Hydrogen Sulfide (H₂S) and Polysulfide (H₂Sₙ) Signaling: The First 25 Years

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Abstract: Since the first description of hydrogen sulfide (H₂S) as a toxic gas in 1713 by Bernardino Ramazzini, most studies on H₂S have concentrated on its toxicity. In 1989, Warenycia et al. demonstrated the existence of endogenous H₂S in the brain, suggesting that H₂S may have physiological roles. In 1996, we demonstrated that hydrogen sulfide (H₂S) is a potential signaling molecule, which can be produced by cystathionine β-synthase (CBS) to modify neurotransmission in the brain. Subsequently, we showed that H₂S relaxes vascular smooth muscle in synergy with nitric oxide (NO) and that cystathionine γ-lyase (CSE) is another producing enzyme. This study also opened up a new research area of a crosstalk between H₂S and NO. The cytoprotective effect, anti-inflammatory activity, energy formation, and oxygen sensing by H₂S have been subsequently demonstrated. Two additional pathways for the production of H₂S with 3-mercapto pyruvate sulfurtransferase (3MST) from L- and D-cysteine have been identified. We also discovered that hydrogen polysulfides (H₂Sₙ, n ≥ 2) are potential signaling molecules produced by 3MST. H₂Sₙ regulate the activity of ion channels and enzymes, as well as even the growth of tumors. S-Sulfuration (S-sulfhydration) proposed by Snyder is the main mechanism for H₂S/H₂Sₙ underlying regulation of the activity of target proteins. This mini review focuses on the key findings on H₂S/H₂Sₙ signaling during the first 25 years.

Keywords: hydrogen sulfide; polysulfides; S-sulfuration; nitric oxide; hydrogen peroxide; S-nitrosylation; S-sulfenylation; 3MST

1. Identification of H₂S as a Signaling Molecule

Patients that recover from H₂S poisoning show cognitive decline, and the levels of neurotransmitters in the brains of animals exposed to H₂S change, suggesting that the brain is vulnerable to H₂S toxicity [1]. Warenycia et al. measured the levels of H₂S accumulated in the brain of rats exposed to H₂S when they discovered a certain amount of H₂S in the brain even without exposure to H₂S [2]. Although the concentrations were overestimated, the existence of endogenous H₂S was identified in the brain.

Pyridoxal 5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), have been suggested to regulate several pathways. CBS catalyzes the first step of the transsulfuration pathway in which cystathionine is produced from serine and homocysteine, and cystathionine is further catalyzed by CSE to cysteine. An alternate pathway exists in which CBS catalyzes the condensation of cysteine with homocysteine to generate cystathionine and H₂S [3,4]. CSE catalyzes an elimination reaction which metabolizes cysteine to pyruvate, NH₃, and H₂S [3,4]. However, rather than being recognized as a physiologically active molecule, in these early studies, H₂S was merely thought to be a byproduct of the metabolic pathways.

The observations that H₂S is produced by enzymes and exists in the brain prompted us to study a physiological role of this molecule. The activities of CBS and CSE have been intensively studied in the liver and kidney, but little is known about them in the brain. We found CBS in the brain and confirmed the production of H₂S, which is augmented by S-adenosyl methionine (SAM) [3].
Other gaseous signaling molecules NO and carbon monoxide (CO) induce hippocampal long-term potentiation (LTP), a synaptic model of memory formation, as retrograde messengers, which are produced at postsynapse and released to presynapse to facilitate a release of a neurotransmitter glutamate from presynapse [6–10]. We examined whether or not H\textsubscript{2}S has a similar effect. H\textsubscript{2}S facilitated the induction of LTP by enhancing the activity of N-methyl-d-aspartate (NMDA) receptors but not as a retrograde messenger [5].

NMDA receptors are activated by a reducing substance dithiothreitol (DTT) through the reduction of a cysteine disulfide bond located at the hinge of the ligand-binding domain [11]. Because H\textsubscript{2}S is a reducing substance, it is likely to be a mechanism for facilitating the induction of LTP. However, H\textsubscript{2}S with one-tenth of the concentration of DTT exerted a greater effect than that of DTT [5]. This observation suggested that there is an additional mechanism for LTP induction by H\textsubscript{2}S. The prominent neuroscientist Solomon Snyder commented the following in \textit{Science News}: “They have very impressive evidence that H\textsubscript{2}S is a potential neurotransmitter. It is an exciting paper that should stimulate a lot of people’s interest” [12].

The synaptic transmission is regulated not only by events at synapses such as a release of transmitters and the sensitivity of receptors but also by astrocytes, a type of glia, which surround synapses. Astrocytes release gliotransmitters to regulate synaptic activity. We found that H\textsubscript{2}S induces Ca\textsuperscript{2+} influx in astrocytes, which was greatly suppressed by La\textsuperscript{3+}, Gd\textsuperscript{3+}, and ruthenium red, broad-spectrum inhibitors known for transient receptor potential (TRP) channels, suggesting that H\textsubscript{2}S activates TRP channels [13]. H\textsubscript{2}S was reported to activate TRPA1 channels in urinary bladder and in sensory neurons, but concentrations greater than 1 mM were required for inducing responses [14,15].

2. Identification of H\textsubscript{2}S\textsubscript{n} as Signaling Molecules

During this study, we found that a batch of NaHS, i.e., the sodium salt of H\textsubscript{2}S, with yellowish color was much more potent than the colorless batch. We successfully reproduced a solution with a similar color by dissolving elemental sulfur into Na\textsubscript{2}S solution according to a report by Searcy and Lee [16]. The color came from H\textsubscript{2}S\textsubscript{n}, which induces Ca\textsuperscript{2+} influx in astrocytes much more potently than H\textsubscript{2}S [17–19]. H\textsubscript{2}Sn are natural inorganic polymeric sulfur–sulfur species or sulfane sulfur, which we later found to be produced by 3-mercaptoppyruvate sulfurtransferase (3MST) from 3-mercaptopyruvate [20–22] and the partial oxidation of H\textsubscript{2}S [19], such as via the chemical interaction with NO [23,24]. H\textsubscript{2}S\textsubscript{2} (2.6 µM) exists in the brain almost equivalent to the level of H\textsubscript{2}S (3 µM) [25]. Ca\textsuperscript{2+} influx induced in astrocytes by AITC, cinnamaldehyde, selective activators of TRPA1 channels, and Na\textsubscript{2}S\textsubscript{3} was greatly suppressed by HC030031 and AP-18, selective inhibitors of TRPA1 channels. In astrocytes transfected with TRPA1-siRNA, Ca\textsuperscript{2+} influx was not efficiently induced by Na\textsubscript{2}S\textsubscript{3} [19]. The EC\textsubscript{50} value for H\textsubscript{2}S was 116 µM, while that for H\textsubscript{2}S\textsubscript{3} was 91 nM, suggesting that H\textsubscript{2}S\textsubscript{n} rather than H\textsubscript{2}S are ligands for TRPA1 channels [13,17–19]. The amino terminus of TRPA1 channels has 24 cysteine residues [26], and two cysteine residues Cys422 and Cys634 are sensitive to H\textsubscript{2}S\textsubscript{n} [27].

S-Sulfuration (S-sulfuhydration) was proposed by Snyder and colleagues to regulate the activity of target proteins by H\textsubscript{2}S [28]. This proposal needs a minor revision to highlight H\textsubscript{2}S\textsubscript{n} but not H\textsubscript{2}S S-sulfurate cysteine residues. In contrast, H\textsubscript{2}S S-sulfurates oxidized cysteine residues such as those S-nitrosylated and S-sulfenylated [29]. H\textsubscript{2}S\textsubscript{n}, S-sulfurate (S-sulfhydryl) two cysteine residues of TRPA1 channels to induce the conformational changes to activate the channels. As an alternative mechanism, one cysteine residue, which is S-sulfurated, reacts with the remaining cysteine residue to generate a cysteine disulfide bond. Although the conformation has not been examined in detail, the latter mechanism may induce conformational changes more efficiently than the former one.
Various target proteins of $\text{H}_2\text{S}_n$ have been identified such as a tumor suppressor phosphate and tensin homolog (PTEN), protein kinase $\alpha$, and an enzyme responsible for glycolysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [28,30–32]. It has been reported that GAPDH is activated by $\text{H}_2\text{S}$ through $S$-sulfuration of the active site Cys150 [28], while it is suppressed by $\text{H}_2\text{S}_n$ through $S$-sulfuration of Cys156, which is not the active site [32]. Cys150 may be an oxidized residue when $S$-nitrosylated or $S$-sulfenylated, which can be $S$-sulfurated by $\text{H}_2\text{S}$, while Cys156 must be a thiol, which is $S$-sulfurated by $\text{H}_2\text{S}_n$.

3. Synergy and Crosstalk between $\text{H}_2\text{S}$ and NO

$\text{H}_2\text{S}$ relaxes vascular smooth muscle in synergy with NO [33]. A similar result was also obtained in the ileum [34]. Whiteman et al. proposed that the chemical interaction of $\text{H}_2\text{S}$ with NO generate nitrosothiol, which releases NO in the presence of Cu$^{2+}$ [35]. Filipovic et al. reported that $\text{H}_2\text{S}$ and NO produces nitroxyl (HNO) as a major product, as well as $\text{H}_2\text{S}_n$ [36,37], while Cortese-Krott et al. suggested that SSNO$^-$ as a major product with $\text{H}_2\text{S}_n$ as a minor one [38]. We proposed that $\text{H}_2\text{S}_n$ are major products [23]. The effect of $\text{H}_2\text{S}_n$ and that of the products obtained from the mixture of Na$_2$S and diethylamine NONOate, an NO donor, were eliminated when they were exposed to cyanide or DTT [23]. In contrast, HNO is resistant to cyanide, and SSNO$^-$ is resistant to DTT. Based on these observations, $\text{H}_2\text{S}_n$ are potential chemical entities produced from $\text{H}_2\text{S}$ and NO [23,37,38]. Bogdandi et al. recently suggested that $\text{H}_2\text{S}_n$ transiently activate TRPA1 channels at the early phase of the production from $\text{H}_2\text{S}$ and NO, while the more stable product SSNO$^-$ sustainably activates the channels [39].

4. Vascular Tone Regulation by $\text{H}_2\text{S}$ and $\text{H}_2\text{S}_n$

Since $\text{H}_2\text{S}$ relaxes vascular smooth muscle in synergy with NO [33] and activates ATP-dependent $K^+$ ($K_{\text{ATP}}$) channels [40], it has been suggested that $\text{H}_2\text{S}$ is a potential endothelial-derived hyperpolarizing factor (EDHF), which is a component of endothelial-derived relaxing factor (EDRF) [41]. However, previous studies showed that the hyperpolarization induced by EDHF is resistant to glibenclamide, a $K_{\text{ATP}}$ channel blocker [42,43]. The relaxation of vascular smooth muscle in the mesenteric bed, which is mediated predominantly by EDHF, is rather abolished by apamine, a blocker of Ca$^{2+}$-activated $K^+$ channels [44].

$\text{H}_2\text{S}_n$ are potential EDHFs (Figure 1). $\text{H}_2\text{S}_n$ produced by 3MST together with cysteine aminotransferase (CAT), both of which are localized to the vascular endothelium [20,45,46], or $\text{H}_2\text{S}_n$ generated by the chemical interaction between $\text{H}_2\text{S}$ and NO produced by endothelial NO synthase (eNOS) can activate TRPA1 channels [19,23] localized to myoendothelial junctions. The channels induce Ca$^{2+}$ influx, which activate Ca$^{2+}$-activated $K^+$ channels to hyperpolarize the endothelial cell plasma membrane. The change in membrane potential is conducted via myoendothelial gap junctions to hyperpolarize the vascular smooth muscle [47].

$\text{H}_2\text{S}$ has also been demonstrated to relax vascular smooth muscle via the protein kinase G pathway as an endogenous inhibitor of phosphodiesterase and increases the levels of both cyclic GMP and cyclic AMP [48,49], as well as by activating Kv7 potassium channels [50]. Kv7 channels are also involved in CBS-derived $\text{H}_2\text{S}$ induced human malignant hyperthermia syndrome triggered by volatile inhalation anesthetics in skeletal muscle [51].
duction of GSH [56]. H2Sn increase the levels of GSH and protect cells from oxidative stress
mia/reperfusion injury by preserving mitochondrial function [55].

Lefer and colleagues demonstrated that H2S protects the heart from ischemia
via KCNQ1 channels [54]. Kv7 channels are also involved in CBS-derived H2S induced human malignant
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The change in membrane potential is conducted via gap junction to hyperpolarize the smooth muscle plasma membrane.

Figure 1. H2Sn are potential EDHFs. Both 3MST and eNOS are localized to endothelium. H2Sn produced by 3MST or by the chemical interaction between H2S and NO activate TRPA1 channels present in myoendothelial junctions to induce Ca2+ influx, which activates Ca2+-dependent K+ channels. The change in membrane potential is conducted via gap junction to hyperpolarize the smooth muscle plasma membrane.

5. Cytoprotective Effect of H2S, H2Sn, and H2SO3

The impression of H2S as toxic gas led to its cytoprotective effect being overlooked [52]. Expecting that all cells would be killed by H2S, I applied NaHS to cells and incubated for overnight. On the contrary, cells were lively and survived from the toxin. H2S increases the production of glutathione (GSH), a major intracellular antioxidant, by enhancing the activity of cystine/glutamate antiporter, which incorporates cystine into cells, and of glutamate cysteine ligase (GCL), a rate-limiting enzyme for GSH production [52,53]. H2S also facilitates the translocation of GSH into mitochondria [53]. The protective activity of H2S is also exerted through the stabilization of membrane potential by enhancing the activity of KATP channels and cystic fibrosys transmembrane conductance regulator (CFTR) Cl− channels [54]. Lefer and colleagues demonstrated that H2S protects the heart from ischemia/reperfusion injury by preserving mitochondrial function [55].

H2Sn S-sulfurate Keap1 and release Nrf2 from the Keap1/Nrf2 complex to the nucleus, where Nrf2 upregulates antioxidant genes including the GCL gene to increase the production of GSH [56]. H2Sn increase the levels of GSH and protect cells from oxidative stress to a greater level than H2S [57]. Sulfite (H2SO3), a metabolite of H2S and H2Sn, protects neurons by increasing the production of GSH as efficiently as the parental molecules [57].

6. Signaling by H2S, H2Sn through S-Sulfuration and Bound Sulfane Sulfur

In addition to CBS and CSE, 3MST, along with CAT or DAO, was recognized to produce H2S from l- or d-cysteine, respectively [46,58,59]. Subsequently, 3MST was found to produce H2Sn and other S-sulfurated molecules such as cysteine persulfide, GSSH, and S-sulfurated cysteine residues [20,21,60]. Other enzymes such as sulfide-quinone oxidoreductase (SQR), haemoglobin, neuroglobin, catalase, super oxide dismutase (SOD), cysteine tRNA synthetase (CARS), and peroxidases have been identified to produce H2Sn and other S-sulfurated molecules [61–69].

In total, 10–20% of cysteine residues of proteins are S-sulfurated [28], also observed as a part of bound sulfane sulfur, which releases H2S under reducing conditions, including H2Sn, cysteine persulfide, GSSH, and S-sulfurated cysteine residues [70–73].

In cells and
tissues, 5–12% of total protein cysteine residues are oxidized, such as S-nitrosylated (P-CysSNO) and S-sulfenylated (P-CysSOH), and this can be increased to more than 40% under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2). The amount of bound sulfane sulfur and its associated species is distinct among tissues. For example, heart homogenates release H$_2$S under oxidative conditions [74] (Figure 2).

Some cysteine residues are oxidized by H$_2$O$_2$ to generate S-nitrosylated cysteine residues, and some others are S-nitrosylated by NO. These oxidized cysteine residues are S-sulfurated by H$_2$S. In contrast, cysteine residues are S-sulfurated by H$_2$S$_n$.

Figure 2. S-Sulfuration of cysteine residues by H$_2$S and H$_2$S$_n$. Cysteine residues are S-sulfenylated by H$_2$O$_2$ and S-nitrosylated by NO. These oxidized cysteine residues are S-sulfurated by H$_2$S. In contrast, cysteine residues are S-sulfurated by H$_2$S$_n$.

Another role of S-sulfuration is that it enables proteins to recover their functions from over-oxidization. Sulfenic (P-CysSO$_2$H) and sulfonic acids (P-CysSO$_3$H) are not reduced back to P-CysSH by thioredoxin and deteriorate the protein function. In contrast, S-sulfurated proteins P-CysSSO$_2$H and P-CysSSO$_3$H can be reduced by thioredoxin to P-CysSH [75,76].

7. Diseases Caused by the Disturbance of H$_2$S and H$_2$S$_n$

Both an excess and a deficiency of H$_2$S and H$_2$S$_n$ have been suggested in the pathogenesis of schizophrenia. Thiol homeostasis is shifted to oxidized conditions, reflecting significantly less H$_2$S and more disulfide bond formation in patients than normal individuals [77,78]. In contrast, we suggested that excess H$_2$S and H$_2$S$_n$ are involved in the pathogenesis. Mice with high expression of 3MST impaired prepulse inhibition, an endophenotype for schizophrenia, and 3MST levels were positively correlated with symptom severity scores [79].

CBS and H$_2$S may be involved in regulating proliferation and bioenergetics in breast cancer, ovarian cancer, and colorectal cancer [80–82], and high levels of CBS, CSE, and 3MST expression were observed in lung cancer [83].
Gliomas with the highest grades of malignancy contained greater levels of polysulfides than glioma-free brain regions [84], and H$_2$S$_n$ levels were greater in glioblastoma-bearing regions than glioblastoma-free control regions [85]. In contrast, it was reported that CBS is involved in suppressing glioma, whereby glioma with suppressed CBS had high levels of VEGF and HIF-2α and was deeply invaded with dense vascularization and aggressive growth [86].

Parkinson’s disease is a neurodegenerative disorder. Parkin, an E3 ubiquitin ligase responsible for the clearance of misfolded proteins, is suppressed in this disease. Specific cysteine residues of parkin are S-nitrosylated in patients, while they are S-sulfurated in the normal individuals [87]. H$_2$S may be involved in S-sulfurating the S-nitrosylated cysteine residues of parkin.

Down’s syndrome (DS) is characterized by impaired brain growth and maturation that causes mental retardation and is associated with an Alzheimer’s type of dementia in elderly adults. DS involves a trisomy of chromosome 21 where CBS is encoded, and its mRNA level is 12 times greater in myeloblasts of DS children, while CBS protein levels in the brains of patients are approximately three times greater compared to the normal individuals [88,89]. Higher levels of thiosulfate, a metabolite of H$_2$S, were detected in patients [90]. Mice with CBS overexpression showed DS-like neurocognitive deficits [91]. Fibroblasts prepared from DS patients showed profound suppression of mitochondrial electron transport, oxygen consumption, and ATP generation [92].

Ethylmalonyl encephalopathy is an autosomal recessive early-onset disorder, defective in cytochrome c oxidase in the brain and muscle. In this disease, ETHE1, a gene encoding sulfur dioxygenase, which metabolizes H$_2$S in collaboration with SQR, is deficient, and H$_2$S levels are increased to suppress cytochrome c oxidase [93]. High levels of H$_2$S and persulfides also suppress acyl-protein thioesterase in the mouse model of this disease [94,95].

The brain is very sensitive to oxygen deprivation, i.e., hypoxia. During hypoxia, heme oxygenase-2 produces less carbon monoxide, which suppresses the activity of CBS, resulting in the overproduction of H$_2$S that stimulates the carotid body to increase in respiratory rate, heart rate, and blood pressure [96]. On the other hand, the increased levels of H$_2$S, in turn, suppress cytochrome c oxidase to cause hypoxic brain injury [97]. The levels are augmented in SQR-deficient animal models [97].

8. Perspective

Cysteine residues of the target proteins initially found, such as TRPA1 channels, PTEN, and protein kinase G1α, may be thiols whose sulfur has the oxidation state of −2, and that of H$_2$S$_n$ has the oxidation state of −1 or 0 and is able to S-sulfurate thiols to regulate the activity of the targets. On the basis of these observations, S-sulfurated molecules including H$_2$S$_n$ have been recognized as the chemical entities as signaling molecules rather than H$_2$S, which is thought to be a mere precursor of H$_2$S$_n$ or a byproduct of other S-sulfurated molecules. However, depending on the redox conditions of cysteine residues of target proteins, both H$_2$S and S-sulfurated molecules including H$_2$S$_n$ can S-sulfurate the targets. Considering that the endogenous levels of H$_2$S (approximately 3 µM in the brain) and H$_2$S$_n$ (2.6 µM) are well balanced [25] and that the reaction between these signaling molecules and targets is fast [21], both H$_2$S and H$_2$S$_n$ may react with their corresponding targets at a similar frequency. Identifying the redox conditions of target cysteine residues may help understand the functions of H$_2$S or H$_2$S$_n$ as signaling molecules for specific targets.

High concentrations of H$_2$S are toxic, while low concentrations are beneficial. Examples of the former can be observed in Down’s syndrome, ethylmalonyl encephalopathy, and hypoxic brain injury, while examples of the latter can be observed in Parkinson’s disease and Huntington’s disease [87,92,93,96–98]. Similarly, H$_2$S exerts opposite effects depending on its concentration as observed in the effect of acetylcholine on the vascular smooth muscle, where low concentrations exert relaxation, while high concentrations exert contraction. Acetylcholine was previously known to contract vasculature, before Furchgott
and Zawadzki discovered that lower concentrations of acetylcholine relaxed vasculature where endothelial cells were intact to release EDRF (NO) [99]. Recently, Vellecco et al. identified that vascular contraction by low concentrations of H$_2$S (10 nM to 3 µM) is mediated by cyclic IMP [100]. The effect of nanomolar concentrations of H$_2$S has also been found in T-cell activation [101]. The target, which responds to low concentrations of H$_2$S, should be compared with its response to H$_2$S$_n$. It will be interesting to know whether or not the targets are oxidized cysteine residues.

Many compounds which release H$_2$S and H$_2$S$_n$ have been developed for clinical use on the basis of their cytoprotective and anti-inflammatory effects. Some of them have successfully completed phase 2 clinical trials and are proceeding further, while many preclinical compounds are awaiting trials [102,103]. It is hoped that various H$_2$S-based compounds will be translated into the field of clinical therapy over the next decade.

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