cells. It is suggested that the activation of IK\textsubscript{Ca} channels by H\textsubscript{2}S is also due to sulfhydration.\textsuperscript{7}

Because H\textsubscript{2}S is able to stimulate IK\textsubscript{Ca} and SK\textsubscript{Ca} channels in an endothelial-dependent manner, it was speculated for years that H\textsubscript{2}S might act as an endothelial-derived hyperpolarization factor (EDHF). However, just a year ago, solid evidence showed that H\textsubscript{2}S indeed is an EDHF.\textsuperscript{8} A characteristic of an EDHF is that it is produced by endothelial cells and influences the contractility of VSMCs by inducing hyperpolarization. By using advanced techniques it was found that H\textsubscript{2}S yielded significant hyperpolarization in VSMCs in a dose-dependent manner.\textsuperscript{8} Blocking IK\textsubscript{Ca} and SK\textsubscript{Ca} channels abolished this effect of H\textsubscript{2}S, indicating that endothelium-derived H\textsubscript{2}S hyperpolarizes endothelial cells via activation of these channels. Subsequently, hyperpolarization in adjacent VSMCs is induced.\textsuperscript{8}

Although studies are contradictory, large-conductance calcium-activated K⁺ channels (BK\textsubscript{Ca} channels) also seem to be involved in H\textsubscript{2}S signaling. H\textsubscript{2}S inhibits BK\textsubscript{Ca} channels in nonvascular cell types, whereas H\textsubscript{2}S-induced vasodilation of rat aortic rings is not affected by blocking BK\textsubscript{Ca} channels.\textsuperscript{1,9} By blocking BK\textsubscript{Ca} channels in rat mesenteric arteries, H\textsubscript{2}S-induced vasorelaxation is inhibited.\textsuperscript{10} Recently, the same group discovered that H\textsubscript{2}S is able to increase intracellular calcium sparks in VSMCs.\textsuperscript{11} Calcium sparks occur in a spatially and temporally manner when calcium is released from the endoplasmatic reticulum via RyR channels.\textsuperscript{12} After endothelial cell disruption, or inhibition of BK\textsubscript{Ca} or RyR channels, the effect of H\textsubscript{2}S to increase calcium sparks and induce vasorelaxation is impaired. This indicates that H\textsubscript{2}S increases calcium sparks in VSMCs via BK\textsubscript{Ca} channels in the endothelium. Within VSMCs, calcium-sparks activate BK\textsubscript{Ca} channels, which induce vasorelaxation (figure 1A).\textsuperscript{11} This mechanism is another way that endothelium-derived H\textsubscript{2}S contributes to VSMC hyperpolarization and vasodilation.

Finally, H\textsubscript{2}S is able to induce vasorelaxation via stimulation of K\textsubscript{v7} voltage-gated K⁺ channels in VSMCs, which are important in stabilizing the membrane potential. Ex vivo the K\textsubscript{v7} channel blocker XE991 can block H\textsubscript{2}S-mediated dilation of both rodent aorta and mesenteric artery.\textsuperscript{13} More recently, it was confirmed that K\textsubscript{v7} channels are pharmacological targets for H\textsubscript{2}S. In particular, the K\textsubscript{v7.4} subunit of this channel is involved in the vasorelaxing effect of H\textsubscript{2}S (figure 1A).\textsuperscript{14}
Figure 1 - Schematic illustration of mechanisms by which H\textsubscript{2}S induces vasorelaxation (A) and angiogenesis (B).

Arrow-headed lines represent activation, bar-headed lines inhibition and dashed lines diffusion from one cell type to another. Abbreviations: H\textsubscript{2}S, hydrogen sulfide; CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; 3-MST, 3-mercaptoppyruvate sulfurtransferase; NO, nitric oxide; eNOS, endothelial NO-syntase; SKca, small-conductance calcium-dependent potassium-channels; IK\textsubscript{ca}, intermediate conductance calcium-dependent potassium-channels; BK\textsubscript{ca}, large conductance calcium-dependent potassium-channels; EC, endothelial cell; GC, guanyl cyclase; cGMP, cyclic guanosine monophosphate; PDE, phosphodiesterase; ER, endoplasmic reticulum; K\textsubscript{v7}, voltage-gated potassium-channel; K\textsubscript{ATP}, potassium ATP-channels; MAPK, mitogen-activated protein kinase; SPRC, S-propargyl-cysteine; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; PLC, phospholipase C; InsP\textsubscript{3}, inositol trisphosphate.
Signaling pathways involved in the pro-angiogenic actions of hydrogen sulfide

H₂S is also a profound pro-angiogenic agent. Angiogenesis is a process recognized by the sprouting of new blood vessels from preexisting blood vessels. VEGF is an important mediator of angiogenesis and the majority of its biological effects are mediated by the vascular endothelial growth factor receptor 2 (VEGFR2). The ability of H₂S or H₂S-releasing compounds to increase VEGF production was recently confirmed by several groups. We demonstrated that exogenous administration of H₂S increased both circulating and local (kidney) VEGF production and ameliorates hypertension and proteinuria in a rat model of ‘antiangiogenic state’. Agents that release H₂S or modulate endogenous H₂S, such as diallyl trisulfide (derived from garlic), SG-1002, and S-propargyl-cysteine, increase local VEGF production in animal models for ischemia. The mechanisms via which H₂S influences the VEGF-signaling pathway, and consequently exerts its pro-angiogenic effect, are not completely understood. In 2009, it was suggested that H₂S induces angiogenesis by stimulating KATP channels in endothelial cells, which in turn activate MAPK pathways, inducing angiogenesis. Since then, additional mechanisms have been explored, as schematically illustrated in figure 1B.

An interesting interaction between H₂S and VEGFR2 is reported by Tao et al. who found that H₂S, in its anion form HS⁻, is able to break the disulfide (S – S) bond between Cys1045 and Cys1024 within the VEGFR2. When a mutant form of VEGFR2 is used, which is not able to form the S – S bond, H₂S is not able to activate the receptor and induce endothelial cell migration. Moreover, H₂S is able to break the S – S bond and induce endothelial cell migration in the absence of VEGF protein, confirming a direct activation of the receptor by H₂S. Interestingly, another group showed that H₂S-induced vasodilation is impaired after blocking the VEGFR2 ex vivo. This effect is in line with the fact that H₂S is able to directly activate the VEGFR2. However, a positive feedback mechanism in which the VEGFR2 stimulates H₂S production via CSE activation is also described. Subsequently, increased H₂S in cultured endothelial cells stimulates PLC to produce InsP3, leading to a release of intraluminal calcium and the induction of angiogenesis (figure 1B).

H₂S also promotes angiogenesis via (indirect) activation of the transcription factor STAT3, which in turn activates the VEGF promoter in the endothelial cell nucleus (figure 1B). This mechanism was demonstrated using exogenous S-propargyl-cysteine, a
compound that modulates endogenous H$_2$S by inducing CSE activity. In both *in vitro* and *in vivo* settings, S-propargyl-cysteine promotes angiogenesis by increasing H$_2$S, S-propargyl-cysteine induced phosphorylation of the transcription factor STAT3, a potential target of angiogenesis-mediated therapy. A direct effect of H$_2$S on STAT3 was excluded, but the authors found evidence that increased H$_2$S production activates the VEGFR2 that enables STAT3 to translocate to the nucleus wherein it activates downstream promoters such as the VEGF promoter (figure 1B).

Finally, apart from up regulation of VEGF expression, data suggest that H$_2$S is able to release VEGF from cells, as demonstrated in cultured podocytes. The study did not unravel the mechanism through which H$_2$S releases VEGF, but there are several possibilities. The ability of H$_2$S to influence matrix metalloproteinases, which are known to modulate VEGF release from the inside of the cell, could be a possible mechanism. Although, studies showing the effect of H$_2$S on matrix metalloproteinase-2 and matrix metalloproteinase-9 expression are conflicting. It is unknown whether H$_2$S is able to release VEGF from other cell types such as endothelial cells or VSMCs (figure 1B).

The crosstalk between hydrogen sulfide and nitric oxide

H$_2$S shares structure and functions with the gasotransmitter nitric oxide, the most potent endothelium-derived relaxing factor in the vasculature. By stimulating soluble guanylyl cyclase in VSMCs, leading to increased cyclic GMP production, it subsequently induces vasorelaxation. H$_2$S is able to interact with the nitric oxide-signaling pathway in several ways. For example, H$_2$S promotes vasorelaxation and angiogenesis by inhibiting phosphodiesterase, an enzyme breaking down cyclic GMP. Furthermore, H$_2$S increases nitric oxide synthase (NOS) expression in endothelial cells and stimulates the reduction from nitrite to nitric oxide in ischemic tissue. Conversely, nitric oxide is able to regulate endogenous H$_2$S production by increasing both CSE activity and expression. It was reported that H$_2$S and nitric oxide are mutually dependent in controlling vascular function. However, recently it was shown *in vitro* that in addition to a nitric oxide-dependent mechanism, H$_2$S also exerts pro-angiogenic functions in a nitric oxide-independent manner. Moreover, it was shown that depletion of H$_2$S in the kidney resulted in a reduction of nitric oxide metabolites but this was not true the other
way around; reduction of $\text{H}_2\text{S}$ was not observed after nitric oxide depletion.\textsuperscript{29} Therefore, a mutual dependency between the two gases appears at least to be absent in renal tissue.

One of the actions involved in the crosstalk between nitric oxide and $\text{H}_2\text{S}$ is phosphorylation of endothelial nitric oxide synthase (eNOS) by $\text{H}_2\text{S}$.\textsuperscript{26} This finding was confirmed \textit{in vitro} by Altaany et al.\textsuperscript{28} showing that $\text{H}_2\text{S}$ is able to increase eNOS phosphorylation via phosphorylation of p38 and Akt and subsequently increasing nitric oxide production. An interesting \textit{in vivo} study in 2014 evaluated this mechanism as well. Authors observed increased phosphorylation at the inhibitory site (eNOST495) and decreased phosphorylation at the activating site (eNOST1177) in CSE knockout mice. These alterations in eNOS phosphorylation were accompanied by reduced nitric oxide bioavailability.\textsuperscript{30}

### Therapeutic studies of hydrogen sulfide in vascular disorders

Although $\text{H}_2\text{S}$ has been implicated in several vascular disorders, we will focus in this section on the therapeutic studies in hypertension, (ischemic) kidney injury and preeclampsia.

#### Hypertension

$\text{H}_2\text{S}$ is involved in the regulation of blood pressure. Exogenous (slow-) releasers of $\text{H}_2\text{S}$ such as sodium hydrosulfate (NaHS) and GYY4137 have effectively decreased blood pressure in several animal models for disease.\textsuperscript{3,31,32} However, until now, no effective $\text{H}_2\text{S}$-based therapies are available in the clinic. Intensive research is in progress concerning various compounds that release $\text{H}_2\text{S}$ or increase its endogenous production (table 1).

An interesting rediscovered compound is thiosulfate, a major $\text{H}_2\text{S}$ metabolite. There is evidence that thiosulfate can be an endogenous source for $\text{H}_2\text{S}$ signaling.\textsuperscript{33} In the clinical setting, sodium thiosulfate is used as a therapy for calciphylaxis. Snijder et al.\textsuperscript{34} were the first to show that sodium thiosulfate can act as a therapeutic agent in an angiotensin II-induced model for hypertension and associated hypertensive heart disease.

In the past 2 years, Martelli et al.\textsuperscript{35} evaluated the $\text{H}_2\text{S}$-releasing properties and vascular effects of arythioamides and aryl isothiocyanates. They found that the arythioamide p-hydroxybenzothioamide slowly released $\text{H}_2\text{S}$ in the presence of endogenous biomolecules such as L-cysteine. Furthermore, the arythioamide inhibited
vasoconstriction of rat aortic rings ex vivo, induced hyperpolarization in human VSMCs and lowered SBP in normotensive rats.\textsuperscript{35} The same group showed that isothiocyanates also have vasorelaxing effects ex vivo, which is mediated via K\textsubscript{V7} channels.\textsuperscript{36}

Inhibitors of the angiotensin-converting enzyme (ACE) are widely used as antihypertensive drugs. It was observed that zofenopril had extra beneficial effects in patients compared with other ACE inhibitors. The beneficial effect can probably be explained by the potential of zofenopril to release H\textsubscript{2}S from its sulfhydryl moiety. Indeed, it was found that zofenopril had additional vascular effects, which were independently from ACE-inhibition but related to H\textsubscript{2}S release.\textsuperscript{37}

Ischemia–reperfusion and other kidney diseases
Several studies have been published on the direct renal protective effect of H\textsubscript{2}S, possibly independent of blood pressure (table 1). At first, the antioxidant role of endogenous H\textsubscript{2}S derived from CSE was investigated in renal ischemia-reperfusion injury. CSE knockout mice suffered from increased kidney damage and mortality after renal ischemia compared with wild types, an effect that was rescued by H\textsubscript{2}S (administered as NaHS). Both in vivo and in vitro data revealed the antioxidant activity of H\textsubscript{2}S and its cytoprotective effect through anti-necrotic mechanisms.\textsuperscript{38} Furthermore, the compound D-cysteine was found to have a protective role in a mouse model with renal ischemia-reperfusion injury.\textsuperscript{39} Via 3MST and D-amino acid oxidase, H\textsubscript{2}S can be produced from D-cysteine. In the kidney, D-cysteine seems to be an important source of H\textsubscript{2}S. Interestingly, it was found that exogenous D-cysteine increased the renal levels of the sulfane sulphur pool, which is the major physiologically H\textsubscript{2}S pool. Other renal protective effects of D-cysteine, such as ameliorating renal fibrosis and glomerulosclerosis, have not been investigated so far.

In a model of severe kidney damage caused by chronic infusion of angiotensin II, both H\textsubscript{2}S (administered as NaHS) and sodium thiosulfate attenuated hypertension, proteinuria, tubular damage and renal fibroses.\textsuperscript{40} In another hypertensive rat model, (sFlt1-overexpression induced by adenovirus); exogenous H\textsubscript{2}S (using NaHS) had therapeutic effects as well.\textsuperscript{19} Simultaneously to lowering blood pressure in this model, H\textsubscript{2}S reduced proteinuria and kidney damage. Indeed, the blood pressure lowering effects may be the primary mechanism of H\textsubscript{2}S to protect the kidney. However, we also showed that H\textsubscript{2}S therapy increased the expression of VEGF mRNA in the kidney.\textsuperscript{19} As reduced local production of renal VEGF induces massive proteinuria and kidney damage,\textsuperscript{41} H\textsubscript{2}S might also provide blood pressure-independent renal protection.
### Table 1 - Recent advances in H₂S-based therapeutic compounds in (animal models for) vascular disease

<table>
<thead>
<tr>
<th>Condition</th>
<th>Compound</th>
<th>Animal Models</th>
<th>Clinical Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>Sodium Thiosulfate (STS)</td>
<td>Lowers blood pressure in a rat model for angiotensin induced hypertension</td>
<td>Used for calyphyaxis</td>
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<tr>
<td></td>
<td>Aryl Isothiocyanates</td>
<td>Inhibits ex vivo vasocontriction via Kv7-channels</td>
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<tr>
<td></td>
<td>Arylthioamides</td>
<td>Inhibits ex vivo vasocontriction, and lowers blood pressure in spontaneous hypertensive rats</td>
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<tr>
<td></td>
<td>Zofenopril</td>
<td>Lowers blood pressure in spontaneous hypertensive rats by releasing H₂S and independent of ACE inhibition</td>
<td>Used as ACE-inhibitor</td>
</tr>
<tr>
<td>(Ischemic) kidney disease</td>
<td>Sodium Hydrosulfate (NaHS)</td>
<td>Exerts anti-oxidant activity and has cytoprotective effect through anti-necrotic mechanisms and protects against mortality in a mice model for renal ischemia-reperfusion injury</td>
<td></td>
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<tr>
<td></td>
<td>D-Cysteine</td>
<td>Increases renal levels of the sulfate sulphur pool and protects against ischemia-reperfusion injury in mice</td>
<td></td>
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<tr>
<td></td>
<td>Sodium Thiosulfate</td>
<td>Protects against kidney damage and proteinuria in a rat model for angiotensin induced hypertension</td>
<td>Used for calyphyaxis</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>Sodium Hydrosulfate (NaHS)</td>
<td>Lowers blood pressure, proteinuria and circulating sFlt1 levels in a rat model with sFlt1-overexpression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GYY4137</td>
<td>Inhibits circulating sFlt1 and soluble endoglin levels in a mice model with a preeclamptic phenotype</td>
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</tbody>
</table>

ACE, angiotensin-converting enzyme; H₂S, hydrogen sulfide; NaHS, sodium hydrosulfate; sFlt1, soluble FMS-like tyrosine kinase-1.
Preeclampsia

High circulating levels of sFlt1 are related to maternal symptoms of the gestational disease preeclampsia; a severe syndrome that is characterized by both hypertension and de novo proteinuria in the second half of pregnancy. Placentae from pre-eclamptic patients show down regulation of both CBS and CSE and circulating plasma H₂S is impaired in patients suffering from preeclampsia. It is not surprising that H₂S-related therapy is emerging in this field as well (table 1).

Within cultured human endothelial cells, knock down of CSE expression by silencing RNA resulted in increased sFlt1 and soluble endoglin (sEng), the latter is another antiangiogenic factor related to preeclampsia. Overexpression of CSE in the same cells with an adenovirus resulted in decreased sFlt1 and sEng. Furthermore, in an assay wherein in vitro tube formation is inhibited by both recombinant sFlt1 and preeclamptic serum, H₂S is able to increase tube formation. In vivo, the effect of H₂S on angiogenic factors was also observed. After treatment with NaHS, free plasma sFlt1 levels were obviously reduced, whereas levels of free VEGF were increased. Apparently, H₂S is able to shift the angiogenic balance from antiangiogenic to pro-angiogenic. Furthermore, inhibiting CSE expression by using DL-propargylglycine (PAG) in pregnant mice induced a preeclampsia-like phenotype. After treatment with the slow H₂S-releaser GYY4137, the mice were rescued from the preeclampsia-like phenotype. These two studies imply that H₂S can act as a therapeutic agent in preeclampsia.

Conclusion

H₂S is an endogenous gas with important physiological functions. Within the vasculature, main functions of H₂S are vasodilation and promoting new vessel growth. Based on the discussed in vitro and in vivo rodent data, H₂S is aligned to have therapeutic potential in diseases such as hypertension, renal ischemia-reperfusion disorders and hypertension. Therefore, several compounds such as the H₂S-donor NaHS, synthetic slow releasers such as aryl isothiocyanates, arylthioamides and GYY4137, and also clinical available compounds such STS and zofenopril, are in the running to become H₂S-based therapies for vascular disease.
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Conflicts of interest

S.A.K. is a coinventor on patents related to angiogenic biomarkers, has financial interest in Aggamin Therapeutics, is a consultant to Siemens Diagnostics, and receives research funding from Thermofisher.
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