**Review**

**Hydrogen sulfide and polysulfides as signaling molecules**

By Hideo Kimura*1,†

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**Abstract:** Hydrogen sulfide (H2S) is a familiar toxic gas that smells of rotten eggs. After the identification of endogenous H2S in the mammalian brain two decades ago, studies of this molecule uncovered physiological roles in processes such as neuromodulation, vascular tone regulation, cytoprotection against oxidative stress, angiogenesis, anti-inflammation, and oxygen sensing. Enzymes that produce H2S, such as cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfurtransferase have been studied intensively and well characterized. Polysulfides, which have a higher number of inner sulfur atoms than that in H2S, were recently identified as potential signaling molecules that can activate ion channels, transcription factors, and tumor suppressors with greater potency than that of H2S. This article focuses on our contribution to the discovery of these molecules and their metabolic pathways and mechanisms of action.

**Keywords:** hydrogen sulfide, polysulfides, neuromodulator, vascular relaxant, cytoprotectant, TRPA1

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1. Background: From toxic gas to signaling molecule

In 1988, nitric oxide (NO) was discovered as a gaseous signaling molecule that regulates vascular tone and neuronal activity in the brain.1–4 It was later found to regulate synaptic activity for memory formation and learning.5 In 1993, carbon monoxide (CO) was discovered as a second gaseous signaling molecule that facilitated memory formation.6–8 Given these findings, we searched for a novel gaseous molecule that regulates neuronal activity and is involved in memory formation. Since the first description of hydrogen sulfide (H2S) as a toxic gas by Ramazzini in 1713,9 many toxicity studies of this molecule have been undertaken. Memory loss is common in survivors of H2S poisoning, and victims have coordination and psychiatric disturbances.10 In animal models, acute intoxication induced by high concentrations of H2S changes neurotransmitter levels at synapses in the brain and activates Ca2+ channels as well as Ca2+-activated K+ channels in dorsal raphe serotonergic cells.11,12 These observations suggest that H2S has a significant effect on neuronal activity.

In 1989, Warenycia et al.13,14 discovered endogenous sulfide in mammalian brains while studying how much exogenously applied H2S remains in the brain. After the application of H2S via inhalation or intraperitoneal injection of sodium hydrosulfide (NaHS), a salt of H2S, they observed that the amount of H2S in the brain increased in a dose-dependent manner. Surprisingly, H2S was detected even without the application of H2S, which indicated the presence...
of endogenous H$_2$S. This discovery urged us to study the physiological roles of this molecule in the nervous system.

H$_2$S-producing enzymes were studied extensively between the 1950s and 1970s; however, H$_2$S was thought to be merely a by-product of metabolic pathways or a marker for the activities of specific enzymes.\textsuperscript{15)–18)} In 1996, we demonstrated that H$_2$S facilitates the induction of hippocampal long-term potentiation (LTP) by enhancing the activity of N-methyl D-aspartate (NMDA) receptors, and that cystathionine $\gamma$-synthase (CBS) produces H$_2$S in the brain\textsuperscript{19)} (Figs. 1 and 2). Using these observations, we proposed a possible role for H$_2$S as a neuromodulator in the brain. Solomon Snyder of the Johns Hopkins University commented in an interview in Science News that our evidence supported the categorization of H$_2$S as a neurotransmitter.\textsuperscript{20)} In 2009, Snyder et al.\textsuperscript{21)} demonstrated a mode of action for H$_2$S, suggesting that it adds sulfur to cysteine residues of target proteins—a process called sulfhydration—which induces conformational changes in the proteins and alters their activity. In the same year, we showed that this sulfur addition reaction generates bound sulfane sulfur, which we proposed as the intracellular storage molecule of H$_2$S.\textsuperscript{22)}

H$_2$S also apparently has a role in the vasculature. We found that cystathionine $\gamma$-lyase (CSE) is an H$_2$S-producing enzyme in smooth muscle and that H$_2$S is a smooth muscle relaxant\textsuperscript{23)} (Fig. 3). The idea to investigate the possibility of these roles originated in the results of studies of NO, which has roles in both the nervous and the vascular systems. After the discovery of vascular system roles for NO, Garthwaite et al.\textsuperscript{4)} demonstrated that NO is also released through the NMDA receptors of brain neurons in a Ca$^{2+}$-dependent manner. Breit and Snyder\textsuperscript{24)} subsequently identified NO synthase in the brain.

H$_2$S relaxes the thoracic aorta, portal vein, and ileum.\textsuperscript{23)} However, the relaxation effect on the thoracic aorta is significantly weaker than that on the portal vein and the ileum. We found the effect of H$_2$S is greatly augmented by the presence of NO,
which suggests a synergy between these two molecules (see Fig. 3). This synergistic effect was confirmed in the twitch responses of the ileum by Teagues et al. With respect to the mechanism of H$_2$S effects on the vasculature, Zhao et al. identified ATP-sensitive K$^+$ channels as H$_2$S targets for relaxation and hyperpolarization in vascular smooth muscle. Mustafa et al. later identified endothelial Ca$^{2+}$-sensitive intermediate conductance and small conductance K$^+$ channels as target molecules as well. Further highlighting of the various roles of H$_2$S continues in the following discussion of the basic properties of this molecule and its enzymatic production.

### 2. Basic properties of H$_2$S

NO, CO, and O$_2$ barely dissolve in water; 100 mL of water dissolves 5.6 mg, 2.76 mg at 20 °C, and 3.93 mg at 25 °C, respectively. By contrast, 413 mg H$_2$S dissolves in 100 mL of water at 20 °C. In solution, H$_2$S dissociates into H$^+$ and HS$^-$ and further to S$^{2-}$ under alkaline conditions. Under physiological conditions (i.e., 37 °C and pH 7.4), approximately 20% of H$_2$S exists as a gas and the remainder as HS$^-$ with a trace amount of S$^{2-}$.

\[
H_2S \rightarrow H^+ + HS^- \quad (pK_1 \approx 7.04) \quad [1]
\]

\[
HS^- \rightarrow H^+ + S^{2-} \quad (pK_2 \approx 11.96) \quad [2]
\]

pK values were obtained from Reiffenstein et al. Note also that Meyer et al. reported a pK$_2$ of 13.78.

Because it is difficult to determine which form of H$_2$S (H$_2$S, HS$^-$, or S$^{2-}$) is active, the term “hydrogen sulfide” is used to include all forms. The term “gasotransmitter” has often been used for hydrogen sulfide, which can mislead readers into believing that H$_2$S gas is the only active form.

Given that intracellular pH is approximately 7.0 in the cytosol, 6.0–6.7 in the Golgi apparatus, and 4.7 in lysosomes, the ratio of H$_2$S/HS$^-$ likely varies among subcellular compartments. Although the precise ratio of H$_2$S/HS$^-$ in each organelle has not been measured, it is likely higher (i.e., a greater
amount of undissociated H$_2$S) in subcellular compartments in which the pH is more acidic.

H$_2$S has a molecular structure similar to that of H$_2$O; the distance between H and S in the former is 0.134 nm, whereas that between H and O in the latter is 0.0957 nm. The angle of H-S-H is 92°, whereas that of H-O-H is 104.52°. Despite these structural similarities, H$_2$S does not pass through aquaporins or water channels. H$_2$S also dissolves well in lipids and readily passes through lipid bilayers, even those containing cholesterol and sphingomyelin, both of which decrease membrane diffusion. The lower limit of lipid bilayer permeability to H$_2$S is 0.5 cm/s, which is greater than that to NH$_3$ (0.016 cm/s) but lower than that to CO$_2$ (3.2 cm/s). Another diffusion model proposed by Cuevasanta et al. estimates the permeability of H$_2$S at 0.85 cm/s. Considering that intracellular pH is lower than extracellular pH, H$_2$S passes more quantitatively through plasma membranes in the intracellular to extracellular direction.

Theoretical calculations by Mathai et al. indicate that transport of HS$^-$ by anion channels is unlikely to occur under physiological conditions. However, the anion-exchange protein AE1 was recently found to transport HS$^-$ in exchange for Cl$^-$ in erythrocytes. H$_2$S and HS$^-$ rapidly enter erythrocytes, which may act as a sink for H$_2$S and regulate its local extracellular concentrations (Fig. 4).

An HS$^-$ channel has also been identified in bacteria. Because the extracellular environment of bacteria is acidic (pH 6.0) and intracellular pH is 7.4, HS$^-$ concentrations are much greater inside the cells than outside, where H$_2$S gas is dominant. Therefore, H$_2$S passes through the bacterial membrane from the extracellular into the intracellular environment. However, in contrast to H$_2$S production in mammals, H$_2$S produced in bacteria dissociates to HS$^-$, which can pass through HS$^-$ channels to the extracellular environment and be released as H$_2$S gas (see Fig. 4).

![Graphs showing synergistic effect of H$_2$S with NO on vascular smooth muscle relaxation](image-url)
3. Endogenous H\(_2\)S

In addition to being present as free H\(_2\)S, H\(_2\)S is produced by enzymes and released from cellular stores.\(^{22}\) Two stored forms of H\(_2\)S have been identified: acid-labile sulfur and bound sulfane sulfur. Acid-labile sulfur is mainly found in an iron-sulfur complex attached to enzymes belonging to the respiratory chain. This complex releases H\(_2\)S under acidic conditions. Conversely, bound sulfane sulfur, which exists as the sulfurated cysteine residues of proteins, releases H\(_2\)S under reducing conditions (Fig. 5).

3.1. Acid-labile sulfur. The concentrations of endogenous free H\(_2\)S reported in initial studies\(^{13},^{14}\) were later found to be overestimations due to inappropriate methods of measurement.\(^{22},^{34},^{35}\) Specifically, the measurements were made under strong acidic conditions in which acid-labile sulfur releases H\(_2\)S in much greater amounts than those observed naturally. Nevertheless, we wanted to determine whether acid-labile sulfur functions as an H\(_2\)S store in cells. The pH at which H\(_2\)S is released from acid-labile sulfur is 5.4.\(^{22}\) Given that the enzymes containing the iron-sulfur complex—which contains acid-labile sulfur—are mainly localized to the mitochondria, in which the pH is approximately 8 and acid-labile sulfur is abundant,\(^{36}\) H\(_2\)S may not be released from acid-labile sulfur under physiological conditions (see Fig. 5). Although iron-sulfur complexes also release H\(_2\)S when they are detached from enzymes by detergents and protein denaturants, this may not occur under physiological conditions.\(^{22}\)

3.2. Bound sulfane sulfur. Bound sulfane sulfur is incorporated into proteins as persulfide or polysulfide, which release H\(_2\)S under reducing conditions (see Fig. 5). We observed the absorption of H\(_2\)S in tissues and found that the rate of absorption depends on tissue type. Absorbed H\(_2\)S can be recovered with a reducing substance such as dithiothreitol (DTT).\(^{22},^{36}\) H\(_2\)S must be oxidized to bind to proteins as a persulfide or polysulfide.\(^{37}\) Because

Fig. 4. H\(_2\)S passes through the membrane and HS\(^{-}\) through HS\(^{-}\) channels. H\(_2\)S dissociates to HS\(^{-}\) and H\(^{+}\) with a trace amount of S\(^{2-}\) under physiological conditions. At pH 7.4 and 37°C, approximately 20% H\(_2\)S exists as a gas, and remaining 80% as HS\(^{-}\). In mammalian cells, H\(_2\)S passes through the plasma membrane and then dissociates under the extracellular environment where pH is slightly higher than inside the cell. The anion exchange protein AE1 transports HS\(^{-}\) in exchange for Cl\(^{-}\). In bacteria, HS\(^{-}\) is released through HS\(^{-}\) channels to the extracellular environment, while H\(_2\)S enters into cells through the plasma membrane similar to that seen for mammalian cells.
exogenously applied H\(_2\)S is absorbed and stored in proteins, we predicted that endogenous H\(_2\)S produced by enzymes might also be incorporated into proteins. To this end, we found that cells expressing 3-mercaptopropionate sulfrurtransferase (3MST) and cysteine aminotransferase (CAT) contain increased levels of bound sulfane sulfur.\(^{38}\) By contrast, cells expressing a defective mutant 3MST, in which active-center cysteine 247 is replaced with a serine residue, do not produce H\(_2\)S (i.e., the levels of bound sulfane sulfur remain at control levels). We concluded that H\(_2\)S produced by enzymes is stored as bound sulfane sulfur in cells.

Does bound sulfane sulfur release enough H\(_2\)S with appropriate timing for the exertion of H\(_2\)S activity? Lysates of neurons and astrocytes release H\(_2\)S in the presence of endogenous concentrations of cysteine and glutathione at pH 8.4 or when dihydro-lipoic acid (DHLA) is present at pH 8.0.\(^{22,39}\) When neurons are excited, K\(^+\) is released and the extracellular concentration of K\(^+\) reaches 10–12 mM, which depolarizes the membrane potential of surrounding astrocytes. During membrane repolarization, Na\(^+\)/HCO\(_3^-\) transporters are activated, shifting the intracellular pH to alkaline, which releases H\(_2\)S from bound sulfane sulfur.\(^{40}\) However, we were unable to detect the release of H\(_2\)S from astrocytes.\(^{22}\) Further investigations are required to determine whether bound sulfane sulfur is an H\(_2\)S store in cells.

### 3.3. Free H\(_2\)S

The cellular concentration of free H\(_2\)S can change significantly in a short period because (1) H\(_2\)S is produced and degraded by enzymes, (2) the sulfur of H\(_2\)S is incorporated into cysteine residues after being oxidized to H\(_2\)Sn\(_h\), and (3) the reaction between thiol and H\(_2\)Sn\(_h\) releases H\(_2\)S. Although local changes in H\(_2\)S concentration are not well understood, steady state concentrations have been measured. Furne et al.\(^{34}\) vigorously mixed brain homogenates with phosphate buffer (pH 5.7) and measured the released gases with gas chromatography. At this pH, more than 95% of H\(_2\)S remained as H\(_2\)S gas without the release of H\(_2\)S from acid-labile
sulfur. The endogenous concentration of H$_2$S in the brain was determined to be 14 nM via this method. Using a modified method, Furne et al.$^{31}$ later found approximately 1 µM in the aorta and approximately 7 nM in the blood. The accuracy of the measurements obtained using this method depends on the efficiency of H$_2$S transfer from tissues into the gas space. By contrast, polarographic measurements by Whitfield et al.$^{42}$ detected no sulfide in the blood of several species.

Wintner et al.$^{35}$ detected 7 µM H$_2$S in blood by mixing monobromobimane, a fluorescent dye that binds to thiols, with blood and analyzing it with high-performance liquid chromatography (HPLC). Shen et al.$^{33,44}$ used this method to measure H$_2$S concentrations in mice and humans and detected 0.7 and 0.2 µM, respectively. These values are approximately 30–1000 times greater than those obtained by Furne et al.$^{31,44}$. However, monobromobimane complexed with other thiols, which has the same HPLC retention time as monobromobimane complexed with H$_2$S, may cause overestimation of the value.$^{35}$

We mixed brain homogenates with silver powder, which reacts with H$_2$S to produce silver sulfide on the surface of the powder. After washing for complete removal of proteins, which may contain acid-labile sulfur, the silver powder was exposed to thiourea and H$_2$SO$_4$ to recover H$_2$S from silver sulfide. The amount of H$_2$S was then measured with gas chromatography. In this method, the steady-state level of H$_2$S in the brain was under the detectable concentration of 25 nM/tube, corresponding to 9.2 µM in the brain.$^{22}$

The results obtained with these three methods show that free H$_2$S, like NO, is maintained at low steady-state concentrations. The repetitive application of H$_2$S to astrocytes causes desensitization and a drop in Ca$^{2+}$ influx. Thus, free H$_2$S must apparently be maintained in cells at low levels for proper cellular response to H$_2$S.$^{45}$

### 4. H$_2$S production

H$_2$S is produced by three enzymes — CBS, CSE, and 3MST — along with CAT, which is identical to aspartate aminotransferase. H$_2$S is also produced from D-cysteine via the D-amino acid oxidase (DAO)/3MST pathway (Fig. 6).

#### 4.1. CBS

Since our demonstration that CBS is an H$_2$S-producing enzyme,$^{19}$ the properties of this enzyme have been studied extensively. CBS catalyzes pyridoxal 5’-phosphate (PLP)-dependent β-replacement reactions to produce H$_2$S.$^{46,47}$

Cysteine + homocysteine → cystathionine + H$_2$S [3]
Cysteine → serine + H$_2$S [4]
Cysteine → lanthionine + H$_2$S [5]

As described in the previous section, free H$_2$S is maintained at low concentrations in the steady state. For H$_2$S to function as a signaling molecule, its concentrations must be controlled by physiological stimuli, including substances that regulate enzyme activity.

One of the greatest effectors of H$_2$S production is S-adenosyl methionine (SAM).$^{19,48}$ Shan et al.$^{49}$ demonstrated the mechanism through which SAM enhances CBS activity. The catalytic site, which is located at the center of the enzyme, is covered by the carboxyl-terminal SAM domain in the absence of SAM. SAM binding releases the domain and exposes the catalytic site, thereby activating the enzyme. H$_2$S production is also greatly enhanced in the presence of homocysteine and cysteine compared with cysteine alone, as shown in Eq. [3].$^{46,47}$

CBS activity is regulated through glutathionylation, which is a post-transcriptional modification of protein cysteine residues with the addition of glutathione. Glutathionylation of cysteine 346 increases CBS activity threefold.$^{50}$ By contrast, the binding of NO or CO to the heme group at the amino terminus of CBS suppresses CBS activity.$^{51}$ This regulation plays a key role in microcirculation in the brain.$^{52}$ During hypoxia, the suppression of CBS by CO is reversed by decreased production of CO by heme oxygenase-2 in neurons. H$_2$S produced by CBS localized in astrocytes, which surround capillaries, relaxes capillary walls to increase blood flow. Thus, this mechanism may compensate for deficiencies in O$_2$ supply. Combinations of these enhancers and suppressers may cause dynamic changes in the activity of CBS.

The balance between H$_2$S production and clearance also plays an important role in controlling H$_2$S concentration.$^{53,54}$ H$_2$S is cleared by mitochondrial enzymes such as sulfide-quinone oxidoreductase (SQR), sulfur dioxygenase, and rhodanese. However, the regulation of these enzymes is poorly understood.$^{55,57}$

We examined the developmental and pathological changes in the localization of CBS in the brain. CBS is mainly localized to cerebellar Bergmann glia and astrocytes.$^{58,59}$ At early developmental stages, CBS is expressed in neuroepithelial cells in the ventricular zone, but radial glial cells and astrocytes express CBS during the late embryonic and neonatal
periods. CBS expression is up-regulated in reactive astrocytes. This up-regulation is induced by epidermal growth factor, transforming growth factor-β, cyclic adenosine monophosphate, and dexamethasone. In CBS knockout mice, which show abnormal lipid metabolism in the liver, cerebellar morphological abnormalities are significant. Although the localization of CBS to cerebellar Purkinje cells and hippocampal neurons has also been reported, we were unable to find CBS in these neurons even with the antibody used by Robert et al. CBS is encoded on chromosome 21 (21q22.3), which is trisomy in Down syndrome (DS). Therefore, CBS expression is expected to be 1.5 times higher in people with DS than in normal individuals. However, our measurements showed that CBS in DS brains is approximately three times higher than that in normal individuals. Moreover, DS patients are predisposed to Alzheimer’s disease, and CBS is localized to astrocytes and astrocytes surrounding senile plaques in Alzheimer brains in individuals with DS. A polymorphism in the CBS allele is significantly underrepresented in children with a high intelligence quotient, suggesting that CBS may influence cognitive function. The overexpression of CBS may cause developmental abnormalities in cognition in children with DS that may in turn lead to Alzheimer’s disease in adulthood.

4.2. CSE. Since our demonstration that CSE is an H₂S-producing enzyme in smooth muscle tissues, the localization and activity of this enzyme have been studied extensively. CSE produces H₂S via the PLP-dependent α,β-elimination reaction with cysteine. Yang et al. reported that the activity of CSE is regulated by Ca²⁺/calmodulin. However, although CSE is localized in the cytosol, they examined its regulation in the presence of 1–2 mM Ca²⁺, which is the extracellular Ca²⁺ concentration. The intracellular Ca²⁺ concentration is approximately 100 nM.
in steady-state cells and increases up to 3 μM in cells such as excited neurons. Our re-evaluation showed that CSE activity is regulated by Ca\(^{2+}\) but in a manner different from that reported in the Yang et al. study and that calmodulin is not involved in this regulation.\(^{66}\) In the presence of PLP, the H\(_2\)S-producing activity of CSE is at its maximum potential in the absence of Ca\(^{2+}\). It is suppressed by Ca\(^{2+}\) in a concentration-dependent manner up to 300 nM, and this suppressing state is maintained at higher Ca\(^{2+}\) concentrations. These observations suggest that H\(_2\)S may be constitutively produced by CSE in steady-state cells, whereas production is suppressed when intracellular Ca\(^{2+}\) concentrations are elevated.\(^{66}\)

CSE has been found in vascular smooth muscle but not in the endothelium.\(^{23,26,67}\) A study by Yang et al.\(^{65}\) reported that CSE is also localized to the vascular endothelium; however, their previous in situ hybridization and western blot analyses, as well as our immunohistochemical analysis and that of Olson et al.\(^{68}\) showed that CSE is localized only to the smooth muscle, not the endothelium.\(^{26,67,68}\) This finding was confirmed by showing that the lysates of endothelium did not produce H\(_2\)S with cysteine alone, which would have occurred if CSE were present. By contrast, endothelial lysates required α-ketoglutarate and cysteine, both of which are substrates for CAT, for H\(_2\)S production.\(^{67}\) These observations suggest that 3MST and CAT are localized to the endothelium to produce H\(_2\)S.

Because H\(_2\)S relaxes vascular smooth muscle, CSE knockout mice were expected to be hypertensive. Of two lines of CSE knockout mice studied, one was hypertensive and the other was not.\(^{65,69}\) It is reasonable that the knockout of a single H\(_2\)S-producing enzyme, CSE, is inadequate to change blood pressure significantly because the H\(_2\)S-producing 3MST/CAT pathway and CBS reportedly localize in the vascular endothelium.\(^{67,68}\) The 3MST/CAT pathway, CBS, or both may compensate for the loss of CSE. Further studies are required to determine which enzymes are involved in the regulation of vascular tone (see Fig. 3).

We found that the promoter region of the CSE gene has an SP1 binding site that is activated by the multifunctional, proinflammatory cytokine tumor necrosis factor α.\(^{70}\) This site was later found to play a critical role not only in the expression of CSE but also in the antiapoptotic action of H\(_2\)S and the differentiation of smooth muscle\(^{71,72}\) (Fig. 7). Tumor necrosis factor α increases the production of CSE and activates IκB kinase, which phosphorylates IκB to release its binding partner nuclear factor-κB (NF-κB) and expose its nuclear localization signal. H\(_2\)S produced by CSE adds sulfur to the cysteine residues of NF-κB, which facilitates the nuclear translocation of NF-κB and activation of the transcription of antiapoptotic genes.\(^{72}\)

Because CSE is not detected with western blot analysis in the brain and H\(_2\)S production in the brain is not suppressed by propargylglycine, an inhibitor selective to CSE, it appears that this enzyme contributes little to H\(_2\)S production in the brain.\(^{19,70}\) However, contradictory observations have recently been reported. Specifically, CSE expression in the brains of Huntington model mice is markedly lower than that in wild-type brains.\(^{74}\)

### 4.3. 3MST and CAT.

Initially, only CBS and CSE were recognized as H\(_2\)S-producing enzymes. Because CSE was not found in the brain, we thought that CBS was a unique H\(_2\)S-producing enzyme in brain tissue.\(^{19,70}\) However, we found that the brains of CBS knockout mice produced H\(_2\)S, which suggested that a third H\(_2\)S-producing enzyme is present in the brain. The activity for this enzyme was localized to mitochondria and synaptosomes, and cysteine and another factor (present with molecular weight less than 3 kDa in cytosol) were required to produce H\(_2\)S. A possible alternative to CBS for H\(_2\)S production in the brain was 3MST.\(^{75}\) A substrate of 3MST, 3-mercaptoppyruvate (3MP), is provided via the metabolism of cysteine and α-ketoglutarate by CAT. 3MST and CAT were found in both synaptosomes and mitochondria, and the factor present with molecular weight less than 3 kDa was α-ketoglutarate.\(^{38}\)

3MST produces H\(_2\)S from 3MP, which is produced from cysteine and α-ketoglutarate by CAT (see Fig. 6).

\[
\text{CAT: cysteine} + \alpha-\text{keto-glutarate} \\
\rightarrow \text{3MP} + \text{glutamate} \quad [9] \\
3\text{MST: 3MP} \rightarrow \text{pyruvate} + \text{H}_2\text{S} \quad [10]
\]

Although 3MST is a ubiquitous enzyme, its levels vary among tissues. High expression occurs in the liver, large intestine, and kidney.\(^{78}\) In the brain, it localizes to neurons such as cerebellar Purkinje cells, mitral cells in the olfactory bulb, hippocampal pyramidal neurons, and astrocytes.\(^{38,79}\) Unlike the cytosol, mitochondria contain concentrations of cysteine that are sufficiently high (approximately 1 mM) for H\(_2\)S production via the 3MST/CAT pathway.\(^{80,81}\)
CBS and CSE do not require a reducing substance such as DTT for H₂S production, whereas 3MST does. Because the required endogenous reducing substance was unknown until we identified thioredoxin, the 3MST/CAT pathway was not recognized as a H₂S-producing pathway. However, the 3MST orthologue of *Trichomonas vaginalis* catalyzes the formation of thioredoxin persulfide, and 3MST interacts with thioredoxin, which has two redox-active cysteine residues in its active site. Given these observations, we hypothesized that thioredoxin may be the endogenous reducing substance associated with 3MST that allows for H₂S production. Thioredoxin has two forms, thioredoxin 1 and 2, that are localized in the cytosol and mitochondria, respectively. The two cysteine residues at the active site are conserved among different species, and similar to the bacterial thioredoxin that we used, thioredoxin 2 is resistant to oxidative stress. Given that thioredoxin is readily oxidized, it requires a reductase to maintain its reduced form. The mammalian thioredoxin reductase is a selenoprotein that cannot be produced in bacteria. Therefore, we used lysates of A549 human lung adenocarcinoma cells, which have an abundant supply of thioredoxin reductase, with nicotinamide adenine dinucleotide phosphate (NADPH) to reduce thioredoxin. Approximately 20 µM thioredoxin is present in cells, and H₂S is produced in fourfold greater amounts in the presence of thioredoxin than in the presence of the same concentration of DTT (Figs. 6 and 8).

DHLA, which is present at levels of approximately 40 µM in the brain, exhibits efficiency similar to that of DTT for the production of H₂S by 3MST. Other physiological reducing substances such as glutathione (GSH), cysteine, NADPH, nicotinamide adenine dinucleotide, and coenzyme A show no effect on the production of H₂S by 3MST. Dithiols such as DTT and DHLA have redox potentials that range from −0.29 to −0.33 V, and the redox potential of the active-site dithiol of thioredoxin is −0.29 V. The reducing potentials of monothiols such as GSH, cysteine, and coenzyme A range from −0.22 to −0.35 V, and those of

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**Fig. 7.** Transcription factor SP-1 up-regulates the CSE gene. H₂S produced by CSE, whose transcription is increased by TGF-α mediated through SP1 binding activation, sulfurates (sulfhydrates) NF-κB to make it translocate into the nucleus and up-regulate antiapoptotic genes. Increased production of CSE by the activation of SP1 also plays an important role in the vascular smooth muscle differentiation.
nicotinamide adenine dinucleotide and NADPH are $-0.320$ and $-0.324\, \text{V}$, respectively. These data show that reducing potential has no correlation with the capacity of these substances to affect $\text{H}_2\text{S}$ production by $3\text{MST}$, but dithiols such as DTT, DHLA, and thioredoxin have critical effects.

4.4. 3MST and DAO. Because mammalian enzymes generally metabolize L-amino acids with few exceptions (D-serine and D-aspartate), we applied D-cysteine to brain homogenates as a negative control to L-cysteine for $\text{H}_2\text{S}$ production. Unexpectedly, $\text{H}_2\text{S}$ was produced from D-cysteine. Although the production of $\text{H}_2\text{S}$ from D-cysteine has been studied in microorganisms and plants, its production in mammals is not well understood. The production of $\text{H}_2\text{S}$ from L-cysteine and D-cysteine has significantly different dependencies on pH and PLP. Because mitochondrial 3MST produces $\text{H}_2\text{S}$ from 3MP, we searched for an enzyme that produces 3MP from D-cysteine. DAO has been proposed to produce 3MP in hepatocytes. Indeed, our study showed that in the absence of DAO, $\text{H}_2\text{S}$ was not produced from D-cysteine and that indole-2-carboxylate, a DAO inhibitor, suppressed $\text{H}_2\text{S}$ production from D-cysteine.

D-Cysteine is metabolized by DAO to achiral 3MP, which is a substrate of 3MST for the production of $\text{H}_2\text{S}$ (see Fig. 6).

Fig. 8. 3MST produces $\text{H}_2\text{S}$ with thioredoxin as an acceptor of sulfane sulfur. 3MST receives sulfur from 3MP to produce 3MST persulfide, which is transferred to one of the thiols in thioredoxin to generate thioredoxin persulfide. The remaining thiol reacts with persulfide to release $\text{H}_2\text{S}$. Oxidized thioredoxin is reduced back to its reduced form by thioredoxin reductase.
As a source of D-cysteine, approximately 20–40% of L-cysteine is converted to D-cysteine by heat and alkaline conditions during food processing. This D-cysteine is easily absorbed through the gastrointestinal tract and readily enters the bloodstream. Alternatively, L-cysteine may be converted to D-cysteine by a racemase via a process that has not been elucidated.

Recently, a bioluminescent method was developed to measure the real-time activity and localization of DAO. In this method, the highly selective condensation reaction between D-cysteine and 6-hydroxy-2-cyanobenzothiazole to form D-luciferin is used for the sensitive measurement of DAO activity.

5. From toxin to cytoprotectant

Because H₂S is a toxic gas, it was generally not predicted to have a cytoprotective effect. However, our research indicated that high concentrations of H₂S in the primary cultures of neurons did not cause cell death but rather protected them from oxidative insults or oxidative glutamate toxicity. There are two forms of glutamate toxicity: excitotoxicity and oxidative toxicity. Excitotoxicity is caused by the long-lasting activation of NMDA receptors through which Ca²⁺ enters cells at levels beyond cell tolerance. Oxidative toxicity is caused by high concentrations of glutamate suppressing the cystine/glutamate antiporter, which imports cystine into cells with reciprocal export of glutamate. Cystine is reduced to cysteine inside cells. The decrease in the intracellular cysteine concentration decreases the production of GSH, a major intracellular antioxidant, which makes cells vulnerable to oxidative stress.

Embryonic neurons, which do not express NMDA receptors, provide a good model for oxidative glutamate toxicity because they are not disturbed by excitotoxicity. Cultures of embryonic neurons die 24 h after being exposed to high concentrations of glutamate but survive in the presence of H₂S. This cryoprotection by H₂S is effective even 8 h after the application of glutamate, although the efficiency declines with time. Because H₂S readily evaporates from culture medium (i.e., half-life of approximately 10 min), its effect of making cells commit to resist oxidative stress occurs during this initial short exposure.

H₂S exerts its cytoprotective effect by increasing GSH production and scavenging reactive oxygen species. GSH, a tripeptide consisting of glutamate, cysteine, and glycine, is produced by two enzymes, glutamate-cysteine ligase (GCL), also called γ-glutamyl cysteine synthetase, and GSH synthetase, which adds glycine to the GCL product γ-glutamyl cysteine. H₂S enhances GCL activity and potentiates the activity of the cystine/glutamate antiporter as well as the cysteine transporter, both of which increase intracellular concentrations of cysteine. Through these effects, H₂S increases the production of GSH (Fig. 9).

H₂S produced by 3MST in the mitochondrion suppresses oxidative stress in this organelle. The endogenous concentrations of H₂S range from approximately 10 nM to 3 µM, whereas those of GSH are 1–10 mM. Therefore, H₂S suppresses oxidative stress mainly by increasing GSH production (see Fig. 9).

The discovery of the neuroprotective effect of H₂S led to the identification of protective effects from ischemic insults in the heart and kidney and from high-fat diet-induced glucotoxicity in pancreatic β cells. H₂S limits infarct size and preserves cardiac function by inhibiting myocardial inflammation and apoptosis and preserving mitochondrial structure and function. With respect to renal protection during ischemic insult, H₂S attenuates the phosphorylation of mitogen-activated protein kinases as well as the activation of NF-κB and caspase-3 and suppression of Bcl-2 expression.

Other mechanisms for the cytoprotective effect of H₂S have also been identified. For instance, retinal tissue is susceptible to oxidative stress because it consumes large quantities of O₂ and is constantly exposed to light. Exposure of the retina to excessive light results in photoreceptor cell damage, which is caused by various factors, including elevated intracellular concentrations of Ca²⁺ and reactive oxygen species. In the retina, H₂S activates vacuolar-type H⁺-ATPase in horizontal cells to release H⁺, which suppresses Ca²⁺ channels in photoreceptor cells to maintain intracellular concentrations of Ca²⁺ at appropriately low levels. Moreover, the regulation of endoplasmic reticulum (ER) stress contributes to the cytoprotective effect of H₂S. After being produced in the ER, proteins are trafficked to the Golgi apparatus. Misfolded proteins trigger the unfolded protein response to start proapoptotic cascades. ER stress increases the production of H₂S, which inhibits protein tyrosine phosphatase (PTP). In turn, PTP inactivates protein kinase-like ER kinase to inhibit global translation by phosphorylating eIF2α, which suppresses ER stress responses.

In addition to its cytoprotective effects, H₂S regulates bacterial resistance to antibiotics. Shatalin
...demonstrated that the inactivation of the bacterial homologues of CBS, CSE, and 3MST decreases the production of H₂S, which increases the vulnerability of bacteria to antibiotics. The cytoprotective effect of H₂S appears to be a universal defense mechanism in organisms from bacteria to mammals. Bacteria also produce NO, and both H₂S and NO synergistically protect bacteria from antibiotics. Because bacterial CBS, CSE, and 3MST are evolutionarily distinct from their mammalian counterparts, they may be appropriate targets for the development of new classes of antibiotics.¹¹⁵

6. Regulation of synaptic transmission

6.1. Enhancing the activity of NMDA receptors. To explore the mechanism for the facilitation of LTP induction by H₂S, we examined the target of H₂S at the synapse. The activation of NMDA receptors is required to induce LTP. Our observations showed that H₂S enhanced the activity of NMDA receptors but not that of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, another type of glutamate receptor¹⁹) (see Fig. 1).

Aizenman et al.¹¹⁶) demonstrated that the reduction by DTT of the cysteine disulfide bond at the hinge of the ligand-binding domain of NMDA receptors enhances the activity of the receptors. By contrast, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors lack a corresponding cysteine disulfide bond. Because H₂S can reduce the cysteine disulfide bond, the enhancement of NMDA receptor activity is one of the H₂S effects that facilitates LTP induction. However, this was not the only effect of H₂S. We found an inconsistency that introduces difficulty in explaining the induction of LTP solely by the reducing activity of H₂S. DTT,
which is a more efficient reducing agent than H₂S, facilitated the induction of LTP, but a lower concentration of H₂S further facilitated the induction of LTP even after DTT treatment. This discrepancy suggests that H₂S has an additional effect that DTT does not (see Fig. 1).

6.2. Activation of astrocytes surrounding synapses. Astrocytes, which are a type of glia, surround synapses and have neurotransmitter receptors. Typically, presynaptic neurons release a neurotransmitter to postsynaptic neurons and activate surrounding astrocytes as well. These activated astrocytes in turn release gliotransmitters into synapses to modulate their activity. We hypothesized that H₂S may have an effect on astrocytes that in turn modulates synaptic activity, which leads to the facilitation of LTP. We first observed that H₂S induces Ca²⁺ influx in astrocytes. The responses were abolished in the absence of extracellular Ca²⁺ and by broad-spectrum transient receptor potential (TRP) channel inhibitors such as La³⁺ and ruthenium red, suggesting that H₂S activates TRP channels to induce Ca²⁺ influx. Only glial fibrillary acidic protein-positive mature astrocytes respond to H₂S; immature or reactive astrocytes induced by acidic protein-positive mature astrocytes respond to H₂S, but immunohistochemical studies have not indicated the location of TRPA1 channels.

6.3. Identification of H₂Sₙ. During the study of the effect of H₂S on astrocytes, we found that compared with NaHS (a sodium salt of H₂S), sodium tri- and tetrasulfide (Na₂S₃ and Na₂S₄; sodium salts of H₂S₂ and H₂S₃) induce Ca²⁺ influx in astrocytes much more potently. Unlike H₂S, H₂Sₙ does not exist as a gas under physiological conditions. Therefore, Na₂Sₙ is dissolved in water and dissociates to H⁺ and HS⁻. Given that the applied concentrations of Na₂Sₙ are low, the effect of Na⁺ can be ignored.

\[ \text{H}_2\text{S}_n \rightarrow \text{H}^+ + \text{HS}_n^- \]  \hspace{1cm} [12]

Comparison of the half-maximal effective concentration (EC₅₀) for the induction of Ca²⁺ influx of Na₂S₃ (EC₅₀ = 91 nM) and NaHS (EC₅₀ = 116 μM) has shown that Na₂S₃ is approximately 1000 times more potent than NaHS. 45)–118) (Fig. 10).

While investigating the effects of NaHS on the induction of Ca²⁺ influx in astrocytes, we noticed that some solutions of NaHS, which are typically colorless, were yellowish. We realized that compared with colorless solutions, the yellow NaHS solutions had a greater effect on inducing Ca²⁺ influx. We speculated that the yellow color originated from elemental sulfur and that a synergy exists between H₂S and elemental sulfur that activates TRP channels. When elemental sulfur was dissolved in a solution of NaHS, the solution turned yellow and potently induced Ca²⁺ influx in astrocytes. Searcy and Lee 121) have reported that elemental sulfur dissolved in Na₂S solution, another sodium salt of H₂S, generates H₂Sₙ species (Na₂S₃ and sodium pentasulfide), and in 2006, we demonstrated that Na₂S₃ and Na₂S₄ induce Ca²⁺ influx much more potently than NaHS does by activating TRP channels. 118) Thus, it appears that it is not the synergy between elemental sulfur and H₂S but the effect of the H₂Sₙ products from both agents that potently induces Ca²⁺ influx.

6.4. H₂Sₙ activates TRP ankyrin 1 (TRPA1) channels in astrocytes. In 2008, Streng et al. 122) demonstrated that H₂S activates the TRPA1 channels of sensory neurons in the urinary bladder and that Chinese hamster ovary cells overexpressing TRPA1 channels respond to H₂S. Ogawa et al. 123) found a similar result in dorsal root ganglion cells using TRPA1 knockout mice and TRPA1-overexpressing human embryonic kidney 293 cells. However, high concentrations of NaHS (1–10 mM) were applied in these studies. H₂S is oxidized to H₂Sₙ species with a varying number of sulfur atoms until the number of sulfur atoms reaches eight, at which point the sulfur molecules cyclize and precipitate. 37),124) The number of sulfur atoms on H₂Sₙ species under physiological conditions remains undetermined.

\[ 2\text{nH}_2\text{S} + 1/2(2\text{n} - 1)\text{O}_2 \rightarrow \text{H}_2\text{S}_n + (2\text{n} - 1)\text{H}_2\text{O} \] \hspace{1cm} [13]

\[ \text{HS}^- \leftrightarrow \text{HSS}^- \leftrightarrow \text{HSSS}^- \leftrightarrow \ldots \leftrightarrow \text{HS}_7^- \rightarrow \text{S}_8 \] \hspace{1cm} [14]

Therefore, a portion of NaHS may have been oxidized to Na₂Sₙ, which activated TRPA1 channels in previous studies. Taking these results and our previous observation into account, we hypothesized that H₂Sₙ species activate TRPA1 channels in astrocytes. A transcriptional database showed that TRPA1 messenger RNA is present below detectable levels in astrocytes, but immunohistochemical studies have thus far not indicated the location of TRPA1 channels in astrocytes. 125),126) However, in 2012, Shigetomi et al. 127) demonstrated the existence of TRPA1 channels in astrocytes with western blot and functional analyses. Furthermore, the application of the TRPA1 channel-selective agonists allyl isothiocyanate and cinnamaldehyde induced Ca²⁺
influx and confirmed the existence of these channels in astrocytes. The responses to Na$_2$S$_3$ and Na$_2$S$_4$ were suppressed by the TRPA1 channel-selective antagonists HC-030031 and AP-18 as well as by small interfering RNA selective to TRPA1 channels, which suggested that H$_2$Sn species activate TRPA1 channels in astrocytes.

Ogawa et al. determined the site within TRPA1 channels that was sensitive to high H$_2$S concentrations. They revealed that replacing two cysteine residues at the amino terminus of TRPA1 channels with serine causes an insensitivity to NaHS. The observation that the effect of NaHS was suppressed by the reducing agent DTT suggests that these two sensitive cysteine residues are sulfurated by H$_2$Sn generated from NaHS, which results in a conformational change in the TRPA1 channels that leads to their activation. When the sulfurated cysteine residues are removed with DTT or glutathione, the channels return to their quiescent conformation (Fig. 11).

6.5. Endogenous H$_2$Sn in the brain. We identified H$_2$Sn in the brain by using HPLC to analyze samples derivatized with monobromobimane. Because H$_2$Sn species are mixture with various numbers of sulfur atoms in equilibrium, even the standard Na$_2$S$_3$ and Na$_2$S$_4$ exert several peaks at the same retention times. Brain samples contain H$_2$Sn (Figures in 120 were modified).

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Fig. 10. Existence of H$_2$Sn in the brain and the induction of Ca$^{2+}$ influx in astrocytes. H$_2$S$_3$ induces Ca$^{2+}$ influx in astrocytes in a dose-dependent manner with EC$_{50} = 91$ nM (A and B). Because H$_2$Sn are mixture of molecules with different number of sulfur atoms in equilibrium as shown in the Eq. [14], even the standard Na$_2$S$_3$ and Na$_2$S$_4$ exert several peaks at the same retention times. Brain samples contain H$_2$Sn (Figures in 120 were modified).
receptors, but this reaction does not fully explain the mechanism for its facilitation of LTP induction. Shigetomi et al. demonstrated that Ca$^{2+}$ influx through the activated TRPA1 channels facilitates the release of the gliotransmitter d-serine, which enhances the activity of NMDA receptors, in turn leading to the induction of LTP. H$_2$Sn activates TRPA1 channels, whereas H$_2$S activates NMDA receptors by reducing the cysteine disulfide bond. The combined activity of H$_2$S and H$_2$Sn facilitates the induction of LTP more efficiently than does DTT, which reduces cysteine disulfide bonds but does not activate TRPA1 channels (Fig. 12).

7. Modification of protein function by sulfuration

7.1. Sulfuration (sulfhydration) by H$_2$S. The modification of enzyme activities through the addition of sulfur to specific cysteine residues of enzymes was studied extensively between the 1960s and 1980s. We found that H$_2$S is absorbed and stored in cells as bound sulfane sulfur, which releases H$_2$S in response to reducing agents such as DTT. Mustafa et al. have defined sulfhydration as a process in which sulfur provided by H$_2$S attaches to reactive cysteine residues in target proteins. Toohey has suggested calling it sulfuration, as only sulfur is transferred in the process.

Sulfuration of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by H$_2$S increases its catalytic activity, and DTT treatment removes the added sulfur to diminish activity. This observation is supported by evidence that the GAPDH activity is reduced in CSE knockout mice. Actin polymerization is also enhanced by H$_2$S and reversed by DTT. ATP-dependent K$^+$ channels, which are involved in vascular smooth muscle relaxation, are activated by sulfuration at cysteine 43 on the Kir6.1 subunit. Sulfuration of NF-$\kappa$B by H$_2$S facilitates its translocation to the nucleus to up-regulate anti-apoptotic genes, and protein kinase-like ER kinase retains its activity due to the sulfuration of the PTP that regulates ER stress. Parkin, an E3 ubiquitin ligase that is suppressed in Parkinson’s disease, is also activated by sulfuration.

Thus, the sulfuration of cysteine residues by H$_2$S seems to play a key role in the regulation of many target proteins. However, it does not. Because atoms with the same oxidation state do not exchange electrons (i.e., do not undergo a redox reaction), the sulfuration of cysteine residues cannot occur (i.e., the oxidation state of sulfur in H$_2$S is $-2$, and that in cysteine residues is also $-2$; Fig. 13). Early studies of sulfuration (sulfhydration) likely measured cysteine residues reacted with H$_2$S produced by the oxidation of H$_2$S or the oxidized cysteine residues.
reacted with H₂S in the same reaction for glutathionylation in which oxidized cysteine residues react with glutathione.⁵⁰) Tao et al.¹³³ demonstrated that H₂S reduces cysteine disulfide bonds but does not sulfurate cysteine residues. H₂S induces angiogenesis mediated by vascular endothelial growth factor receptor 2¹³⁴,¹³⁵ by reducing a disulfide bond located between cysteine 1045 and cysteine 1024. Mass spectrometry analysis shows that H₂S reduces the cysteine disulfide bond contained in the synthesized hexapeptide but does not sulfurate any of the 20 free amino acids, including cysteine.¹³³ The sulfuration of cysteine residues is only transiently observed as an intermediate during the reduction of the disulfide bond, and the intermediate is immediately attacked by a second HS-molecule and reduced to cysteine.

### 7.2. Sulfuration by H₂Sn

Because the oxidation state of sulfur in H₂S and cysteine is −2, H₂S is unable to donate electrons to cysteine. By contrast, H₂Sn readily receives electrons from cysteine and transfers sulfur atoms to cysteine. As described in section 6.4, two active cysteine residues at the amino terminus of TRPA1 channels are sulfurated by H₂Sn but not by H₂S.

Another example of the activation of target proteins by sulfuration is Kelch ECH-associating protein 1 (Keap1), which binds to nuclear factor erythroid 2-related factor 2 (Nrf2) to remain in the cytosol. Calvert et al.¹³⁶ initially reported that H₂S facilitates the translocation of Nrf2 to the nucleus, where Nrf2 up-regulates the transcription of antioxidant genes that contribute to the cytoprotective effect of H₂S. Yang et al.¹³⁷ later proposed a mechanism through which H₂S sulfurates (sulfhydrates) cysteine residues of Keap1, which releases Nrf2 to the nucleus. However, H₂S does not sulfurate cysteine residues of Keap1 for the reason described earlier. Instead, we showed that H₂S₄ sulfurates Keap1¹³⁸ (Fig. 14).

### 7.3. Other forms of polysulfides

Compared with GSH, glutathione persulfide (GSSH) has greater...
reducing activity. Massey et al.\textsuperscript{139} initially reported that GSSH generated from glutathione trisulfide reduces cytochrome c more efficiently than GSH and that cysteine persulfide has similar activity. The effect of GSSH on cytochrome c was confirmed by Fukuto et al.\textsuperscript{140} who showed a similar reductive effect of GSSH on papain. The reducing activity of GSSH was further confirmed by showing its scavenging effect on H$_2$O$_2$.\textsuperscript{141}

CBS and CSE were demonstrated to metabolize cystine rather than cysteine to produce cysteine persulfide, which generates GSSH via an exchange reaction with GSH.\textsuperscript{141} However, the physiological relevance of this pathway must be re-evaluated. Although CSE has a high affinity for cystine (the extracellular form of cysteine) with a $K_m$ value of 30–70 µM, CBS and CSE are localized to the cytosol, in which the amount of cystine is insufficient for both enzymes. For instance, only 0.2 µM cystine has been measured in the liver, and other tissues contain even lower concentrations. The cell line A549 contains an exceptionally high concentration of cystine at approximately 12 µM.\textsuperscript{141} Even the extracellular concentration of cystine in human blood is only approximately 40 µM.\textsuperscript{142} Therefore, it may be difficult for CBS and CSE to produce cysteine persulfide under physiological conditions.

H$_2$S is metabolized by SQR in mitochondria, and GSH receives a sulfur atom from SQR to generate GSSH.\textsuperscript{55–57} Libiad et al.\textsuperscript{143} demonstrated that GSSH can be formed via the enzymatic activity of SQR rather than nonenzymatic sulfane sulfur exchange between cysteine persulfide and oxidized glutathione reported by Ida et al.\textsuperscript{144}
8. Cross talk between H2S and NO

When we discovered the relaxation effect of H2S on smooth muscle, we recognized that this effect was much weaker in the thoracic aorta than in the portal vein and the ileum. In the presence of NO donors, however, the effect of H2S was greatly augmented and vice versa (see Fig. 3). This finding was the first demonstration of cross talk between H2S and NO. Moore et al. later confirmed the synergistic effect of both molecules in the ileum, and also showed the opposite effect: H2S induces vasoconstriction by scavenging endothelial NO. Zhao et al. reported that H2S inhibits the vasorelaxation effect of the NO donor sodium nitroprusside (SNP) and showed that SNP increases H2S production and another NO donor, S-nitroso-N-acetylpenicillamine, up-regulates the expression of CSE.

Minamishima et al. demonstrated that the protection of cardiomyocytes by H2S is mediated by nitric oxide synthase 3 (eNOS). The application of Na2S increases the phosphorylation of serine 1179 in eNOS to raise serum nitrite and nitrate levels and attenuates cardiac arrest-induced mitochondrial dysfunction. These effects were confirmed when they were not observed in eNOS knockout mice. King et al. performed additional studies on the mediation of NO in cytoprotection by H2S. The eNOS activation site, serine 1179, is less phosphorylated in CSE knockout mice than in wild-type mice, whereas the inhibitory site, threonine 495, is more phosphorylated in CSE knockout mice. The application of Na2S activates eNOS to increase the bioavailability of NO in wild-type mice, but this effect does not occur in eNOS-deficient mice.

H2S induces angiogenesis mediated by vascular endothelial growth factor. It also promotes the migration of vascular endothelial cells and facilitates microvessel tube formation. Bir et al. demonstrated that the angiogenic effect of H2S is mediated by NO by showing that eNOS knockout mice do not show the effect. By contrast, Szabo et al. proposed that the cooperative action between H2S and NO is essential for angiogenesis by showing that the suppression of the H2S-generating enzyme CSE abolishes angiogenesis. The difference in results likely occurred because hypoxic conditions were used in the study by Bir et al., whereas Coletta et al. made observations under non-hypoxic conditions.
The chemical interaction between H₂S and NO produces several potential intermediates. One study showed that nitrosothiol, the chemical structure of which was not reported, is produced from H₂S and NO. Nitrosothiol does not activate guanylyl cyclase to increase the production of cyclic guanosine monophosphate unless NO is released with Cu²⁺. H₂S releases NO from nitrosoglutathione, S-nitroso-N-acetylpenicillamine, SNP, and brain homogenates, which may contain nitrosothiols and metal nitrosyl complexes, to relax the vasculature. Filipovic et al. demonstrated that H₂S and nitrite produce the intermediate thionitrous acid, which in turn generates either NO and the HS⁻ radical or nitroxy via further reaction with H₂S. They also demonstrated that nitroxy activates TRPA1 channels, which releases calcitonin gene-related peptide, in turn inducing vasodilatation. Cortese-Krott et al. demonstrated that nitrosopersulfide, which is more stable than thionitrous acid, efficiently releases NO and H₂S, which results in effective activation of soluble guanylyl cyclase and relaxation of smooth muscle compared to that observed with the parent nitrosothiol.

Mustafa et al. proposed that the S-nitrosylation of proteins suppresses their activity, whereas sulfuration (sulfhydration) potentiates this activity. Sulfuration of GAPDH increases its catalytic activity, whereas S-nitrosylation of the same cysteine residue abolishes the activity. A similar reciprocal oppositional effect of sulfuration and S-nitrosylation is observed in parkin, in which sulfuration at cysteine 95, a principal site of sulfuration, enhances its activity. Moreover, parkin sulfuration is decreased in the brains of Parkinson’s disease patients, in which S-nitrosylation is increased. Altaany et al. showed that eNOS activity is activated by the sulfuration of cysteine 443, whereas it is suppressed by nitrosylation of this residue. Sulfuration of eNOS decreases S-nitrosylation in eNOS and increases eNOS dimer stability to augment NO bioavailability. Altaany et al. also confirmed results published by Minamishima et al. and King et al. suggesting that NaHS enhances eNOS activity by increasing the phosphorylation of serine 1179.

9. Development of H₂S- and polysulfide-sensitive fluorescent probes

The production and clearance of H₂S and its absorption by proteins occurs rapidly. Therefore, accurate real-time measurements of the concentration and movement of H₂S is difficult. We collaborated with Nagano et al. to develop hydrogen sulfide imaging prove-1 (HSIP-1) a fluorescent probe selective for H₂S. The probe consists of two functional parts, a site sensitive to H₂S and a fluorescence emission fluorophore. HSIP-1 has azamacrocyclic rings that form stable complexes with Cu²⁺, thereby suppressing the emission of fluorescence. H₂S releases Cu²⁺ from the complex, inducing fluorescence. Because the intracellular concentrations of GSH and cysteine are approximately 1–10 mM and 100 µM, respectively, whereas that of H₂S is below a micromolar order, it is crucial for a probe to detect low concentrations of H₂S in the presence of high concentrations of other thiols such as GSH and cysteine. GSH and cysteine do not release Cu²⁺, which enables HSIP-1 to detect H₂S selectively with almost no response to 10 mM GSH and 1 mM cysteine. The fluorescence intensity is increased 50-fold in 5 min in the presence of 10 µM H₂S.

Several other probes were reported in 2011. The sensitivity of SF1, SF2, and DNS-Az to H₂S is based on the reduction of azides to amines by H₂S that triggers changes in the electronic properties of a fluorophore, which then emits fluorescence. The fluorophore of SF1 and SF2 is rhodamine, and that of DNS-Az is a dansyl group. Within 1 h of H₂S exposure, SF1 and SF2 showed increases in fluorescence intensity of 7- and 9-fold, respectively. The detection limit for SF1 and SF2 is 5–10 µM H₂S. The response of DNS-Az is fast, reaching a peak within 10 s and showing a 40-fold increase in fluorescence intensity in the presence of 25 µM H₂S.

SFP-1, SFP-2, and Probe 1 have similar H₂S binding sites at which H₂S reacts with an electrophilic component to form a free SH-containing intermediate, which then reacts with an ester group at the suitable position for cyclization. Upon cyclization, the fluorophores emit fluorescence. The fluorescence intensity of Probe 1 increases by 55- to 70-fold in the presence of 50 µM H₂S after 1 h, whereas that of SFP-1 and SFP-2 increase by 13-fold under the same conditions.

Because H₂S₈ species have recently been identified as a H₂S-related signaling molecule, the polysulfide-sensitive fluorescence probes SSP1 and SSP2 have been developed. The SH group of the probes acts as a nucleophile to trap the reactive sulfur atoms of polysulfides. The resultant intermediates, -SSH adducts, undergo a fast intramolecular cyclization to release the fluorophore. SSP1 and SSP2 are selective to sulfane sulfur species and do not react with other biologically relevant sulfur species such as cysteine and GSH.
The sensitivity and response times of these probes must be improved. Furthermore, the responses of these probes to H$_2$S and sulfane sulfur are irreversible, and the development of more desirable reversible probes is awaited.

10. Other roles of H$_2$S and therapeutic applications

Since the identification of the neuromodulation, vascular relaxation, and cytoprotection roles of H$_2$S, various other roles have been identified, such as those in the regulation of inflammation, reduction of insulin release, suppression of cancer growth, and detection of cellular oxygen levels. H$_2$S regulates inflammatory processes and their resolution. A significant anti-inflammatory effect was initially reported by Zanardo et al., who showed that H$_2$S inhibits leukocyte adherence to the vascular endothelium as well as accumulation and edema formation. The discovery of these beneficial effects spurred the development of several H$_2$S donors and H$_2$S-releasing non-steroidal anti-inflammatory drugs with no significant side effects in the gastrointestinal tract. Many of these drugs have shown considerable promise in relevant animal models and are presently undergoing clinical trials.

H$_2$S inhibits cancer development, induces cell cycle arrest in the G2/M phase, and promotes apoptosis. H$_2$S-releasing non-steroidal anti-inflammatory drugs enhance these chemopreventive effects on cancer cells, and so-called NOSH compounds, which release both H$_2$S and NO, have been developed to suppress cancer growth. Contrary to these observations, CBS expression is up-regulated in colorectal and ovarian cancer cells, in which H$_2$S is involved in promoting cellular growth and migration. In these cells, suppression of CBS activity may be a potential therapy.

It is important to detect concentrations of O$_2$ in the blood and respond to hypoxia by dilating blood vessels to increase blood flow. O$_2$ levels are also relayed to the brainstem to increase breathing and cardiovascular function. Olson et al. demonstrated that the O$_2$-dependent metabolism of H$_2$S may be an effective O$_2$-sensing mechanism. Tissue H$_2$S concentrations are inversely related to O$_2$ concentrations, and this reciprocal relationship is responsible for a similar effect between hypoxia and H$_2$S. H$_2$S production is closely linked to cellular oxidative states. Oxidative stress depletes GSH and cysteine, which in turn enhances the activity of CBS to stimulate the production of H$_2$S, cysteine, and GSH, which protect cells from oxidative stress.

11. Concluding remarks

Two decades ago, what was considered merely a pungent gas was found to be a physiological mediator of cognitive function and vascular tone. Since then, numerous physiological roles for H$_2$S—such as protecting various tissues and organs from ischemic insults or oxidative stress, a universal defense mechanism across a diverse spectrum of species; regulating inflammation; inducing angiogenesis; and oxygen sensing—have been confirmed. Recently identified H$_2$Sn species are considerably potent and induce various physiological responses, some of which were previously ascribed to H$_2$S. The cross talk between H$_2$S and NO, which was initially demonstrated as a synergistic interaction of both molecules, led to the identification of H$_2$S regulation on NO bioavailability and the generation of several potential signaling molecules from both compounds. Understanding of the biochemical nature of these molecules as well as their mechanisms of action, will elucidate the physiological roles and therapeutic potential of H$_2$S and related molecules.

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Dr. Hideo Kimura was born in Osaka in 1956. He graduated from the University of Tokyo in 1980 and received his Ph.D. degree in 1985. He served the National Defense Medical College (1980–1990) and studied neurotransmission in the cerebellum under Dr. Y. Sakai. Dr. Kimura received training in molecular biology at the Cancer Institute (1985–1987) under Dr. Y. Fujii-Kuriyama. Dr. Kimura received further training as a postdoctoral fellow (1988–1991) at the Salk Institute for Biological Studies, where he isolated and characterized a novel growth factor, schwannoma-derived growth factor, under Dr. D. Schubert. Dr. Kimura continued serving as a staff and senior staff scientist at the Salk Institute until 1999, where he discovered that hydrogen sulfide (H$_2$S) acts as a neuromodulator in the brain; this paper was published in 1996. Subsequently, in 1997, he demonstrated another role for H$_2$S, that is, it acts as a smooth muscle relaxant in synergy with nitric oxide. In 1999, he became Director of the Department of Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo. Dr. Kimura discovered a neuroprotective effect of H$_2$S in 2004 that led to the identification of its protective effect against ischemic insults in various tissues and organs. In 2006, he also identified hydrogen polysulfide (H$_2$Sn) as a potential signaling molecule which activates ion channels and was later found to have various other physiological roles, including the regulation of transcription factors, tumor-suppressing factors, and vascular tone. For his accomplishments, he received a Promotion Award from the Japanese Pharmacological Society, a Human Frontier Science Program Award, a First Award from the National Institutes of Health, an Alzheimer Scholar Award from the Alzheimer Association, and a JB Prize from the Japanese Biochemical Society.