Hydrogen sulfide

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CHAPTER 1

INTRODUCTION

Historically, hydrogen sulfide (H_2S) has been perceived as a highly toxic gas with the distinctive smell of rotten eggs (Table 1). Recently, this pungent gas was discovered to be synthesized enzymatically in mammalian and human tissues,[1,2] which positively transformed the apparent image of H_2S into a promising therapeutic compound. In a relatively small period of time, H_2S gained considerable interest and the amount of research on this subject has immensely expanded. Over these past years, many (patho-) physiological functions of H_2S have been discovered.[2] Like nitric oxide (NO) and carbon monoxide (CO), H_2S fulfills all of the criteria to be considered a gasotransmitter. Its functions range from modulation of oxidative stress, vasodilatation as an endothelial derived hyperpolarizing factor to signaling through posttranslational protein modification.[3-6] Beside this wide range of uncovered functions, there are still many effects of H_2S that have not been elucidated yet. However, it is clear that H_2S is an important mediator in cellular physiology.

ENDOGENOUS PRODUCTION AND FUNCTION

H_2S is generated in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway only accounts for a small portion of H_2S production. Among the enzymes involved in the production of H_2S, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) have been investigated most extensively. Both enzymes produce H_2S by using pyridoxal 5'-phosphate (vitamin B6) as a cofactor. CBS converts homocysteine into cystathionine, which is then converted to L-cysteine by CSE. Both enzymes transform L-cysteine to H_2S (Figure 1).[8,9] H_2S is also synthesized from D-cysteine by tandem enzymes cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Figure 1).[10] H_2S is then serially oxidized to persulfide, thiosulfate, sulfite and sulfate (Figure 1). CBS and CSE are

Table 1 – Overview of the known toxic effects of H_2S

Dose (parts per million)	Effect
0.02 ppm - 0.03 ppm	Detectable odor
5 ppm - 10 ppm	Offensive, unpleasant odor
20 ppm	Maximum allowable concentration for daily 8 hour exposure
50 ppm	Irritation of the ocular conjunctiva
100 ppm	Loss of smell / olfactory nerve paralysis
100 ppm - 200 ppm	Upper respiratory tract irritation
300 ppm - 500 ppm	Pulmonary edema
500 ppm	Headache, dizziness, unconsciousness after 30-60 minutes of exposure
500 ppm - 700 ppm	Unconsciousness, respiratory paralysis eventually leading to death
1000 ppm	Rapid collapse, death within minutes

Adapted from Beauchamp et al.[7]

present in a variety of tissues such as blood vessels, heart, liver, kidneys and the brain, whereas MPST expression has been mainly demonstrated in the vascular endothelium. It has been widely suggested that the expression of CSE, CBS, 3-MST (and CAT) in rodents and humans shows a marked degree of tissue specificity. However, as more researchers investigate the emerging role of H_2S in their particular disease model, this simple though convenient distinction is no longer as clear as once thought.

The majority of evidence for a physiological role of H_2S has been obtained from studies in vascular tissue. These studies convincingly show that H_2S is a vasodilatory intermediate. Genetic knockout of CSE in mice leads to an age-dependent increase in blood pressure. This hypertensive phenotype is likely due to the lack of endogenous H_2S, since injection of H_2S rescues these mice from an increasing blood pressure.[5] While homozygous CBS knockout mice are lethal during the embryonic phase, heterozygous variants similarly develop an elevated blood pressure.[11] Mechanisms thought to be responsible for the vasodilatory effects of H_2S include the activation of ATP-sensitive potassium channels (K_{ATP} channels) in vascular smooth muscle cells through direct cysteine-S-sulfhydration.[4] Furthermore, it is thought that the vasodilatory effects of H_2S are partially NO dependent. Inhibition of NO using L-NG-nitroargininemethyl ester (L-NAME) decreased the potency of H_2S in aortic rings,[12] these findings suggest crosstalk between gasotransmitters is involved in the regulation of blood pressure. Last, it has been shown that H_2S is able to prevent renovascular hypertension by affecting the renin-angiotensin-aldosterone system through direct inhibition of renin and angiotensin-converting enzyme activity.[13]

Another important feature of H_2S is its ability to reduce and modulate oxidative stress by directly scavenging reactive oxygen species (ROS), increasing the formation of the intracellular antioxidant glutathione and activating the anti-oxidative transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2).[14-16] An additional strategy in reducing the amount of oxidative stress is through modulation of mitochondrial ROS production[6]. It has become apparent that H_2S is able to protect the integrity of mitochondria and supports mitochondrial function, thereby contributing to the preservation of cellular energetics.[17,18] H_2S can be used as an inorganic energy substrate for mitochondria.[19] Under stress, such as increased intracellular calcium concentrations, H_2S producing enzymes can translocate to the mitochondria.[18,20,21] This translocation might increase mitochondrial H_2S levels to be used as an electron donor in the electron transport chain.

Furthermore, H_2S seems to regulate inflammatory responses. In literature, both pro- and anti-inflammatory effects of H_2S have been reported. These discrepancies may reflect the varying effects of dose–response relationships. At low, physiological concentrations, H_2S is predominantly anti-inflammatory, whereas at high concentrations H_2S promotes inflammation, a pattern that is also observed for NO and CO.[1,22] The mechanisms by which H_2S exerts anti-inflammatory effects are the inhibition of pro-inflammatory transcription factors like nuclear factor kappa B and the inhibition of pro-inflammatory enzyme activity iNOS, cyclooxygenase-2 and tumor necrosis factor-α converting enzyme.[23]

Also, H_2S is essential for vascular endothelial growth factor (VEGF) mediated angiogenesis. VEGF-stimulated in vitro sprouting is reduced in blood vessels after CSE silencing, whereas it is enhanced in vessels overexpressing CSE. The finding that genetic knockout of CSE in mice causes impaired wound healing, further indicates that endogenous H_2S production by CSE is a dynamic regulator of angiogenesis.[24,25] Exogenous treatment with H_2S improves wound healing in rats, and attenuates heart failure by inducing angiogenesis via an increase in VEGF en eNOS/NO.[24,26]

The capacity of H_2S to exert intracellular signals by sulfhydrating target proteins, a form of posttranslational modification, is particularly striking.[27] During this process a SH-group is added to a reactive cysteine residue and thereby the activity of the target protein can be altered. Sulfhydration by H_2S seems to be more widespread than nitrosylation, and the prevalence and ratio of sulfhydration is perhaps comparable to that of phosphorylation. This is a field still in its infancy, but it seems that it can become a meaningful aspect of intracellular signaling pathways. Table 2 gives an overview of the proposed mechanisms of action of H_2S.

EXOGENOUS ADMINISTRATION

The application of H_2S in biomedical experiments is challenging with regard to its chemical properties. The vast majority of studies that have examined the potential role of H_2S in health and disease have invariably utilized commercially available sulfide salts such as sodium sulfide (Na_2S) and sodium hydrosulfide (NaSH). Over the course of the years, it has become clear that these sodium salts have disadvantages, such as the rapid peak in H_2S levels and short half-life when dissolved or injected. This makes it hard to monitor actual H_2S levels and achieve controlled, stable and therapeutic levels of this gas in vitro and in vivo. Interestingly, H_2S donors with a regulated release have been developed recently.[28] With these slow-release H_2S donor molecules, such as GYY4137, it is possible to expose animals, tissue and cells to H_2S generated in a more physiological manner.[29]

Another interesting approach is the use of thiosulfate (TS), an intermediate of sulfur metabolism from cysteine and a metabolite of H_2S that can also produce H_2S through the action of thiosulfate reductase.[30,31] Since TS seems to have similar protective properties as other H_2S donors, this provides us with exciting possibilities for the translation into clinical use. While short-term treatment with STS is well tolerated in humans for the treatment of calciphylaxis,[32] the long-term side effects should be further explored.

Gaseous administration of H_2S is less frequently used than treatment with sulfide salts or other H_2S donor compounds, but has a very interesting accessory feature. In high, sub-toxic concentrations (80-100 ppm) gaseous H_2S can induce a reversible hibernation-like state in mice. During treatment, oxygen consumption and carbon dioxide production can be reduced by 90-95% within minutes. Also, the core body temperature decreases to around 2°C above ambient temperature. After cessation of H_2S exposure, this massive reduction in oxidative metabolism is reversible without

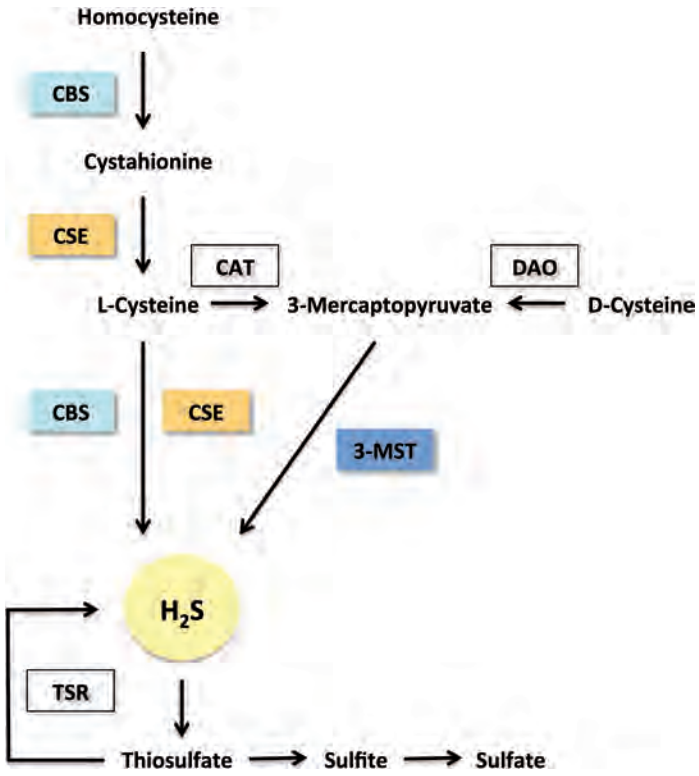

Figure 1 – Overview of endogenous H_2S production. Abbreviations: CSE – cystathionine γ-lyase; CBS – cystathionine – β-synthase (CBS); CAT – cysteine aminotransferase; DAO – D-amino acid oxidase; 3-MST – 3-mercaptopyruvate sulfurtransferase; TSR – thiosulfate reductase

apparent toxic effects.[33,34] This brilliant discovery led to the finding that H_2S-induced hypometabolism is protective during lethal hypoxia possibly by buffering oxygen consumption, altering the redox environment within cells, and preventing a lethal imbalance between energy supply and demand.[35] The hypothesized mechanism behind H_2S-induced hypometabolism is the reversible inhibition of cytochrome c oxidase (COX), the terminal enzyme in the mitochondrial electron transport chain.[36] This capacity of H_2S makes it an extremely interesting protective agent in ischemia-reperfusion related damage where oxygen demand exceeds oxygen availability.

ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion injury (IRI) is one of the major damaging processes responsible for a worsened outcome after organ transplantation. IRI is implicated in other processes than organ transplantation, such as myocardial infarction or surgical procedures like cardiopulmonary bypass. One of the strategies to reduce IRI-related damage is cooling, thereby reducing metabolism and enzymatic activity. However, cooling does

Table 2 – Proposed mechanisms for protective effects of H_2S

Increase in GSH levels
Reduction of mitochondrial superoxide production
Activation of ROS-scavengers (e.g. MnSOD, CuZnSOD)
Preservation of mitochondrial integrity
Acting as an electron donor in the electron transport chain
Modulation of mitochondrial activity / Hypometabolism
Induction of hypothermia
Modulation of vascular proliferation
Modulation of leukocyte adhesion / rolling
Modulation of cytokine production
Activation of KATP-channels
Activation of calcium activated K+ channels
Modulation of protein activity through sulfhydration
Modulation of platelet function
Modulation of hypoxia inducible factor (HIF)
Modulation of Nrf2 signaling
Increase in eNOS / iNOS / HO-1
Increase in HSP90
Modulation of COX-2 activity
Modulation of GSK3beta activity
Modulation of apoptotic signaling
Modulation of autophagy

have detrimental effects as well. Using H_2S to reduce oxidative metabolism might be a promising strategy to circumvent these detrimental effects. In addition, H_2S is also considered protective during other processes critically involved in IRI, such as oxidation, inflammation and apoptosis. Changes in renal CSE and CBS expression and/or activity during ischemia and in the early phase of reperfusion suggest a role for H_2S production in this process.[6,37] Furthermore, CBS and CSE both are involved in the response to oxidative stress. CSE deficient mice are more susceptible to ischemic damage,[6] and overexpression of CSE in cardiac tissue protects from myocardial infarction.[38] The protective effect of H_2S administration against IRI has been shown in various organs, including heart,[39] kidney,[33] liver[40] and brain.[41] In these experimental studies NaSH is often used as a H_2S-donating compound. So far, the exogenous administration, endogenous manipulation and use of genetically modified animals have been successful in demonstrating H_2S-mediated cytoprotection in models of IRI.

HYPERTENSION

H_2S is an important mediator in the vascular system, contributing to vascular regulation and protection against vascular injury that leads to vascular dysfunction. The vasodilatory effects of H_2S were first reported when administration of H_2S donors to anaesthetized rats was found to induce a transient hypotensive effect.[42] Endogenous H_2S shortage is suggested to be involved in the pathogenesis of hypertension and a significant down regulation of the H_2S/CSE system has been reported in several hypertensive models. Spontaneously hypertensive rats (SHR) have been widely used as a model for human essential hypertension because of the similarity of the main cardiovascular characteristics. The CSE – L-cysteine pathway is downregulated in SHR and treating them with a H_2S donor is protective, reducing blood pressure and vascular remodeling.[43] The most compelling evidence for the importance of H_2S in blood pressure regulation is that mice deficient in CSE develop endothelial dysfunction and hypertension within 8 weeks of birth and that H_2S replacement decreases systolic blood pressure in both $CSE^{-/-}$ and $CSE^{+/-}$ mice.[5] H_2S is also reported to have preventive and therapeutic effects on renovascular hypertension by inhibiting plasma renin activity.[13] Because H_2S is a major endothelium derived hyperpolarizing factor and a primary determinant of vasorelaxation in numerous vascular beds, drugs that alter CSE activity or H_2S-mediated channel sulfhydration may be effective therapeutic agents for treating hypertension.

AGING

Aging has been a topic of great interest to the scientific community for a long time. This biological process is characterized by time-dependent progressive decline of physiological functions accompanied by increased incidence of age-associated diseases. One of the proposed theories to explain the process of aging is that aging and age-related diseases are caused by the deleterious consequences of free radicals exposure on cell constituents and connective tissues.[44] Substantial evidence shows that H_2S is involved in aging by inhibiting free-radical reactions, activating SIRT1, and probably interacting with the age-related gene *Klotho*. One study reported that the plasma H_2S level in humans over 50 to 80 years of age declines with age.[45] In Drosophila, CBS is crucial for the increased lifespan linked to dietary restriction. When exposed to H_2S, nematodes are apparently healthy and do not exhibit phenotypes consistent with metabolic inhibition. Instead, animals exposed to H_2S are long-lived, and this phenotype requires Sir2 activity, which may translate environmental changes into physiological alterations to improve survival.[46] Moreover, H_2S has been shown to have therapeutic potential in age-associated diseases, such as hypertension and several neurodegenerative diseases. Decreased levels of H_2S in brain tissue are associated with neurodegenerative age-related diseases like Parkinson's and administration of H_2S has been shown protective in experimental models for this disease.[47,48] The exact mechanisms of action of the role of H_2S in the pathology of

aging have not yet been sufficiently characterized and await elucidation by further studies. A better understanding of the roles of H_2S in aging can provide insights into potential therapeutic interventions against aging and reduce age-associated diseases.

AIM OF THE THESIS

The aim of this thesis was to investigate the therapeutic potential of H_2S and its producing enzymes in the setting of hypertensive renal and heart disease, ischemia and neurodegeneration. Our work underlines the broad implications of H_2S by showing protective effects in several distinct disease models in various organisms.

Since H_2S has been established as the third gasotransmitter alongside NO and CO, many overlapping actions as well as differences have been reported. Crosstalk between the three gasotransmitters has been shown, although the evidence is still scarce. Recently, it has become clear that the vasorelaxant effects of H_2S are partly NO-dependent. In **Chapter 2** an outline of the mechanisms of gas-mediated cytoprotection and complex interactions between gasotransmitters in renal IRI and transplantation is presented. Protective effects of sulfide salts (NaSH and Na_2S) in IRI have been investigated in several organs. Gaseous administration of H_2S has been used only a few times in renal and hepatic IRI. In **Chapter 3** we investigated whether gaseous administration of H_2S is protective in a model for transient myocardial infarction in mice and whether non-hypometabolic concentrations of H_2S have similar protective properties as hypometabolic concentrations. Although the exogenous protective effects of H_2S have been investigated in several IRI models, the role of endogenous H_2S-producing enzymes in IRI has been less researched. **Chapter 4** therefore investigates the antioxidant role of endogenous H_2S production by CSE in ischemic settings in vitro, in vivo and in human renal transplant tissue, using models of CSE deficiency and overexpression. The convincing protective effects of H_2S in IRI and the fact that there are many overlapping underlying damaging processes between IRI and hypertension, led us to investigate its alleged beneficial effects in hypertensive disease. The translation of H_2S to the clinical setting unfortunately is still out of our reach. For that purpose we applied sodium thiosulfate (STS), a novel H_2S-related endogenous substance with clinical potential. STS is an intermediate of sulfur metabolism from cysteine and a metabolite of H_2S that can also release sulfide. In **Chapter 5** and **6** we studied the protective effects of STS in angiotensin II-induced hypertensive renal and cardiac disease. The known role of oxidative stress in aging related neurodegenerative disease instigated a project that led to the creation of CSE overexpressing *Drosophila*. In **Chapter 7** we studied the protective potential of CSE overexpression and STS in a model of spinocerebellar ataxia type 3 in *Drosophila*. In addition, we investigated CSE expression in pontine tissue of SCA3 patients. In **Chapter 8**, all results are summarized and discussed, followed by a view on the future possibilities of H_2S-related research and therapeutic applications.

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