Review

Hydrogen sulfide and polysulfides as signaling molecules

By Hideo KIMURA[*1,†]

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Abstract: Hydrogen sulfide (H_2S) is a familiar toxic gas that smells of rotten eggs. After the identification of endogenous H_2S 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 H_2S, 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 H_2S, were recently identified as potential signaling molecules that can activate ion channels, transcription factors, and tumor suppressors with greater potency than that of H_2S. 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

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 (H_2S) 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 H_2S poisoning, and victims have coordination and psychiatric disturbances.[10)] In animal models, acute intoxication induced by high concentrations of H_2S changes neurotransmitter levels at synapses in the brain and activates Ca^{2+} channels as well as Ca^{2+}-activated K^+ channels in dorsal raphe serotonergic cells.[11),12)] These observations suggest that H_2S 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 H_2S remains in the brain. After the application of H_2S via inhalation or intraperitoneal injection of sodium hydrosulfide (NaHS), a salt of H_2S, they observed that the amount of H_2S in the brain increased in a dose-dependent manner. Surprisingly, H_2S was detected even without the application of H_2S, which indicated the presence

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Abbreviations: CAT: cysteine aminotransferase; CBS: cystathionine β-synthase; CO: carbon monoxide; CSE: cystathionine γ-lyase; DAO: D-amino acid oxidase; DHLA: dihydrolipoic acid; DS: Down syndrome; DTT: dithiothreitol; EC_{50}: half-maximal effective concentration; eNOS: nitric oxide synthase 3; ER: endoplasmic reticulum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GCL: glutamate cysteine ligase; GSH: glutathione; GSSH: glutathione persulfide; H_2S: hydrogen sulfide; H_2S_n: polysulfide; HPLC: high performance liquid chromatography; HSIP-1: hydrogen sulfide imaging prove-1; K_{ATP}: ATP-sensitive K^+; Keap1: Kelch ECH-associating protein 1; LTP: long-term potentiation; 3MP: 3-mercaptopyruvate; 3MST: 3-mercaptopyruvate sulfurtransferase; NADPH: nicotinamide adenine dinucleotide phosphate; NaHS: sodium hydrosulfide; Na_2S_3: sodium trisulfide; Na_2S_4: sodium tetrasulfide; NF-κB: nuclear factor-κB; NMDA: N-methyl D,L-aspartate; NO: nitric oxide; Nrf2: nuclear factor erythroid 2-related factor 2; PLP: pyridoxal 5′-phosphate; PTP: protein tyrosine phosphatase; SAM: S-adenosyl methionine; SNP: sodium nitroprusside; SQR: sulfide-quinone oxidoreductase; TRP: transient receptor potential; TRPA1: TRP ankyrin 1.

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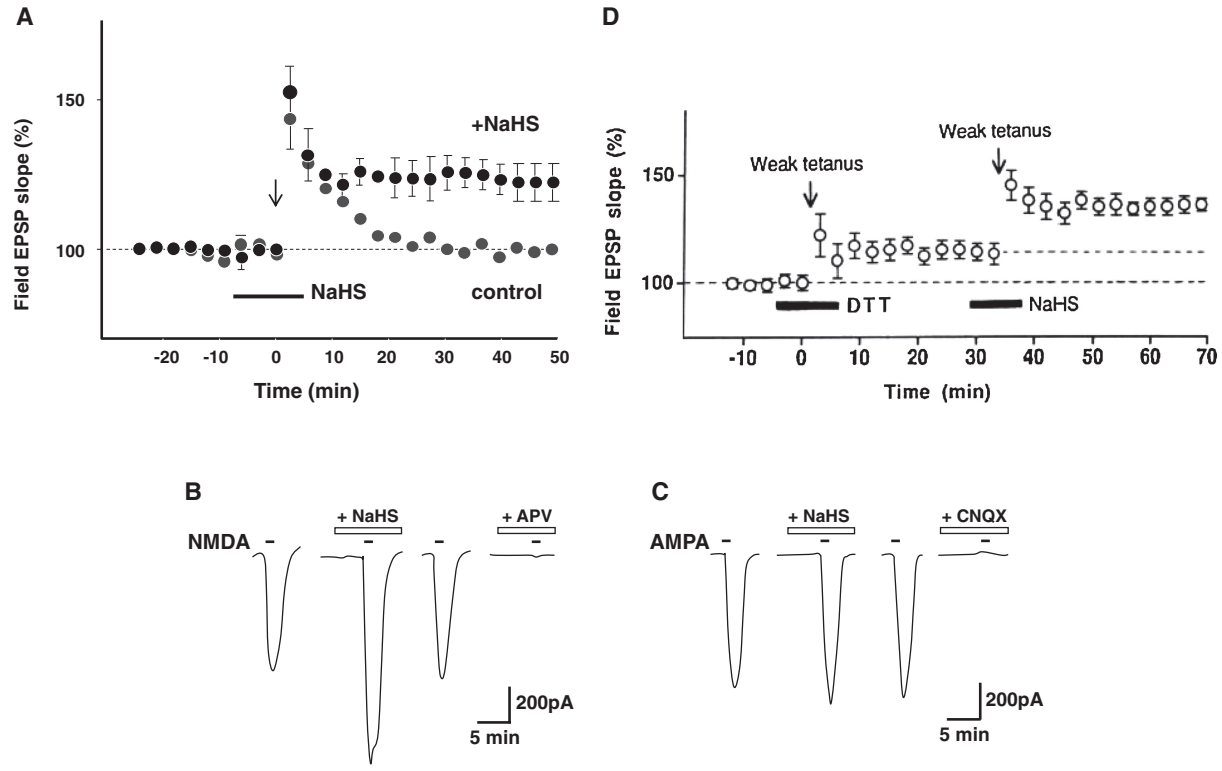

Fig. 1. H_2S facilitates the induction of hippocampal long-term potentiation (LTP) by enhancing the activity of NMDA receptors. A. A weak-tetanic stimulation, which alone does not induce LTP, induces LTP in the presence of H_2S. B and C. H_2S enhances the activity of NMDA receptors but not that of AMPA receptors, another type of ionotropic glutamate receptors. D. A lower concentration of NaHS, a sodium salt of H_2S, further facilitated the induction of LTP even after DTT treatment. (Figures in 19 were modified).

of endogenous H_2S. This discovery urged us to study the physiological roles of this molecule in the nervous system.

H_2S-producing enzymes were studied extensively between the 1950s and 1970s; however, H_2S was thought to be merely a by-product of metabolic pathways or a marker for the activities of specific enzymes.[15)–18)] In 1996, we demonstrated that H_2S facilitates the induction of hippocampal long-term potentiation (LTP) by enhancing the activity of N-methyl D-aspartate (NMDA) receptors, and that cystathionine β-synthase (CBS) produces H_2S in the brain[19)] (Figs. 1 and 2). Using these observations, we proposed a possible role for H_2S 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_2S as a neurotransmitter.[20)] In 2009, Snyder *et al.*[21)] demonstrated a mode of action for H_2S, 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_2S.[22)]

H_2S also apparently has a role in the vasculature. We found that cystathionine γ-lyase (CSE) is an H_2S-producing enzyme in smooth muscle and that H_2S is a smooth muscle relaxant[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.*[4)] demonstrated that NO is also released through the NMDA receptors of brain neurons in a Ca^{2+}-dependent manner. Bredt and Snyder[24)] subsequently identified NO synthase in the brain.

H_2S relaxes the thoracic aorta, portal vein, and ileum.[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_2S is greatly augmented by the presence of NO,

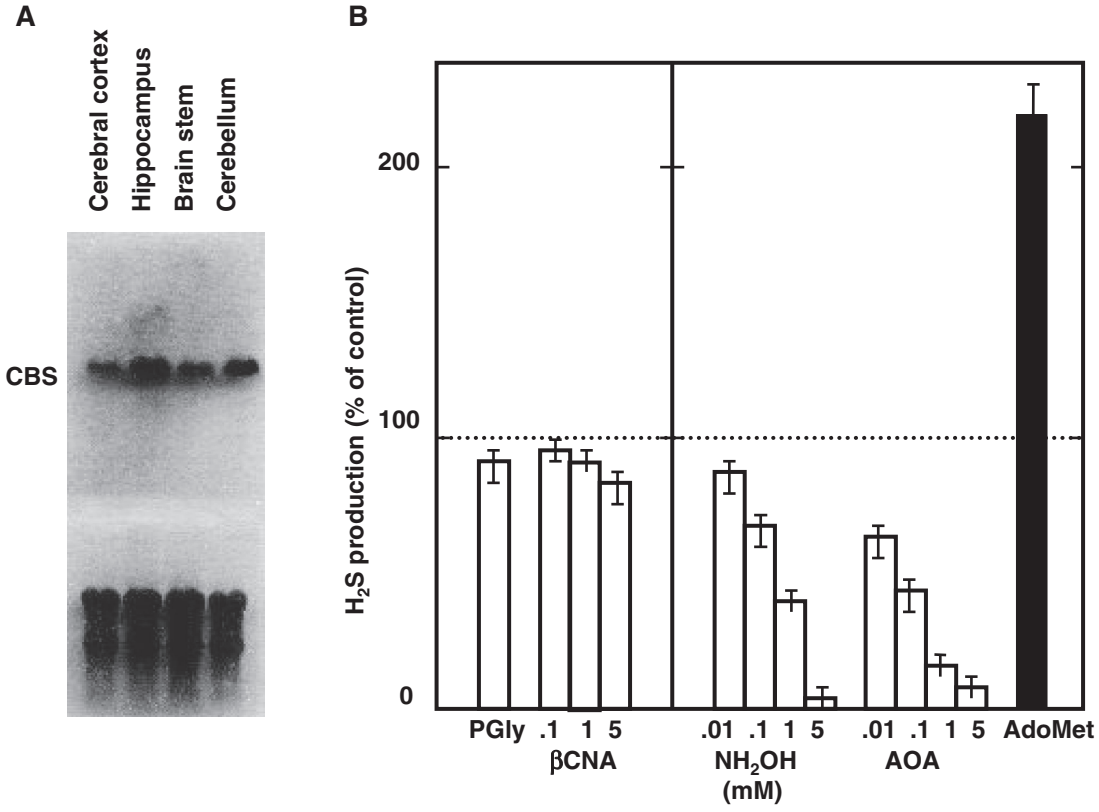

Fig. 2. The expression of CBS in the brain and the production of H_2S. A. CBS is expressed in the brain. B. The production of H_2S is suppressed by hydroxylamine (NH_2OH) and aminooxyacetate (AOA), inhibitors of CBS, but not by propargylglycine (PGly), an inhibitor of CSE. S-adenosyl methionine (AdoMet) enhances the production of H_2S (Figures in 19 were modified).

which suggests a synergy between these two molecules[23] (see Fig. 3). This synergistic effect was confirmed in the twitch responses of the ileum by Teagues *et al.*[25] With respect to the mechanism of H_2S effects on the vasculature, Zhao *et al.*[26] identified ATP-sensitive K^+ channels as H_2S targets for relaxation and hyperpolarization in vascular smooth muscle. Mustafa *et al.*[27] 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_2S continues in the following discussion of the basic properties of this molecule and its enzymatic production.

2. Basic properties of H_2S

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_2S dissolves in 100 mL of water at 20 °C. In solution, H_2S 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_2S exists as a gas and the remainder as HS^- with a trace amount of S^{2-}.

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

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

pK values were obtained from Reiffenstein *et al.*[10] Note also that Meyer *et al.*[28] reported a pK_2 of 13.78.

Because it is difficult to determine which form of H_2S (H_2S, HS^-, or S^{2-}) is active, the term "hydrogen sulfide" is used to include all forms.[19] The term "gasotransmitter" has often been used for hydrogen sulfide, which can mislead readers into believing that H_2S 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_2S/HS^- likely varies among subcellular compartments.[29] Although the precise ratio of H_2S/HS^- in each organelle has not been measured, it is likely higher (*i.e.*, a greater

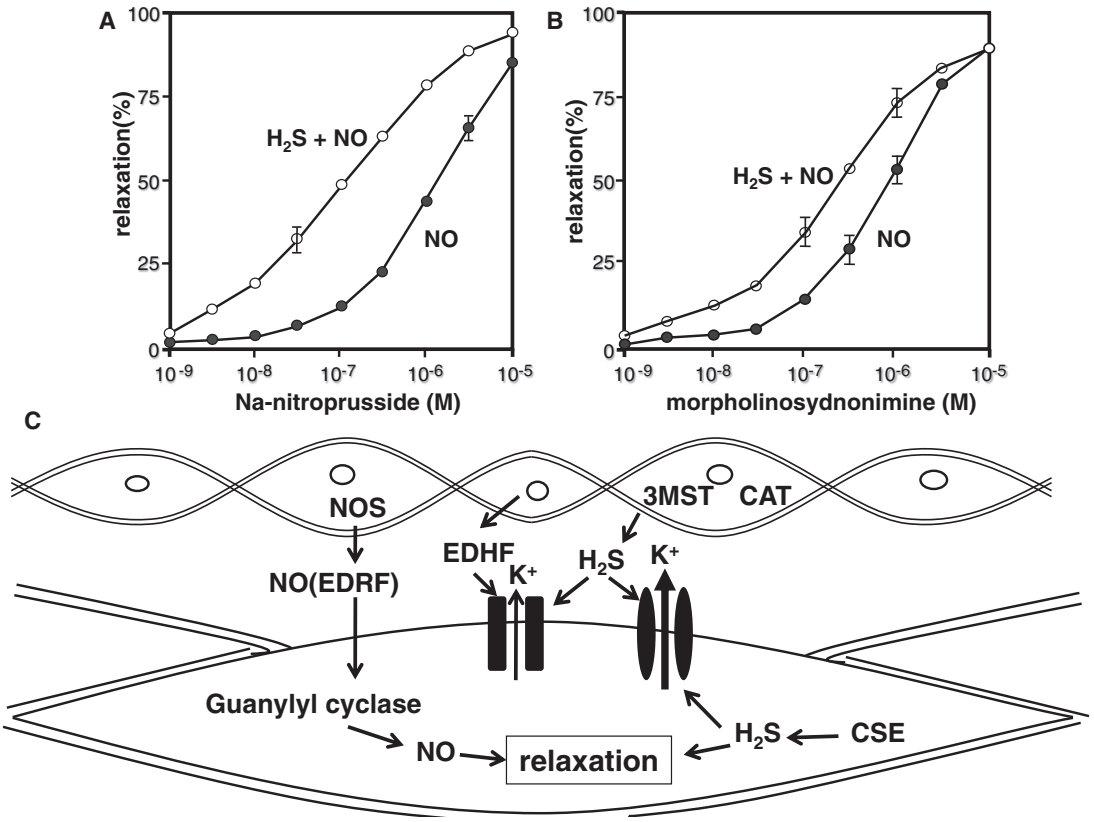

Fig. 3. A synergistic effect of H_2S with NO on vascular smooth muscle relaxation. The vascular relaxation effect of sodium nitroprusside (A) and morpholinosydnonimine (B) is greatly enhanced in the presence of H_2S (Figures in 23 were modified). C. H_2S, which is produced by 3MST together with CAT in endothelium and CSE in smooth muscle, relaxes smooth muscle and hyperpolarizes the membrane potential by activating potassium channels.

amount of undissociated H_2S) in subcellular compartments in which the pH is more acidic.

H_2S has a molecular structure similar to that of H_2O; 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_2S does not pass through aquaporins or water channels.[30] H_2S 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_2S 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).[30] Another diffusion model proposed by Cuevasanta *et al.*[31] estimates the permeability of H_2S at 0.85 cm/s. Considering that intracellular pH is lower than extracellular pH, H_2S passes more quantitatively through plasma membranes in the intracellular to extracellular direction.

Theoretical calculations by Mathai *et al.*[30] 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.[32] H_2S and HS^- rapidly enter erythrocytes, which may act as a sink for H_2S and regulate its local extracellular concentrations (Fig. 4).

An HS^- channel has also been identified in bacteria.[33] 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_2S gas is dominant. Therefore, H_2S passes through the bacterial membrane from the extracellular into the intracellular environment. However, in contrast to H_2S production in mammals, H_2S produced in bacteria dissociates to HS^-, which can pass through HS^- channels to the extracellular environment and be released as H_2S gas (see Fig. 4).

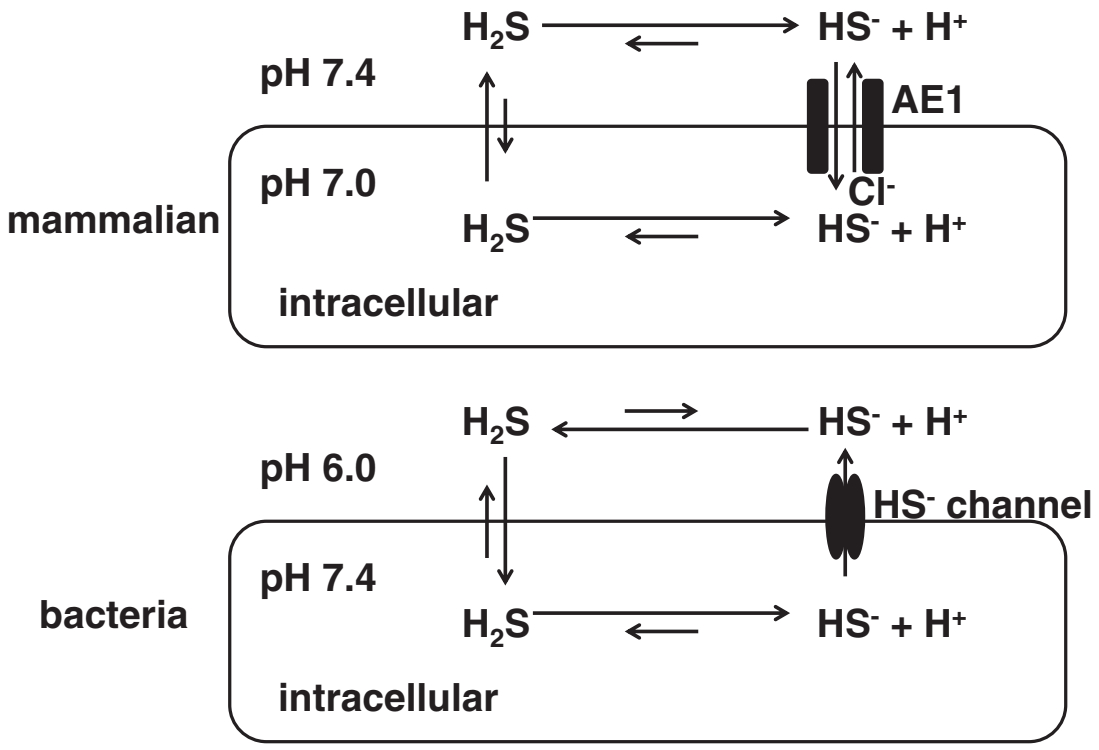

Fig. 4. H_2S passes through the membrane and HS^- through HS^- channels. H_2S dissociates to HS^- and H^+ with a trace amount of S^{2-} under physiological conditions. At pH 7.4 and 37 °C, approximately 20% H_2S exists as a gas, and remaining 80% as HS^-. In mammalian cells, H_2S passes through the plasma 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_2S enters into cells through the plasma membrane similar to that seen for mammalian cells.

3. Endogenous H_2S

In addition to being present as free H_2S, H_2S is produced by enzymes and released from cellular stores.[22] Two stored forms of H_2S 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_2S under acidic conditions. Conversely, bound sulfane sulfur, which exists as the sulfurated cysteine residues of proteins, releases H_2S under reducing conditions (Fig. 5).

3.1. Acid-labile sulfur. The concentrations of endogenous free H_2S 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_2S in much greater amounts than those observed naturally. Nevertheless, we wanted to determine whether acid-labile sulfur functions as an H_2S store in cells. The pH at which H_2S 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_2S may not be released from acid-labile sulfur under physiological conditions (see Fig. 5). Although iron-sulfur complexes also release H_2S 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_2S under reducing conditions (see Fig. 5). We observed the absorption of H_2S in tissues and found that the rate of absorption depends on tissue type. Absorbed H_2S can be recovered with a reducing substance such as dithiothreitol (DTT).[22),36)] H_2S must be oxidized to bind to proteins as a persulfide or polysulfide.[37] Because

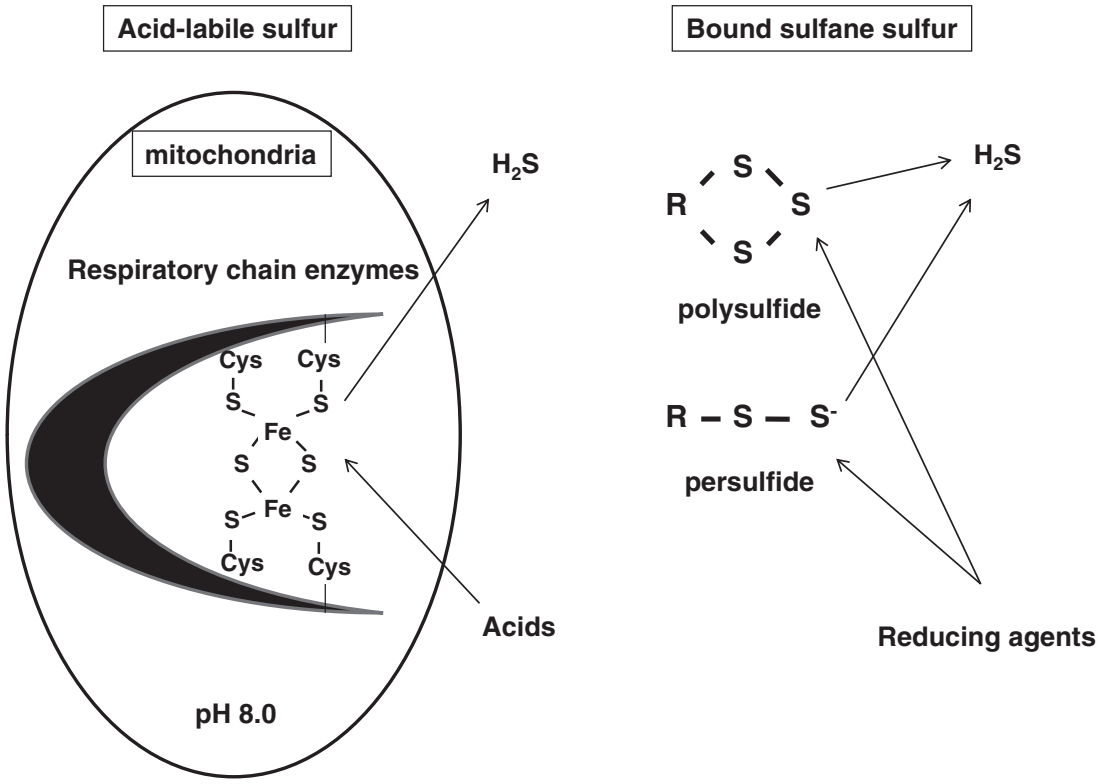

Fig. 5. Two forms of intracellular sulfur that can release H_2S. The iron-sulfur cluster, which localizes to the active center of enzymes in the respiratory chain and releases H_2S under acidic conditions, forms the major acid-labile sulfur in cells. Bound sulfane sulfur, which consists of polysulfide and persulfide bound to proteins, releases H_2S under reducing conditions and may form the intracellular storage for H_2S.

exogenously applied H_2S is absorbed and stored in proteins, we predicted that endogenous H_2S produced by enzymes might also be incorporated into proteins. To this end, we found that cells expressing 3-mercaptopyruvate sulfurtransferase (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_2S (*i.e.*, the levels of bound sulfane sulfur remain at control levels). We concluded that H_2S produced by enzymes is stored as bound sulfane sulfur in cells.

Does bound sulfane sulfur release enough H_2S with appropriate timing for the exertion of H_2S activity? Lysates of neurons and astrocytes release H_2S in the presence of endogenous concentrations of cysteine and glutathione at pH 8.4 or when dihydrolipoic 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_2S from bound sulfane sulfur.[40] However, we were unable to detect the release of H_2S from astrocytes.[22] Further investigations are required to determine whether bound sulfane sulfur is an H_2S store in cells.

3.3. Free H_2S. The cellular concentration of free H_2S can change significantly in a short period because (1) H_2S is produced and degraded by enzymes, (2) the sulfur of H_2S is incorporated into cysteine residues after being oxidized to H_2S_n, and (3) the reaction between thiol and H_2S_n releases H_2S. Although local changes in H_2S 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_2S remained as H_2S gas without the release of H_2S from acid-labile

sulfur. The endogenous concentration of H_2S in the brain was determined to be 14 nM via this method. Using a modified method, Furne *et al.*[41] 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_2S 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_2S 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.*[43),44] used this method to measure H_2S 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.*[34),41] However, monobromobimane complexed with other thiols, which has the same HPLC retention time as monobromobimane complexed with H_2S, may cause overestimation of the value.[35]

We mixed brain homogenates with silver powder, which reacts with H_2S 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_2SO_4 to recover H_2S from silver sulfide. The amount of H_2S was then measured with gas chromatography. In this method, the steady-state level of H_2S 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_2S, like NO, is maintained at low steady-state concentrations. The repetitive application of H_2S to astrocytes causes desensitization and a drop in Ca^{2+} influx. Thus, free H_2S must apparently be maintained in cells at low levels for proper cellular response to H_2S.[45]

4. H_2S production

H_2S is produced by three enzymes — CBS, CSE, and 3MST — along with CAT, which is identical to aspartate aminotransferase. H_2S 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_2S-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_2S.[46),47]

$$\text{Cysteine} + \text{homocysteine} \rightarrow \text{cystathionine} + H_2S \quad [3]$$

$$\text{Cysteine} \rightarrow \text{serine} + H_2S \quad [4]$$

$$\text{Cysteine} \rightarrow \text{lanthionine} + H_2S \quad [5]$$

As described in the previous section, free H_2S is maintained at low concentrations in the steady state. For H_2S 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_2S 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_2S 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_2S 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_2S production and clearance also plays an important role in controlling H_2S concentration.[53),54] H_2S 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

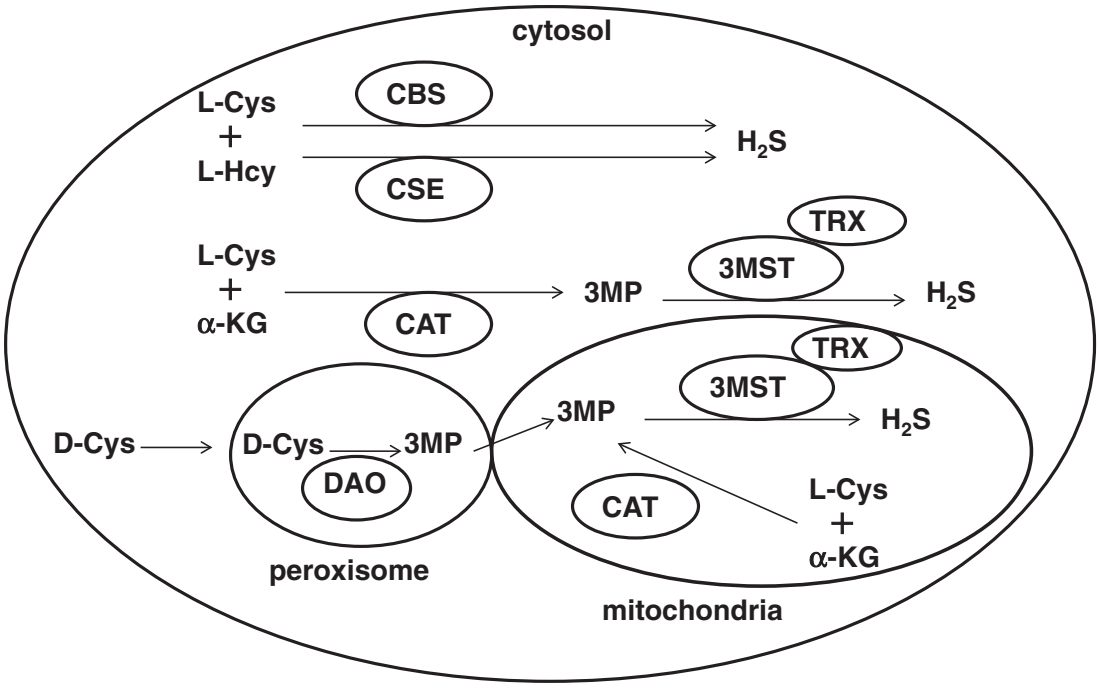

Fig. 6. H_2S-producing pathways. CBS and CSE are localized to cytosol and produce H_2S from L-cysteine alone or L-cysteine along with L-homocysteine. 3MST and CAT are localized to both cytosol and mitochondria. L-cysteine and α–ketoglutarate are metabolized by CAT to 3-MP, which is a substrate for 3MST to produce H_2S. Thioredoxin interacts with 3MST to produce H_2S (see also Fig. 7). D-Cysteine is metabolized in peroxisomes by DAO to 3MP, which is transported into mitochondria via a specific form of the vesicular transport. In mitochondria, 3MP is metabolized by 3MST to H_2S. Peroxisome and mitochondria are in close vicinity or have a physical contact.

periods. CBS expression is up-regulated in reactive astrocytes. This up-regulation is induced by epidermal growth factor, transforming growth factor-α, cyclic adenosine monophosphate, and dexamethasone. In CBS knockout mice, which show abnormal lipid metabolism in the liver, cerebellar morphological abnormalities are significant.[58),60)] Although the localization of CBS to cerebellar Purkinje cells and hippocampal neurons has also been reported,[61)] we were unable to find CBS in these neurons even with the antibody used by Robert *et al.*[58)]

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.[59)] 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.[62)] 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.[59)]

4.2. CSE. Since our demonstration that CSE is an H_2S-producing enzyme in smooth muscle tissues,[23)] the localization and activity of this enzyme have been studied extensively. CSE produces H_2S via the PLP-dependent α,β-elimination reaction with cysteine.[47),63),64)]

$$\text{Cysteine} + \text{homocysteine} \rightarrow \text{cystathionine} + H_2S \quad [6]$$

$$\text{Homocysteine} + \text{homocysteine} \rightarrow \text{homolanthionine} + H_2S \quad [7]$$

$$\text{Cysteine} + \text{cysteine} \rightarrow \text{lanthionine} + H_2S \quad [8]$$

Yang *et al.*[65)] reported that the activity of CSE is regulated by Ca^{2+}/calmodulin. However, although CSE is localized in the cytosol, they examined its regulation in the presence of 1–2 mM Ca^{2+}, which is the extracellular Ca^{2+} concentration. The intracellular Ca^{2+} 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_2S-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_2S 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_2S 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_2S production.[67] These observations suggest that 3MST and CAT are localized to the endothelium to produce H_2S.

Because H_2S 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_2S-producing enzyme, CSE, is inadequate to change blood pressure significantly because the H_2S-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_2S 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_2S 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_2S production in the brain is not suppressed by propargylglycine, an inhibitor selective to CSE, it appears that this enzyme contributes little to H_2S production in the brain.[19),70),73] 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_2S-producing enzymes. Because CSE was not found in the brain, we thought that CBS was a unique H_2S-producing enzyme in brain tissue.[19),70] However, we found that the brains of CBS knockout mice produced H_2S, which suggested that a third H_2S-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_2S. A possible alternative to CBS for H_2S production in the brain was 3MST.[75)–77] A substrate of 3MST, 3-mercaptopyruvate (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_2S from 3MP, which is produced from cysteine and α-ketoglutarate by CAT (see Fig. 6).

$$\text{CAT: cysteine} + \alpha\text{–ketoglutarate} \rightarrow 3\text{MP} + \text{glutamate} \quad [9]$$

$$\text{3MST: 3MP} \rightarrow \text{pyruvate} + H_2S \quad [10]$$

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_2S production via the 3MST/CAT pathway.[80),81]

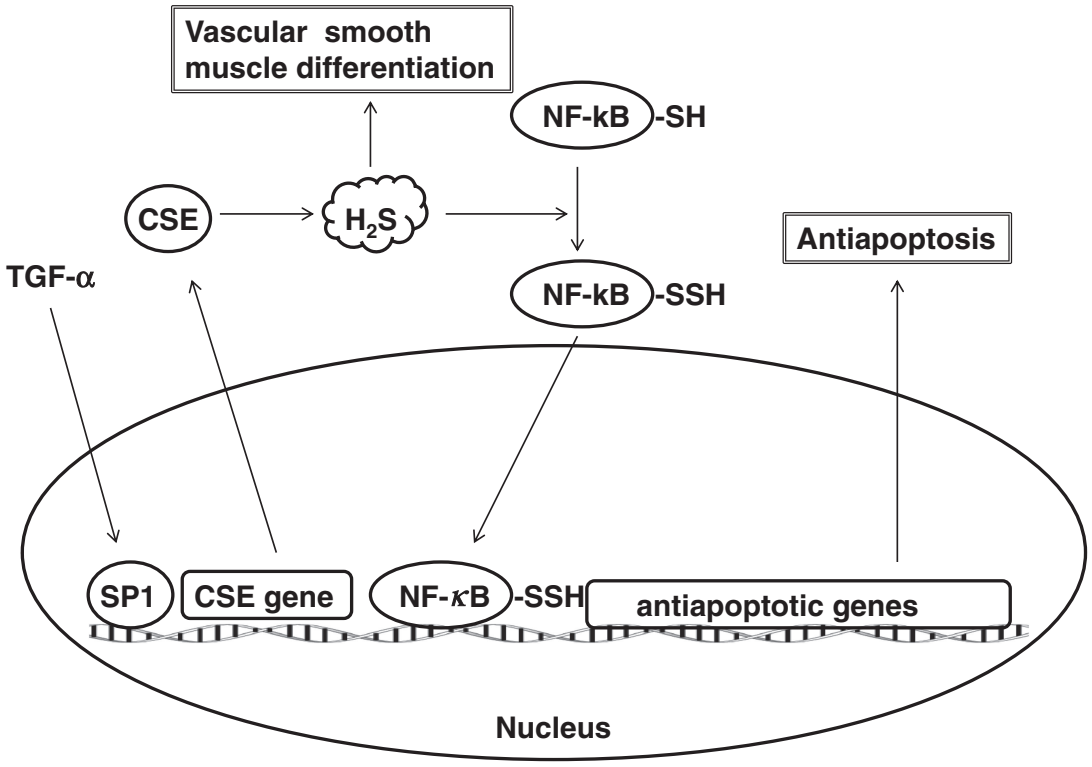

Fig. 7. Transcription factor SP-1 up-regulates the *CSE* gene. H_2S produced by CSE, whose transcription is increased by TGF-α mediated through SP1 binding activation, sulfurates (sulfhydrates) NF-κB to make it translocate into the nucleus and up-regulate antiapoptotic genes. Increased production of CSE by the activation of SP1 also plays an important role in the vascular smooth muscle differentiation.

CBS and CSE do not require a reducing substance such as DTT for H_2S 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 H_2S-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.[82)–84)] Given these observations, we hypothesized that thioredoxin may be the endogenous reducing substance associated with 3MST that allows for H_2S production. Thioredoxin has two forms, thioredoxin 1 and 2, that are localized in the cytosol and mitochondria, respectively.[84),85)] 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.[86)] 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.[87)] Approximately 20 µM thioredoxin is present in cells,[84)] and H_2S is produced in fourfold greater amounts in the presence of thioredoxin than in the presence of the same concentration of DTT[39)] (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 H_2S by 3MST.[39),88),89)] Other physiological reducing substances such as glutathione (GSH), cysteine, NADPH, nicotinamide adenine dinucleotide, and coenzyme A show no effect on the production of H_2S 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.[90),91)] The reducing potentials of monothiols such as GSH, cysteine, and coenzyme A range from -0.22 to -0.35 V,[92)] and those of

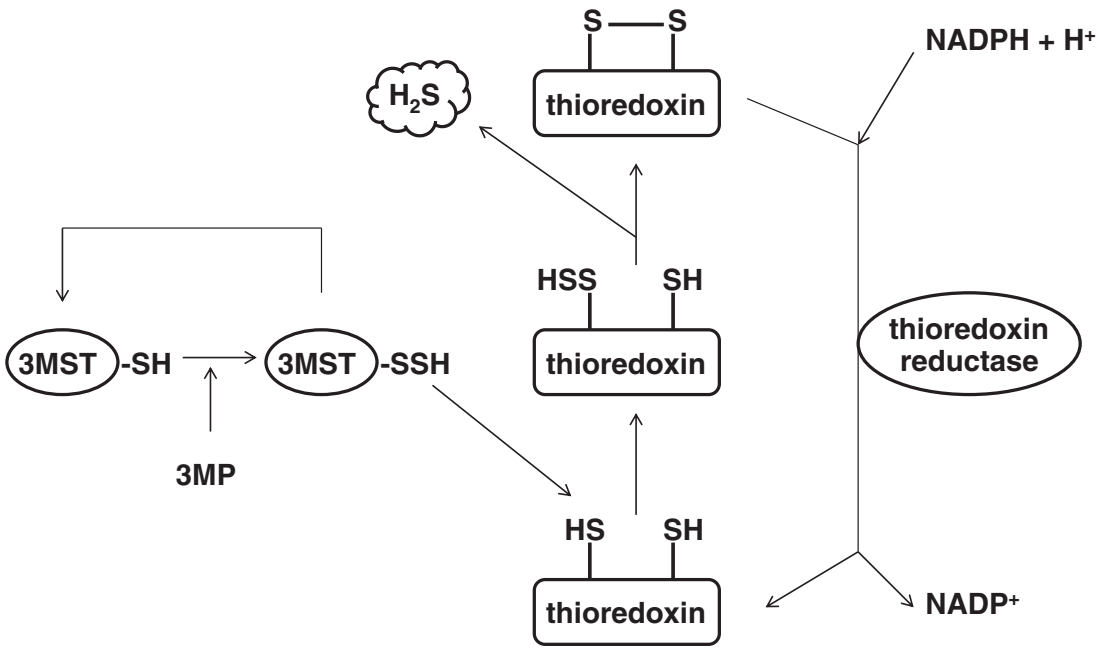

Fig. 8. 3MST produces H_2S with thioredoxin as an acceptor of sulfane sulfur. 3MST receives sulfur from 3MP to produce 3MST persulfide, which is transferred to one of the thiols in thioredoxin to generate thioredoxin persulfide. The remaining thiol reacts with persulfide to release H_2S. Oxidized thioredoxin is reduced back to its reduced form by thioredoxin reductase.

nicotinamide adenine dinucleotide and NADPH are −0.320 and −0.324 V, respectively.[93] These data show that reducing potential has no correlation with the capacity of these substances to affect H_2S production by 3MST, but dithiols such as DTT, DHLA, and thioredoxin have critical effects.[39),83]

4.4. 3MST and DAO. Because mammalian enzymes generally metabolize L-amino acids with few exceptions (D-serine and D-aspartate),[94),95] we applied D-cysteine to brain homogenates as a negative control to L-cysteine for H_2S production. Unexpectedly, H_2S was produced from D-cysteine.[78] Although the production of H_2S from D-cysteine has been studied in microorganisms and plants,[96] its production in mammals is not well understood. The production of H_2S from L-cysteine and D-cysteine has significantly different dependencies on pH and PLP. Because mitochondrial 3MST produces H_2S from 3MP, we searched for an enzyme that produces 3MP from D-cysteine. DAO has been proposed to produce 3MP in hepatocytes.[97] Indeed, our study showed that in the absence of DAO, H_2S was not produced from D-cysteine and that indole-2-carboxylate, a DAO inhibitor, suppressed H_2S production from D-cysteine.[78] D-Cysteine is metabolized by DAO to achiral 3MP, which is a substrate of 3MST for the production of H_2S (see Fig. 6).

$$\text{DAO: D-cysteine} + O_2 + H_2O \rightarrow 3\text{MP} + H_2O_2 + NH_3 \quad [11]$$

$$\text{3MST: 3MP} \rightarrow \text{pyruvate} + H_2S \quad [10]$$

3MST localizes to mitochondria, whereas DAO is present in peroxisomes. Mitochondria and peroxisomes exchange various metabolites via a specific form of vesicular trafficking, and they are usually in close proximity or physical contact with one another.[98] 3MP produced by DAO in peroxisomes is transferred via vesicular trafficking to mitochondria, in which H_2S is produced by 3MST (see Fig. 6).

3MST is a ubiquitous enzyme, whereas DAO is localized to the brain and kidney.[78] H_2S is produced from D-cysteine only in the kidney and the brain (mostly in the cerebellum). The production in the kidney is seven times that in the cerebellum. In the kidney, the production of H_2S from D-cysteine is approximately 80 times that from L-cysteine.[78] Compared with L-cysteine administration, the oral administration of D-cysteine to mice increases the levels of bound sulfane sulfur, a potential intracellular store of H_2S, and protects the kidneys from ischemia-reperfusion injury much more efficiently. D-Cysteine may provide a new therapeutic approach to mitigate ischemia-reperfusion injury by delivering H_2S to specific tissues such as the kidney and cerebellum.

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.[99)–101)] 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.[102)]

5. From toxin to cytoprotectant

Because H_2S is a toxic gas, it was generally not predicted to have a cytoprotective effect. However, our research indicated that high concentrations of H_2S in the primary cultures of neurons did not cause cell death but rather protected them from oxidative insults or oxidative glutamate toxicity.[103),104)] There are two forms of glutamate toxicity: excitotoxicity and oxidative toxicity. Excitotoxicity is caused by the long-lasting activation of NMDA receptors through which Ca^{2+} enters cells at levels beyond cell tolerance.[105)] Oxidative toxicity is caused by high concentrations of glutamate suppressing the cystine/glutamate antiporter, which imports cystine into cells with reciprocal export of glutamate.[106)] 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.[106)] Cultures of embryonic neurons die 24 h after being exposed to high concentrations of glutamate but survive in the presence of H_2S.[103)] This cryoprotection by H_2S is effective even 8 h after the application of glutamate, although the efficiency declines with time.[104)] Because H_2S readily evaporates from culture medium (*i.e.*, half-life of approximately 10 min[42),104)]), its effect of making cells commit to resist oxidative stress occurs during this initial short exposure.

H_2S 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. H_2S 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.[103)] Through these effects, H_2S increases the production of GSH (Fig. 9).

H_2S produced by 3MST in the mitochondrion suppresses oxidative stress in this organelle.[107)] The endogenous concentrations of H_2S range from approximately 10 nM to 3 µM, whereas those of GSH are 1–10 mM. Therefore, H_2S suppresses oxidative stress mainly by increasing GSH production (see Fig. 9).

The discovery of the neuroprotective effect of H_2S 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.[108)–111)] H_2S limits infarct size and preserves cardiac function by inhibiting myocardial inflammation and apoptosis and preserving mitochondrial structure and function.[108)] With respect to renal protection during ischemic insult, H_2S 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.[109)]

Other mechanisms for the cytoprotective effect of H_2S have also been identified. For instance, retinal tissue is susceptible to oxidative stress because it consumes large quantities of O_2 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 Ca^{2+} and reactive oxygen species. In the retina, H_2S activates vacuolar-type H^+-ATPase in horizontal cells to release H^+, which suppresses Ca^{2+} channels in photoreceptor cells to maintain intracellular concentrations of Ca^{2+} at appropriately low levels.[112)] Moreover, the regulation of endoplasmic reticulum (ER) stress contributes to the cytoprotective effect of H_2S. After being produced in the ER, proteins are trafficked to the Golgi apparatus. Misfolded proteins trigger the unfolded protein response to start proapoptotic cascades.[113)] ER stress increases the production of H_2S, which inhibits protein tyrosine phosphatase (PTP). In turn, PTP inactivates protein kinase-like ER kinase to inhibit global translation by phosphorylating elF2α, which suppresses ER stress responses.[114)]

In addition to its cytoprotective effects, H_2S regulates bacterial resistance to antibiotics. Shatalin

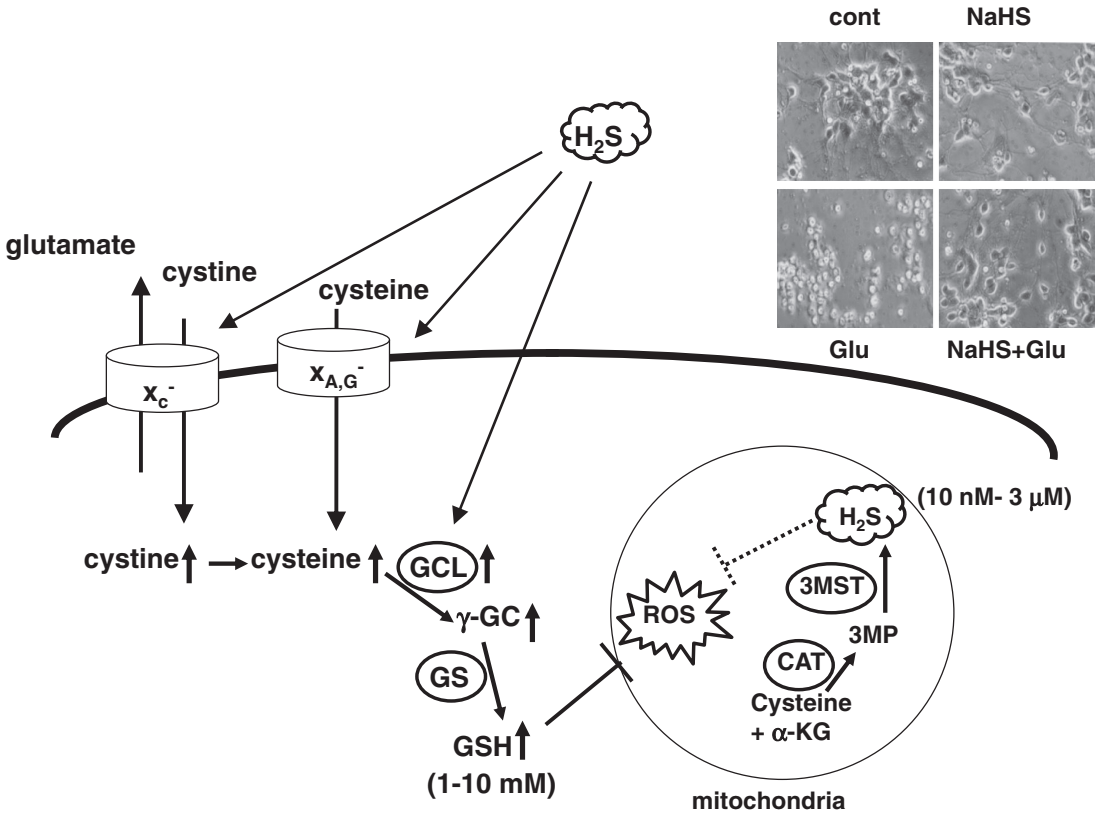

Fig. 9. Cytoprotective effect of H_2S. H_2S protects embryonic neurons from oxidative stress induced by high concentrations of glutamate. H_2S enhances the activity of the cystine/glutamate antiporter and the cysteine transporter to increase the intracellular concentrations of cysteine. H_2S also enhances the activity of glutamate cysteine lygase (GCL) to produce γ–glutamylcysteine to which glycine is added by glutathione synthetase (GC) to generate glutathione (GSH). H_2S produced via the 3-mercaptopyruvate sulfurtransferase (3MST)/(CAT) pathway scavenges reactive oxygen species (ROS), which are abundantly generated in mitochondria. The intracellular concentrations of GSH are 1–10 mM, while those of H_2S are only 10 nM to 3 μM. H_2S efficiently suppresses oxidative stress by increasing the production of GSH rather than by scavenging ROS.

et al.[115] demonstrated that the inactivation of the bacterial homologues of CBS, CSE, and 3MST decreases the production of H_2S, which increases the vulnerability of bacteria to antibiotics. The cytoprotective effect of H_2S appears to be a universal defense mechanism in organisms from bacteria to mammals. Bacteria also produce NO, and both H_2S 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 H_2S, we examined the target of H_2S at the synapse. The activation of NMDA receptors is required to induce LTP. Our observations showed that H_2S enhanced the activity of NMDA receptors but not that of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, another type of glutamate receptor[19] (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 H_2S can reduce the cysteine disulfide bond, the enhancement of NMDA receptor activity is one of the H_2S effects that facilitates LTP induction. However, this was not the only effect of H_2S. We found an inconsistency that introduces difficulty in explaining the induction of LTP solely by the reducing activity of H_2S. DTT,

which is a more efficient reducing agent than H_2S, facilitated the induction of LTP, but a lower concentration of H_2S further facilitated the induction of LTP even after DTT treatment.[19] This discrepancy suggests that H_2S 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 H_2S may have an effect on astrocytes that in turn modulates synaptic activity, which leads to the facilitation of LTP. We first observed that H_2S induces Ca^{2+} influx in astrocytes.[45] The responses were abolished in the absence of extracellular Ca^{2+} and by broad-spectrum transient receptor potential (TRP) channel inhibitors such as La^{3+}, Gd^{3+}, and ruthenium red, suggesting that H_2S activates TRP channels to induce Ca^{2+} influx.[45] Only glial fibrillary acidic protein-positive mature astrocytes respond to H_2S; immature or reactive astrocytes induced by epidermal growth factor, transforming growth factor-α, cyclic adenosine monophosphate, or interleukin-1β do not.[117]

6.3. Identification of H_2S_n. During the study of the effect of H_2S on astrocytes, we found that compared with NaHS (a sodium salt of H_2S), sodium tri- and tetrasulfide (Na_2S_3 and Na_2S_4; sodium salts of H_2S_3 and H_2S_4) induce Ca^{2+} influx in astrocytes much more potently.[45),118)–120] Unlike H_2S, H_2S_n does not exist as a gas under physiological conditions. Therefore, Na_2S_n is dissolved in water and dissociates to H^+ and $HS_n{}^-$. Given that the applied concentrations of Na_2S_n are low, the effect of Na^+ can be ignored.

$$H_2S_n \rightarrow H^+ + HS_n{}^- \quad [12]$$

Comparison of the half-maximal effective concentration (EC_{50}) for the induction of Ca^{2+} influx of Na_2S_3 ($EC_{50} = 91\,nM$) and NaHS ($EC_{50} = 116\,\mu M$) has shown that Na_2S_3 is approximately 1000 times more potent than NaHS[45),120] (Fig. 10).

While investigating the effects of NaHS on the induction of Ca^{2+} 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 Ca^{2+} influx. We speculated that the yellow color originated from elemental sulfur and that a synergy exists between H_2S and elemental sulfur that activates TRP channels. When elemental sulfur was dissolved in a solution of NaHS, the solution turned yellow and potently induced Ca^{2+} influx in astrocytes. Searcy and Lee[121] have reported that elemental sulfur dissolved in Na_2S solution, another sodium salt of H_2S, generates H_2S_n species (Na_2S_3 and sodium pentasulfide), and in 2006, we demonstrated that Na_2S_3 and Na_2S_4 induce Ca^{2+} 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 H_2S but the effect of the H_2S_n products from both agents that potently induces Ca^{2+} influx.

6.4. H_2S_n activates TRP ankyrin 1 (TRPA1) channels in astrocytes. In 2008, Streng *et al.*[122] demonstrated that H_2S activates the TRPA1 channels of sensory neurons in the urinary bladder and that Chinese hamster ovary cells overexpressing TRPA1 channels respond to H_2S. 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. H_2S is oxidized to H_2S_n 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 H_2S_n species under physiological conditions remains undetermined.

$$2nH_2S + 1/2(2n-1)O_2 \rightarrow H_2S_{2n} + (2n-1)H_2O \quad [13]$$

$$HS^- \leftrightarrow HSS^- \leftrightarrow HSSS^- \leftrightarrow \ldots \leftrightarrow HS_7{}^- \rightarrow S_8 \quad [14]$$

Therefore, a portion of NaHS may have been oxidized to Na_2S_n, which activated TRPA1 channels in previous studies. Taking these results and our previous observation into account, we hypothesized that H_2S_n 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 Ca^{2+}

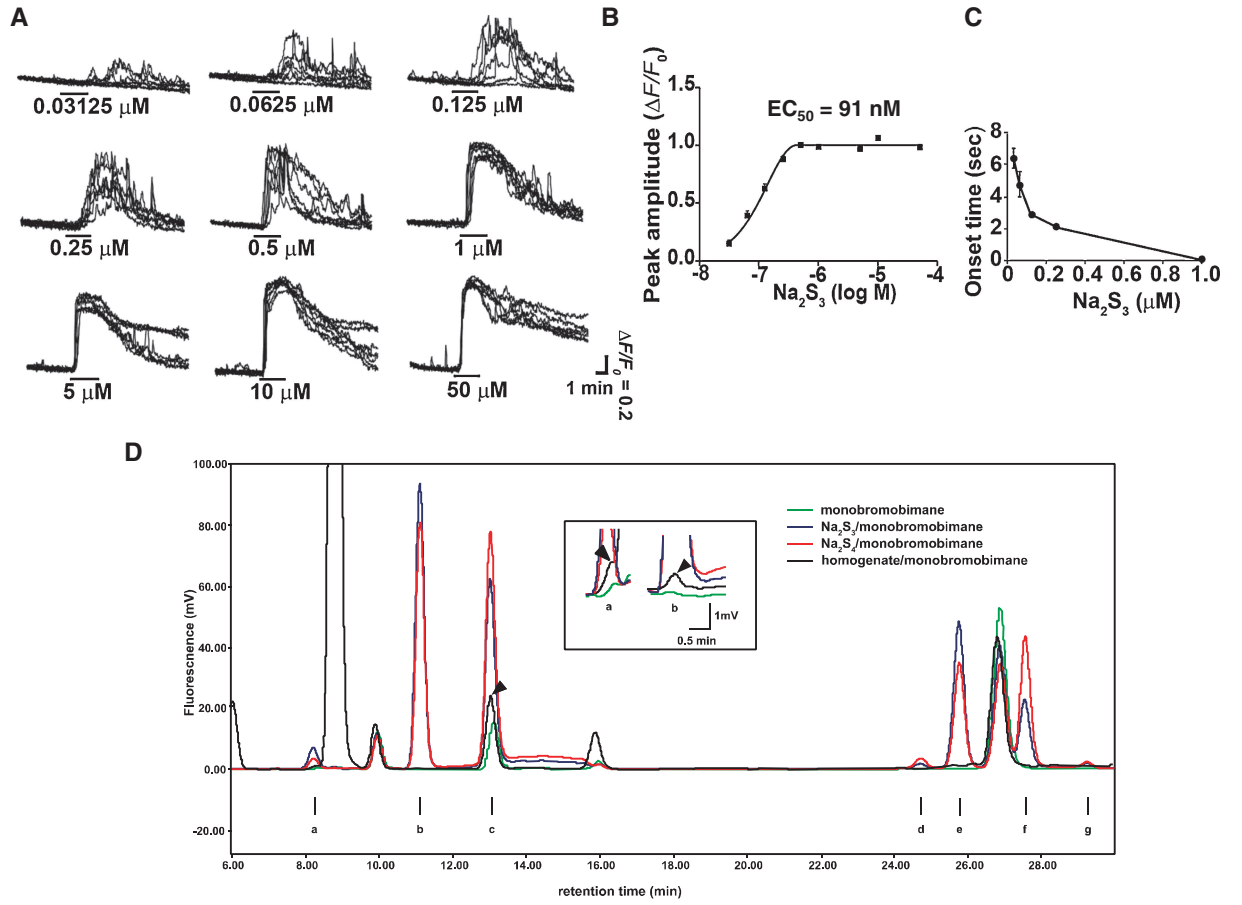

Fig. 10. Existence of H_2S_n in the brain and the induction of Ca^{2+} influx in astrocytes. H_2S_3 induces Ca^{2+} influx in astrocdytes in a dose-dependent manner with $EC_{50} = 91$ nM (A and B). Because H_2S_n are mixture of molecules with different number of sulfur atoms in equilibrium as shown in the Eq. [14], even the standard Na_2S_3 and Na_2S_4 exert several peaks at the same retention times. Brain samples contain H_2S_n (Figures in 120 were modified).

influx and confirmed the existence of these channels in astrocytes.[119),120)] The responses to Na_2S_3 and Na_2S_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_2S_n species activate TRPA1 channels in astrocytes.[120)]

Ogawa *et al.*[123)] determined the site within TRPA1 channels that was sensitive to high H_2S 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_2S_n 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_2S_n in the brain. We identified H_2S_n in the brain by using HPLC to analyze samples derivatized with monobromobimane.[120)] Because H_2S_n species are mixture with various numbers of sulfur atoms in equilibrium, even the standard Na_2S_3 and Na_2S_4 exhibit several peaks. The endogenous level of a major H_2S_n (*i.e.*, a major peak observed in HPLC) is approximately 20 μM in the brain (see Fig. 10). Identification of this H_2S_n is under investigation using liquid chromatography tandem-mass spectrometry.

6.6. Mechanism for the facilitation of LTP. As described in section 6.1, H_2S reduces cysteine disulfide bonds at the ligand-binding domain of NMDA receptors to enhance the activity of the

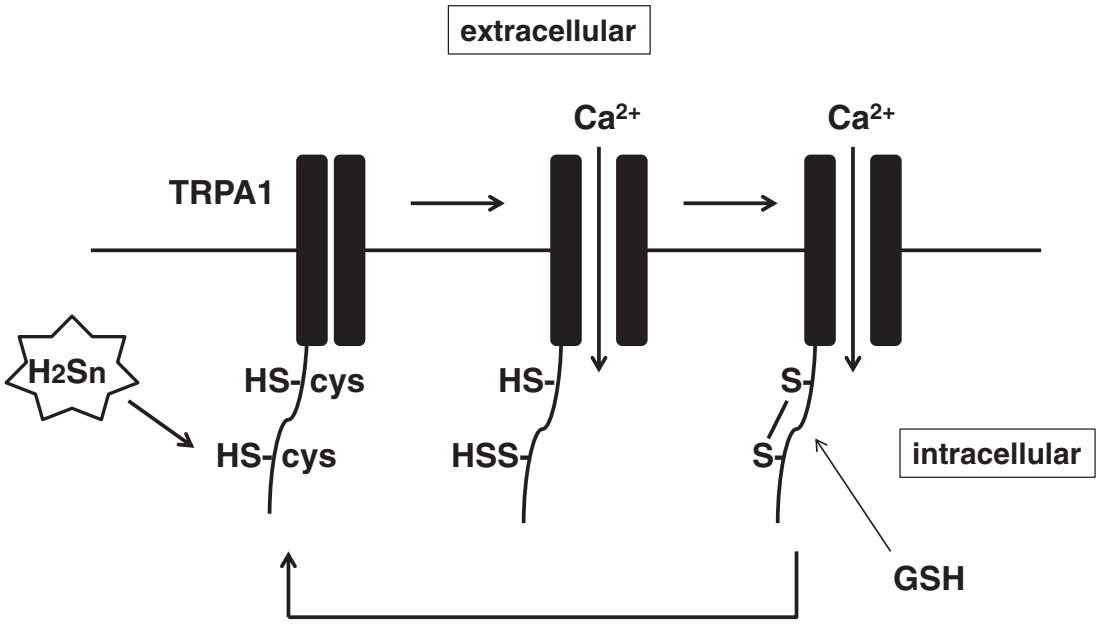

Fig. 11. H_2S_n activates TRPA1 channels. TRPA1 channels are activated by H_2S_n by sulfurating either one or both of the two cysteine residues at their amino terminus, which then form cysteine disulfide bonds. The conformational changes in TRPA1 channels induce Ca^{2+} influx.

receptors, but this reaction does not fully explain the mechanism for its facilitation of LTP induction. Shigetomi *et al.*[128] 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_2S_n activates TRPA1 channels, whereas H_2S activates NMDA receptors by reducing the cysteine disulfide bond.[120] The combined activity of H_2S and H_2S_n 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_2S. The modification of enzyme activities through the addition of sulfur to specific cysteine residues of enzymes was studied extensively between the 1960s and 1980s.[129)–131)] We found that H_2S is absorbed and stored in cells as bound sulfane sulfur, which releases H_2S in response to reducing agents such as DTT.[22] Mustafa *et al.*[21] have defined sulfhydration as a process in which sulfur provided by H_2S attaches to reactive cysteine residues in target proteins. Toohey[37] has suggested calling it sulfuration, as only sulfur is transferred in the process.

Sulfuration of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by H_2S increases its catalytic activity, and DTT treatment removes the added sulfur to diminish activity.[21] This observation is supported by evidence that the GAPDH activity is reduced in CSE knockout mice. Actin polymerization is also enhanced by H_2S and reversed by DTT.[21] ATP-dependent K^+ channels, which are involved in vascular smooth muscle relaxation, are activated by sulfuration at cysteine 43 on the Kir6.1 subunit.[27] Sulfuration of NF-κB by H_2S 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.[72),114)] Parkin, an E3 ubiquitin ligase that is suppressed in Parkinson's disease, is also activated by sulfuration.[132]

Thus, the sulfuration of cysteine residues by H_2S 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_2S is -2, and that in cysteine residues is also -2; Fig. 13). Early studies of sulfuration (sulfhydration) likely measured cysteine residues reacted with H_2S_n produced by the oxidation of H_2S or the oxidized cysteine residues

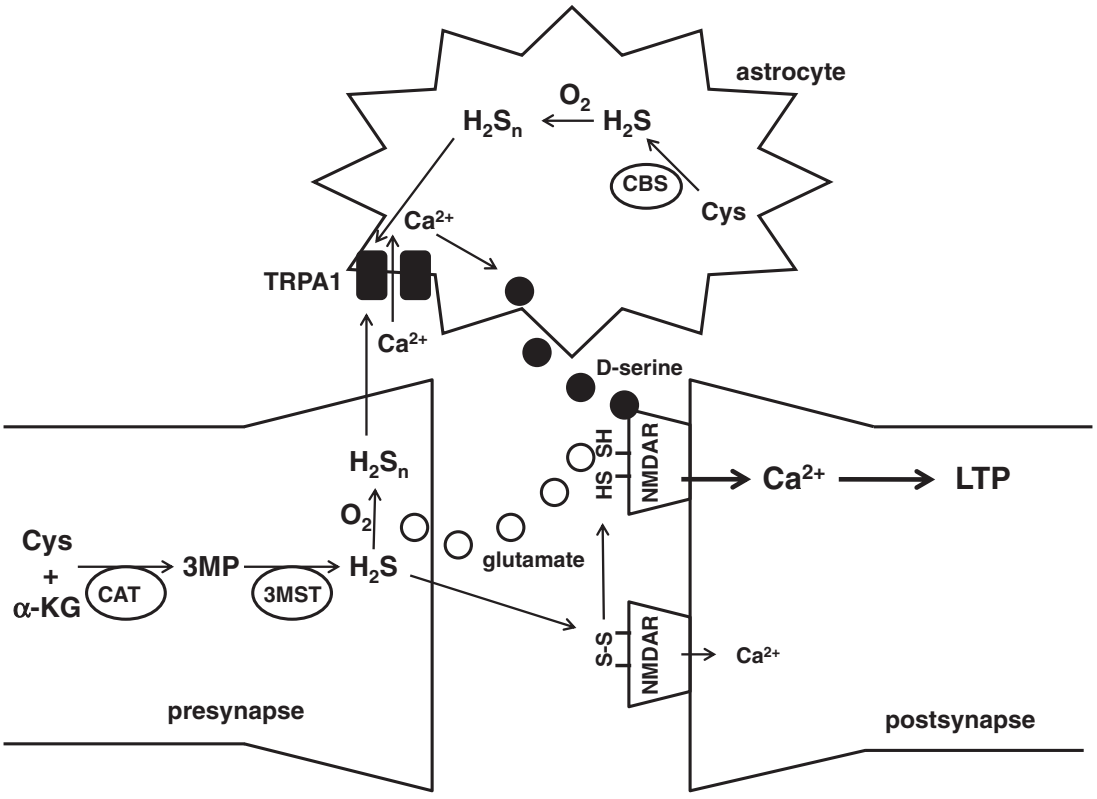

Fig. 12. H_2S together with H_2S_n facilitates the induction of LTP. H_2S enhances the activity of NMDA receptors by reducing a cysteine disulfide bond at the hinge of the ligand-binding domain of the receptors. H_2S_n activates TRPA1 channels in astrocytes to induce Ca^{2+} influx, which facilitates the release of the gliotransmitter, D-serine, to the synaptic cleft. D-serine enhances the activity of NMDA receptors. By these effects of H_2S and H_2S_n LTP is effectively induced.

reacted with H_2S in the same reaction for glutathionylation in which oxidized cysteine residues react with glutathione.[50)]

Tao *et al.*[133)] demonstrated that H_2S reduces cysteine disulfide bonds but does not sulfurate cysteine residues. H_2S induces angiogenesis mediated by vascular endothelial growth factor receptor 2[134),135)] by reducing a disulfide bond located between cysteine 1045 and cysteine 1024. Mass spectrometry analysis shows that H_2S 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 H_2S_n. Because the oxidation state of sulfur in H_2S and cysteine is -2, H_2S is unable to donate electrons to cysteine. By contrast, H_2S_n 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 H_2S_n but not by H_2S.

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 H_2S facilitates the translocation of Nrf2 to the nucleus, where Nrf2 up-regulates the transcription of antioxidant genes that contribute to the cytoprotective effect of H_2S. Yang *et al.*[137)] later proposed a mechanism through which H_2S sulfurates (sulfhydrates) cysteine residues of Keap1, which releases Nrf2 to the nucleus. However, H_2S does not sulfurate cysteine residues of Keap1 for the reason described earlier. Instead, we showed that H_2S_4 sulfurates Keap1[138)] (Fig. 14).

7.3. Other forms of polysulfides. Compared with GSH, glutathione persulfide (GSSH) has greater

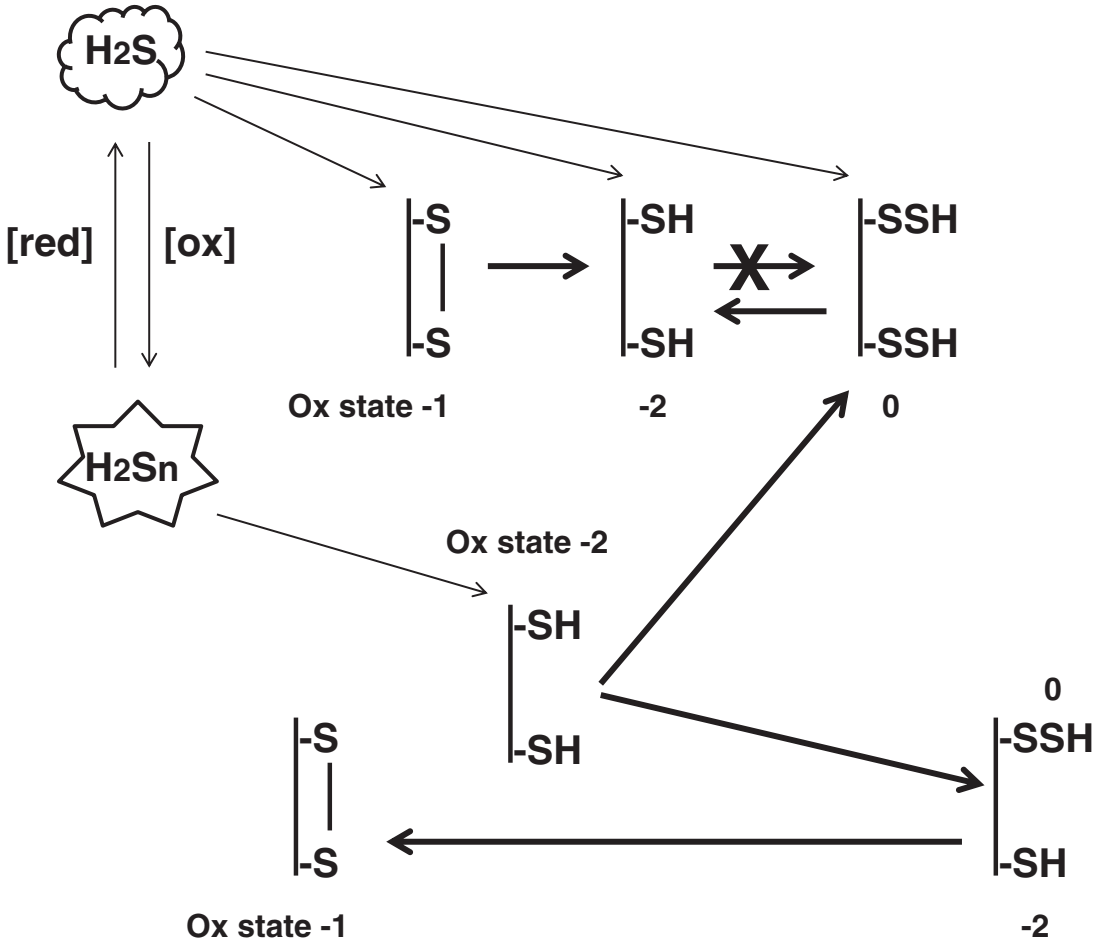

Fig. 13. H_2S reduces cysteine disulfide bond while H_2S_n sulfurates cysteine residues. The atoms with the same oxidation state are not able to transfer electrons and take part in a redox reaction. H_2S reduces sulfur in the cysteine disulfide bond (oxidation state −1) and that in persulfide (oxidation state 0) to cysteine residues (oxidation state −2), while H_2S_n (oxidation state 0) sulfurates cysteine residues. The resulting persulfide reacts with thiols (oxidation state −2) to produce cysteine disulfide bonds.

reducing activity. Massey *et al.*[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.*,[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_2O_2.[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.[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.[141] Even the extracellular concentration of cystine in human blood is only approximately 40 μM.[142] Therefore, it may be difficult for CBS and CSE to produce cysteine persulfide under physiological conditions.

H_2S is metabolized by SQR in mitochondria, and GSH receives a sulfur atom from SQR to generate GSSH.[55)–57)] Libiad *et al.*[143] demonstrated that GSSH can be formed via the enzymatic activity of SQR rather than nonenzymatic sulfane sulfur exchange between cysteine persulfide and oxidized gluthathione reported by Ida *et al.*[141]

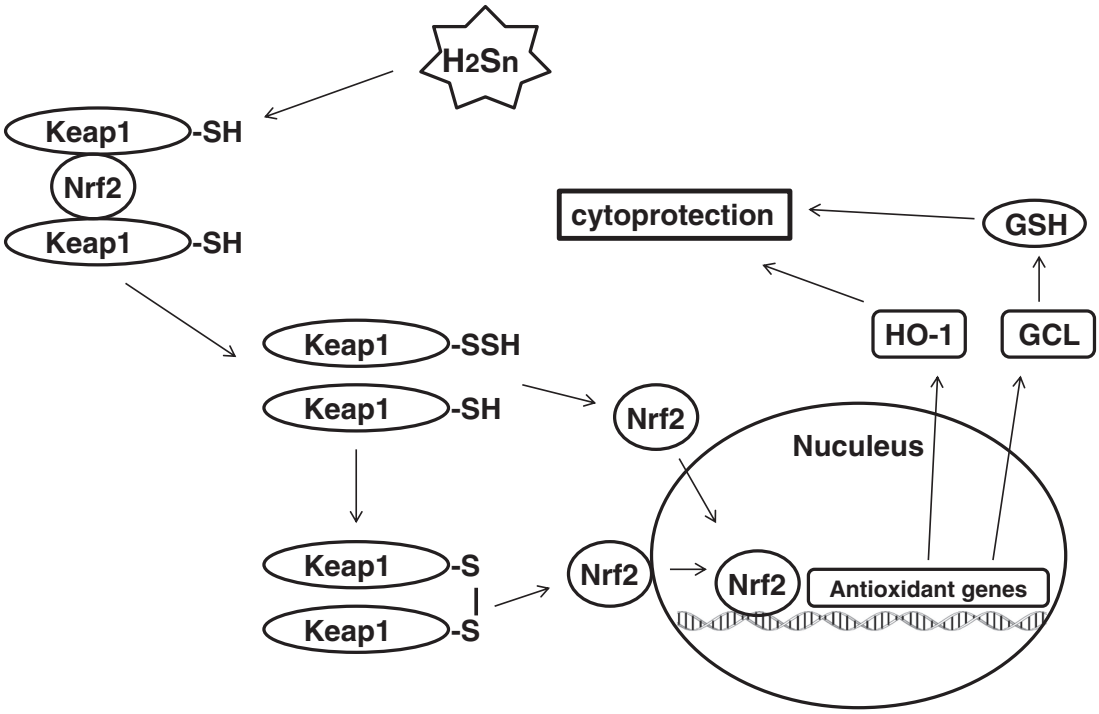

Fig. 14. H_2S_n facilitates the nuclear translocation of Nrf2 to up-regulate antioxidant genes. H_2S_n sulfurates Keap1 to release Nrf2, which translocates to the nucleus and up-regulates the transcription of antioxidant genes such as heme oxygenase 1 and glutamate cysteine ligase to increase the production of glutathione. By these effects on Keap1/Nrf2 complex, H_2S_n exerts its cytoprotective activity.

8. Cross talk between H_2S and NO

When we discovered the relaxation effect of H_2S 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 H_2S was greatly augmented and vice versa[23] (see Fig. 3). This finding was the first demonstration of cross talk between H_2S and NO. Moore *et al.*[25),144] later confirmed the synergistic effect of both molecules in the ileum, and also showed the opposite effect: H_2S induces vasoconstriction by scavenging endothelial NO. Zhao *et al.*[26] reported that H_2S inhibits the vasorelaxation effect of the NO donor sodium nitroprusside (SNP) and showed that SNP increases H_2S production and another NO donor, *S*-nitroso-*N*-acetylpenicillamine, up-regulates the expression of CSE.[26]

Minamishima *et al.*[145] demonstrated that the protection of cardiomyocytes by H_2S is mediated by nitric oxide synthase 3 (eNOS). The application of Na_2S 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.*[146] performed additional studies on the mediation of NO in cytoprotection by H_2S. 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.[146] The application of Na_2S activates eNOS to increase the bioavailability of NO in wild-type mice, but this effect does not occur in eNOS-deficient mice.

H_2S induces angiogenesis mediated by vascular endothelial growth factor.[134),135] It also promotes the migration of vascular endothelial cells and facilitates microvessel tube formation. Bir *et al.*[147] demonstrated that the angiogenic effect of H_2S is mediated by NO by showing that eNOS knockout mice do not show the effect. By contrast, Szabo *et al.*[148] proposed that the cooperative action between H_2S and NO is essential for angiogenesis by showing that the suppression of the H_2S-generating enzyme CSE abolishes angiogenesis. The difference in results likely occurred because hypoxic conditions were used in the study by Bir *et al.*,[147] whereas Coletta *et al.* made observations under non-hypoxic conditions.

The chemical interaction between H_2S and NO produces several potential intermediates. One study showed that nitrosothiol, the chemical structure of which was not reported, is produced from H_2S and NO.[149] Nitrosothiol does not activate guanylyl cyclase to increase the production of cyclic guanosine monophosphate unless NO is released with Cu^{2+}.[149] H_2S releases NO from nitrosoglutathione, S-nitroso-N-acetylpenicillamine, SNP, and brain homogenates, which may contain nitrosothiols and metal nitrosyl complexes, to relax the vasculature.[150] Filipovic *et al.*[151] demonstrated that H_2S and nitrite produce the intermediate thionitrous acid, which in turn generates either NO and the $HS^{\bullet}$ radical or nitroxyl via further reaction with H_2S. They also demonstrated that nitroxyl activates TRPA1 channels, which releases calcitonin gene-related peptide, in turn inducing vasodilatation.[152] Cortese-Krott *et al.*[153] demonstrated that nitrosopersulfide, which is more stable than thionitrous acid, efficiently releases NO and H_2S_n, 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.*[21] 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.[132] Altaany *et al.*[154] 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.*[154] also confirmed results published by Minamishima *et al.*[145] and King *et al.*[146] suggesting that NaHS enhances eNOS activity by increasing the phosphorylation of serine 1179.

9. Development of H_2S- and polysulfide-sensitive fluorescent probes

The production and clearance of H_2S and its absorption by proteins occurs rapidly. Therefore, accurate real-time measurements of the concentration and movement of H_2S is difficult. We collaborated with Nagano *et al.* to develop hydrogen sulfide imaging prove-1 (HSIP-1) a fluorescent probe selective for H_2S.[155] The probe consists of two functional parts, a site sensitive to H_2S and a fluorescence emission fluorophore. HSIP-1 has azamacrocyclic rings that form stable complexes with Cu^{2+}, thereby suppressing the emission of fluorescence. H_2S releases Cu^{2+} 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 H_2S is below a micromolar order, it is crucial for a probe to detect low concentrations of H_2S in the presence of high concentrations of other thiols such as GSH and cysteine. GSH and cysteine do not release Cu^{2+}, which enables HSIP-1 to detect H_2S 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 H_2S.

Several other probes were reported in 2011. The sensitivity of SF1, SF2, and DNS-Az to H_2S is based on the reduction of azides to amines by H_2S that triggers changes in the electronic properties of a fluorophore, which then emits fluorescence.[156),157] The fluorophore of SF1 and SF2 is rhodamine, and that of DNS-Az is a dansyl group. Within 1 h of H_2S 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 H_2S. 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 H_2S.

SFP-1, SFP-2, and Probe 1 have similar H_2S binding sites at which H_2S 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.[158),159] The fluorescence intensity of Probe 1 increases by 55- to 70-fold in the presence of 50 µM H_2S after 1 h, whereas that of SFP-1 and SFP-2 increase by 13-fold under the same conditions.

Because H_2S_n species have recently been identified as a H_2S-related signaling molecule, the polysulfide-sensitive fluorescence probes SSP1 and SSP2 have been developed.[160] 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_2S and sulfane sulfur are irreversible, and the development of more desirable reversible probes is awaited.

10. Other roles of H_2S and therapeutic applications

Since the identification of the neuromodulation, vascular relaxation, and cytoprotection roles of H_2S, 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.[161)–165)] H_2S regulates inflammatory processes and their resolution. A significant anti-inflammatory effect was initially reported by Zanardo *et al.*,[163)] who showed that H_2S 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_2S donors and H_2S-releasing non-steroidal anti-inflammatory drugs with no significant side effects in the gastrointestinal tract.[166)] Many of these drugs have shown considerable promise in relevant animal models and are presently undergoing clinical trials.

H_2S inhibits cancer development, induces cell cycle arrest in the G2/M phase, and promotes apoptosis.[167)] H_2S-releasing non-steroidal anti-inflammatory drugs enhance these chemopreventive effects on cancer cells, and so-called NOSH compounds, which release both H_2S and NO, have been developed to suppress cancer growth.[164),168)] Contrary to these observations, CBS expression is up-regulated in colorectal and ovarian cancer cells, in which H_2S is involved in promoting cellular growth and migration.[169)] 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.*[165),170)] demonstrated that the O_2-dependent metabolism of H_2S may be an effective O_2-sensing mechanism. Tissue H_2S concentrations are inversely related to O_2 concentrations, and this reciprocal relationship is responsible for a similar effect between hypoxia and H_2S. H_2S production is closely linked to cellular oxidative states.[50),171)] Oxidative stress depletes GSH and cysteine, which in turn enhances the activity of CBS to stimulate the production of H_2S, cysteine, and GSH, which protect cells from oxidative stress.[50)]

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_2S — 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_2S_n species are considerably potent and induce various physiological responses, some of which were previously ascribed to H_2S. The cross talk between H_2S and NO, which was initially demonstrated as a synergistic interaction of both molecules, led to the identification of H_2S 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_2S and related molecules.

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Profile

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_2S) acts as a neuromodulator in the brain; this paper was published in 1996. Subsequently, in 1997, he demonstrated another role for H_2S, 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_2S 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_2S_n) 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.