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# Production and Physiological Effects of Hydrogen Sulfide

Hideo Kimura

**Abstract**

***Significance:*** Hydrogen sulfide ($H_2S$) has been recognized as a physiological mediator with a variety of functions. It regulates synaptic transmission, vascular tone, inflammation, transcription, and angiogenesis; protects cells from oxidative stress and ischemia-reperfusion injury; and promotes healing of ulcers. ***Recent Advances:*** In addition to cystathionine $\beta$-synthase and cystathionine $\gamma$-lyase, 3-mercaptopyruvate sulfurtransferase along with cysteine aminotransferase was recently demonstrated to produce $H_2S$. Even in bacteria, $H_2S$ produced by these enzymes functions as a defense against antibiotics, suggesting that the cytoprotective effect of $H_2S$ is a universal defense mechanism in organisms from bacteria to mammals. ***Critical Issues:*** The functional form of $H_2S$—undissociated $H_2S$ gas, dissociated HS ion, or some other form of sulfur—has not been identified. ***Future Directions:*** The regulation of $H_2S$ production by three enzymes may lead to the identification of the physiological signals that are required to release $H_2S$. The identification of the physiological functions of other forms of sulfur may also help understand the biological significance of $H_2S$. *Antioxid. Redox Signal.* 20, 783–793.

## Introduction

The study of hydrogen sulfide ($H_2S$) as a physiological mediator began with the discovery of endogenous sulfide in the brain (28, 64, 77). Although the levels were re-evaluated to be much lower than initially reported, this discovery served to confirm the existence of $H_2S$ in tissues (24, 32). Survivors of sulfide poisonings commonly suffered from memory loss, suggesting that $H_2S$ may be involved in memory formation under physiological conditions. We found that $H_2S$ facilitates the induction of hippocampal long-term potentiation, a synaptic model of memory formation, by enhancing the activity of *N*-methyl-D-aspartate (NMDA) receptors (1). Based on this, as well as the additional finding that cystathionine $\beta$-synthase (CBS) is expressed in the brain, we proposed that $H_2S$ is a neuromodulator. Nitric oxide (NO) was discovered as an endothelium-derived vascular smooth muscle relaxation factor (23), and it was later found to also function in the brain (27). This finding led to the identification of NO synthase in the brain (7). It was also possible that $H_2S$ might function in other tissues to which the producing enzymes are localized. Based on the observations that $H_2S$ relaxed vascular smooth muscle, the ileum, and the portal vein, and that either cystathionine $\gamma$-lyase (CSE) or CBS or both were expressed in these tissues (31), we proposed that $H_2S$ functions as a smooth muscle relaxant. $H_2S$ was later found to activate the adenosine triphosphate (ATP)-sensitive, intermediate conductance, and small conductance-potassium channels (53, 83).

We and others discovered the cytoprotective effect of $H_2S$; it protects neurons from oxidative stress by recovering glutathione levels decreased by oxidative stress (40). Although scavenging of reactive oxygen species (ROS) by $H_2S$ is less efficient than the glutathione cascade induced by $H_2S$, the scavenging effect may also contribute to its neuroprotective effect (39, 78). These findings led to the identification of the cardioprotective effect of $H_2S$, which preserves mitochondrial function against ischemia-reperfusion injury (21). The cytoprotective effect of $H_2S$ has also been observed in bacteria. $H_2S$ produced by the bacterial counterparts of CBS, CSE, and 3-mercaptopyruvate sulfurtransferase (3MST) was discovered to be a key molecule involved in bacterial resistance to antibiotics (66). The cytoprotective effect of $H_2S$ is a universal defense mechanism from bacteria to mammals.

Various other functions of $H_2S$ have been demonstrated: (i) $H_2S$ modulates inflammation; it suppresses leukocyte adherence and infiltration as well as edema formation (81). The proinflammatory activity of $H_2S$ has been observed in endotoxic shock (45). (ii) $H_2S$ suppresses the release of insulin by stimulating ATP-sensitive $K^+$ channels or by suppressing glucose-induced oscillations in intracellular concentrations of

NCNP, National Institute of Neuroscience, Kodaira, Japan.

$Ca^{2+}$ in pancreatic beta cells (36, 80). It was later found that $H_2S$ protects pancreatic beta cells from apoptotic cell death due to high glucose (35). (iii) $H_2S$ induces angiogenesis, as observed in endothelial cell proliferation, adhesion, and the formation of tube-like structures by Akt phosphorylation *in vitro*, and neovascularization in mice *in vivo* (8). Vascular endothelium growth factor-induced angiogenesis is also mediated by $H_2S$ (60). (iv) $H_2S$ functions as an oxygen sensor; this was initially demonstrated by Olson and colleagues and was recently confirmed by other researchers (59, 62).

$H_2S$ dissolves well in water and dissociates to $H^+$, $HS^-$, and $S^{2-}$. The undissociated form ($H_2S$) also dissolves well in lipids. However, it is not well understood which form—$H_2S$, $HS^-$, or $S^{2-}$—is physiologically relevant. $H_2S$ is a reducing substance and can reduce the cysteine disulfide bond, thereby changing the conformation and activity of enzymes, channels, and receptors. It can also modify cysteine residues in proteins by sulfhydration or the production of trisulfide bridge and modulate their function. Since other forms of sulfur, which are produced from $H_2S$, are more stable than $H_2S$, they have been proposed to be able to elicit physiological functions.

## Production of $H_2S$

CBS, CSE, and 3MST, along with cysteine aminotransferase (CAT), were found to have a capacity to produce $H_2S$ *in vitro* in1950s. However, $H_2S$ was simply considered a by-product of metabolic pathways or a marker for the evaluation of enzyme activity, rather than being recognized as a physiologically active molecule. We demonstrated the expression of CBS in the brain, and of CSE in smooth muscle, including in the vascular system (1, 31). Since then, both enzymes have been studied as major $H_2S$ producing enzymes in a variety of tissues. We recently demonstrated that 3MST, along with CAT, is a third $H_2S$-producing enzyme in neurons, vascular endothelium, and the retina (49, 50, 68, 69). 3MST requires a reducing substance such as dithiothreitol (DTT) for the production of $H_2S$, but the endogenous reducing substance has not been identified. Optimal activity of 3MST is achieved under alkaline conditions. For these reasons, this pathway was not recognized to be functional. We recently found that thioredoxin and dihydrolipoic acid (DHLA) are endogenous reducing substances that cause 3MST to release $H_2S$ (49).

## $H_2S$ Production by CBS and CSE

CBS produces $H_2S$ by a $\beta$-replacement reaction with cysteine. CBS also catalyzes a $\beta$-replacement of cysteine by homocysteine to produce $H_2S$ (12, 34).

$$\text{Cysteine} + H_2O \rightarrow \text{serine} + H_2S$$
$$\text{Cysteine} + \text{homocysteine} \rightarrow \text{homolanthionine} + H_2S$$

CSE produces $H_2S$ by the $\alpha$, $\beta$-elimination reaction with cysteine. In the presence of high concentrations of homocysteine as in hyperhomocysteinemia, the $\gamma$-replacement reaction between two molecules of homocysteine becomes dominant in the production of $H_2S$ (34).

$$\text{Cysteine} + H_2O \rightarrow \text{pyruvate} + H_2S + NH_3$$
$$\text{Homocysteine} + \text{homocysteine} \rightarrow \text{homolanthionine} + H_2S$$

Banerjee and colleagues intensively studied the contributions of both enzymes to the production of $H_2S$ in the liver, kidney, and brain. CSE is more abundant than CBS in the liver and kidney; whereas in the brain, CBS is abundant but CSE has much lower levels. CBS and CSE have equal capacity for $H_2S$ production in the liver, while CBS is a major contributor to $H_2S$ production in the brain and kidney (34). $H_2S$ is metabolized by sulfur dioxygenase, which is present in higher levels in the liver and kidney than in the brain. Even small deviations in the rates of $H_2S$ production and clearance may lead to rapid and dramatic changes in the levels of $H_2S$, providing an essential feature for a signaling molecule (76).

## $H_2S$ Production by 3MST and CAT

3MST produces $H_2S$ from 3-mercaptopyruvate (3MP), which is produced by CAT from cysteine and $\alpha$-ketoglutarate (16, 48, 68, 69). CAT is identical to aspartate aminotransferase (75).

$$\text{CAT} : \text{cysteine} + \alpha - \text{ketoglutarate} \rightarrow 3\text{MP}$$
$$\text{3MST} : 3\text{MP} \rightarrow H_2S + \text{pyruvate}$$

### *3MST localizes to mitochondria and the cytosol*

CBS and CSE are localized in the cytosol, while 3MST is localized in mitochondria as well as the cytosol (54, 69). Although the total activity of 3MST is greater in the cytosol compared with that in mitochondria, the specific activity is several-fold greater in mitochondria than in the cytosol. 3MST is highly homologous (60% amino acid identity) to rhodanese, which is predominantly localized in mitochondria and has a similar functional structure (4, 54, 56). The majority of mitochondrial proteins have a signal sequence at their amino terminus that targets these proteins to the mitochondria. After proteins are imported into the matrix, the signal sequence is usually removed. However, a group of mitochondrial proteins that have signal sequences lack the sequence which is essential for processing. These proteins, including rhodanese, remain in mitochondria without their signal sequence being processed. The amino-terminal residues 11–22 of rhodanese form an $\alpha$–helix, which is a putative signal for mitochondrial transport. Two positively charged amino acids, $Lys^{13}$ and $Arg^{23}$, positioned on one side of the $\alpha$-helix, are considered important for the function of the signal sequence to allow targeting to mitochondria. $Arg^{23}$ is conserved in both 3MST and rhodanese, while $Lys^{13}$ is replaced with $Gln^{13}$ in 3MST. Therefore, the lack of $Lys^{13}$ may cause 3MST to localize to both mitochondria and the cytosol (Fig. 1). Although rat, hamster, mouse, bovine, and chicken rhodanese have the conserved $Lys^{13}$, human rhodanese has $Gln^{13}$, which is similar to 3MST (55). A repetitive sequence $(Gly\text{-}Lys\text{-}X)^2$ at the carboxyl terminal of rhodanese is also proposed to be a signal for mitochondrial retention. The sequence is modified in 3MST, suggesting that the signal may determine the differences in mitochondrial retention seen between rhodanese and 3MST (51).

In contrast to 3MST, there are two forms of CAT, that is, mitochondrial and cytosolic. These two CATs share 48% identity in their amino acid sequences (3, 20, 75), and produce 3MP, which is provided to 3MST for $H_2S$ production.

### *3MST requires thioredoxin or DHLA for $H_2S$ production*

3MST has not been recognized as a $H_2S$-producing enzyme because of its requirement for a reducing substance such as

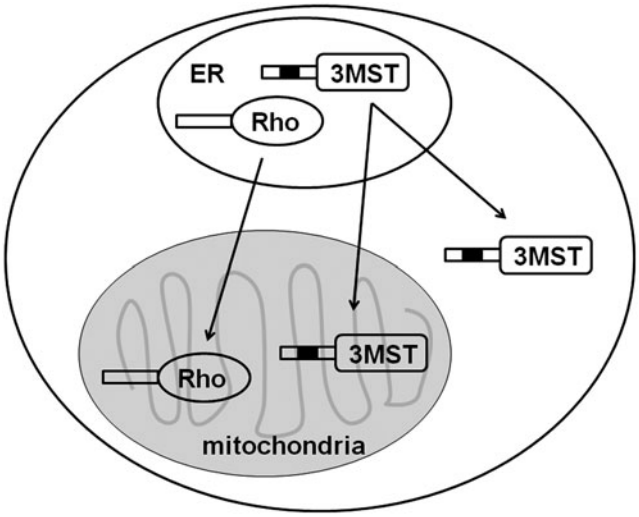


**FIG. 1. Localization of 3MST and rhodanese.** The signal sequence of 3MST for targeting mitochondria is homologous to that of rhodanese. One of the two positively charged amino acids in the sequence is replaced with a neutral amino acid in 3MST. Rhodanese is localized to mitochondria, while 3MST is localized to both mitochondria and cytosol. 3MST, 3-mercaptopyruvate sulfurtransferase; ER, endoplasmic reticulum.

DTT, the endogenous counterpart of which has not been identified. We recently found that 3MST produced $H_2S$ from 3MP in the presence of thioredoxin (30, 49) (Fig. 2). The endogenous level of thioredoxin (20 $\mu M$) is four times as potent as DTT in releasing $H_2S$ from 3MST (49).

There are two forms of thioredoxin in mammals. Thioredoxin 1 is localized in the cytosol, while thioredoxin 2 is localized in mitochondria (70). The CysXXCys motif, which contains cysteine residues at the active site, is highly conserved among different species, and similar to bacterial thioredoxin, thioredoxin 2 is resistant to oxidative stress. For

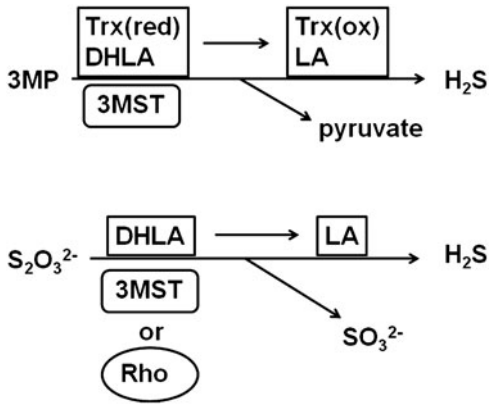


**FIG. 2. $H_2S$ production by 3MST from 3MP and thiosulfate.** 3MST produces $H_2S$ from 3MP in the presence of thioredoxin or DHLA as cofactors. 3MST and rhodanese produce $H_2S$ from thiosulfate in the presence of DHLA. Rhodanese is more specific to thiosulfate than is 3MST. $H_2S$, hydrogen sulfide; 3MP, 3-mercaptopyruvate; DHLA, dihydrolipoic acid.

these reasons, bacterial thioredoxin is widely used as a replacement of mammalian thioredoxin 2. Since thioredoxin is readily oxidized, it has to be reduced using thioredoxin reductase before it is used for experiments. Mammalian thioredoxin reductase is a selenoprotein, but the bacterial protein synthetic machinery does not appropriately incorporate selenium into the product (5). Therefore, A549 human lung adenocarcinoma cells, which contain abundant thioredoxin reductase, are often used as a source of the enzyme (22).

Another reducing substance, DHLA, exists $\sim 40\,\mu M$ in brain mitochondria (37, 63). DHLA is as potent as DTT in causing 3MST to release $H_2S$ (Fig. 2). Other reducing substances such as cysteine, glutathione, nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NADH), and CoA do not have any effect on the production of $H_2S$ by 3MST, even at high concentrations (1 m$M$) (49).

Thioredoxin, DTT, and DHLA have redox potentials in the range of $-0.26$ to $-0.33$ V. The redox potentials of monothiols such as glutathione, cysteine, and CoA are between $-0.22$ and $-0.35$ V, and those of NADH and NADPH are between $-0.320$ and $-0.324$ V. There is no correlation between the reducing potential and the ability to produce $H_2S$. DTT and DHLA are dithiols, and thioredoxin has two cysteine residues at its active site, which may function as a dithiol. Therefore, the presence of a dithiol is a critical factor for these reducing substances to induce the release of $H_2S$ by 3MST (49). A possible mechanism for the production of $H_2S$ by 3MST in the presence of dithiols is that 3MST receives sulfide from 3MP to produce 3MST persulfide, which is then transferred to one of the thiol residues in thioredoxin or DHLA to produce thioredoxin persulfide or DHLA persulfide. The remaining thiol in thioredoxin or DHLA attacks the persulfide to release sulfide.

### *$H_2S$ production from thiosulfate*

Thiosulfate is an intermediate of sulfur metabolism from cysteine, which results in the production of sulfite; sulfite is further oxidized to sulfate by sulfite oxidase (74). It has been proposed that rhodanese metabolizes thiosulfate to produce $H_2S$ and sulfite (41). As predicted from the structural similarity to rhodanese and from the fact that 3MST interacts with thiosulfate, 3MST was found to have the ability to produce $H_2S$ from thiosulfate in the presence of a high concentration (1 m$M$) of DHLA (49). 3MST produces $H_2S$ from both 3MP and thiosulfate, while rhodanese is more specific to thiosulfate than to 3MP (Fig. 2). However, neither the endogenous concentration (20 $\mu M$) of thioredoxin nor the same concentration of DTT was seen to support $H_2S$ production from thiosulfate by 3MST. Considering the requirement for high concentrations of DHLA, the production of $H_2S$ from thiosulfate under physiological conditions remains to be elucidated.

### *$Ca^{2+}$ regulation on 3MST/CAT pathway*

We found that $H_2S$ production by the 3MST/CAT pathway is regulated by $Ca^{2+}$. In the absence of $Ca^{2+}$, the production of $H_2S$ is maximal, and the production is suppressed by $Ca^{2+}$ in a concentration-dependent manner (50). Based on the observation that the application of calmodulin or a calmodulin-specific inhibitor, W-7, did not change the activity, we concluded that calmodulin is not involved in the regulation of the $H_2S$-producing activity of the 3MST/CAT pathway by

$Ca^{2+}$. Based on the following observations, we determined that the enzyme regulated by $Ca^{2+}$ is CAT and not 3MST. First, the production of $H_2S$ from 3MP in the presence of 3MST was not affected by $Ca^{2+}$. Second, $H_2S$ production from cysteine and α-ketoglutarate in the presence of 3MST and CAT was decreased by $Ca^{2+}$ in a concentration-dependent manner. The best way to demonstrate the regulation of CAT by $Ca^{2+}$ is to measure the levels of 3MP, the product of CAT, but 3MP is unstable and difficult to measure. The development of a method that can accurately measure 3MP levels is awaited.

The regulation of enzyme activities by $Ca^{2+}$ without the involvement of calmodulin or any $Ca^{2+}$-binding domain has been demonstrated for three mitochondrial dehydrogenases: pyruvate dehydrogenase, NAD-isocitrate dehydrogenase, and oxoglutarate dehydrogenase (18). The other example is serine racemase, a pyridoxal 5′-phosphate (PLP)-dependent enzyme similar to CAT, which has glutamate and aspartate residues bound to $Ca^{2+}$; however, CAT does not have such a $Ca^{2+}$-binding site (15). In contrast to these enzymes being activated by $Ca^{2+}$, CAT is suppressed by $Ca^{2+}$. CAT is regulated by $Ca^{2+}$ *via* a novel but unknown mechanism.

## Active Forms of $H_2S$

### *$H_2S$ gas, $HS^-$, and sulfane sulfur*

$H_2S$ dissolves well in water and dissociates into $H^+$, $HS^-$, and $S^{2-}$.

$$H_2S \rightarrow H^+ + HS^- \ (pK_1\ 7.02)$$
$$HS^- \rightarrow H^+ + S^{2-} \ (pK_2\ 13.9)$$

Under physiological extracellular conditions at pH 7.4, ~1/5 of the total $H_2S$ exists in an undissociated form ($H_2S$) and the remaining 4/5 as HS- with a trace amount of $S^{2-}$. The basal intracellular pH is different among organelles. The pH of the cytosol, mitochondria, Golgi apparatus, and lysosomes is 7.0–7.2, 8.0, 6.0–6.7, and 4.7, respectively (10). The ratio of $H_2S/HS^-$ differs depending on their pH. For example, $HS^-$ is more dominant in mitochondria than in the cytosol.

$H_2S$ also dissolves well in lipids. $H_2S$ readily passes through the lipid bilayer of the plasma membrane, which contains diffusion-resistant substances such as cholesterol and sphingomyelin; while $HS^-$ does not pass through either the lipid bilayer or negative ion channels such as $Cl^-$ channels (47). The distribution of $H_2S$ and $HS^-$ on the two sides of the plasma membrane depends on the pH of the cytosol and the extracellular fluid. Since the extracellular environment (pH 7.4) is slightly more alkaline than the cytosol (pH 7.0–7.2), $H_2S$ may preferentially flow from the cytosol to the extracellular compartment.

The situation is different in bacteria. A $HS^-$ channel was recently discovered in bacteria (17). Since the extracellular environment of bacteria is acidic (pH 6.0) while the intracellular pH is 7.4, the $HS^-$ concentration is much greater in the interior of the cells than in the extracellular environment where $H_2S$ gas is dominant. Therefore, extracellular $H_2S$ passes through the bacterial membrane into the interior of the cells. Once $H_2S$ enters into cells, the equilibrium between $H_2S$ and $HS^-$ shifts toward $HS^-$. The increased intracellular $HS^-$, in turn, passes through $HS^-$ channels to the extracellular environment.

The products of the sulfur compounds in garlic, such as diallyl disulfide and diallyl trisulfide, contain sulfane sulfur. However, whether similar substances are produced in mammals is not well understood. The identification of such substances in mammals and the clarification of their functions may help understand the physiological roles of sulfur compounds, including $H_2S$.

## Regulation of $Ca^{2+}$ Influx by $H_2S$

$H_2S$ induces $Ca^{2+}$ influx in neurons and astrocytes in the brain, whereas it suppresses $Ca^{2+}$ influx in the retinal photoreceptor cells. These contrasting effects are caused by differences in the receptors or channels that are involved in responses to $H_2S$ (Fig. 3).

### *Induction of $Ca^{2+}$ influx in neurons and glia*

$H_2S$ enhances the activity of NMDA receptors. $H_2S$ alone has no effect on NMDA receptors; however, the responses of NMDA receptors to their ligands, the neurotransmitter glutamate, or its synthetic agonist NMDA are augmented by $H_2S$ (1). The reduction or oxidation of disulfide bonds in proteins, including NMDA receptors, regulates their function (2). DTT enhances the activity of NMDA receptors by reducing cysteine disulfide bonds in these receptors. In spite of having lower negative reducing potential compared with DTT, 10-fold lower concentrations of $H_2S$ further enhance the activity of NMDA receptors even after enhancement by DTT. Similar to DTT, $H_2S$ reduces cysteine disulfide bonds in NMDA receptors to activate them. The difference between DTT and $H_2S$ is that $H_2S$ can further sulfhydrate the receptors or make a trisulfide bridge in them (25, 73). Sulfhydration, which has been proposed to modify the function of proteins, including ATP-activated $K^+$ channels, may be a possible mechanism for the enhancement of NMDA receptor activity (25). Alternatively, the trisulfide bridge formation may be involved in the modification of the receptor activity (69).

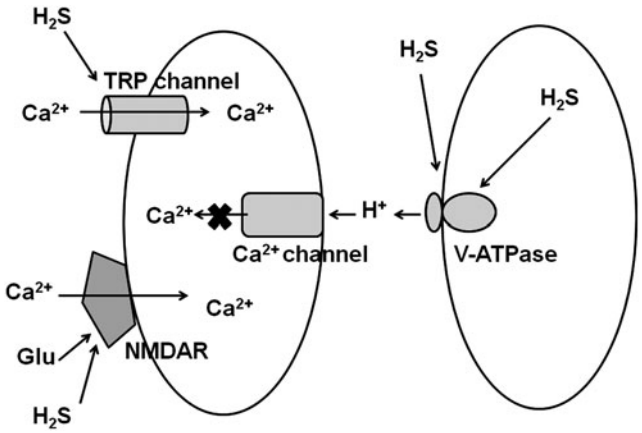


**FIG. 3. Regulation of $Ca^{2+}$ influx by $H_2S$.** $H_2S$ enhances the activity of NMDA receptors activated by the neurotransmitter glutamate and thereby increases $Ca^{2+}$ influx in neurons. $H_2S$ activates TRP channels to increase $Ca^{2+}$ influx in astrocytes. In contrast, in retinal neurons, $H_2S$ suppresses $Ca^{2+}$ influx by activating V-ATPase, which releases $H^+$ to suppress $Ca^{2+}$ channels. NMDA, *N*-methyl-D-aspartate; TRP, transient receptor potential; V-ATPase, vacuolar-type proton ATPase.

The repetitive application of 100 to 300 $\mu M$ $H_2S$ to cultures of cerebellar granule neurons increases the intracellular $Ca^{2+}$ concentrations to the toxic range. Nimodipine and nifedipine, L-type $Ca^{2+}$ channel blockers, and MK-801 and 2-amino-5-phosphonovalerate, inhibitors of NMDA receptors, attenuate the effect of $H_2S$, suggesting the involvement of L-type $Ca^{2+}$ channels and NMDA receptors (26). Since $H_2S$ evaporates from culture medium, the concentrations of $H_2S$ in the medium return to the basal level immediately after the repetitive application. However, the repetitive application of $H_2S$ causes the accumulation of bound sulfane sulfur in the cells that may influence the cellular reponses (32). It is necessary to re-evaluate the effect of $H_2S$.

Astrocytes, a type of glia, were thought to be quiescent cells that surround and support neurons. Recently, however, astrocytes have been found to have receptors for neurotransmitters, such as glutamate, acetylcholine, noradrenaline, dopamine, $\gamma$-aminobutyric acid (GABA), and ATP (6). The transmitters released during synaptic activity spill out of the cleft at concentrations sufficient to activate receptors on the astrocytes surrounding the synapse. Stimulation of astrocytes induces increases in intracellular $Ca^{2+}$ concentrations that propagate into neighboring astrocytes as intercellular $Ca^{2+}$ waves. The mechanisms for increasing intracellular $Ca^{2+}$ concentrations are different among the various neurotransmitters involved. For example, ATP activates purinergic receptors to induce $Ca^{2+}$ release from the intracellular $Ca^{2+}$ stores; whereas glutamate depolarizes the membrane, which, in turn, activates voltage-gated $Ca^{2+}$ channels to induce $Ca^{2+}$ influx. When the intracellular concentrations of $Ca^{2+}$ reach sufficient levels in astrocytes, the fusion of vesicles containing gliotransmitters such as glutamate and ATP is induced, and the gliotransmitters are released by exocytosis (84) (Fig. 4).

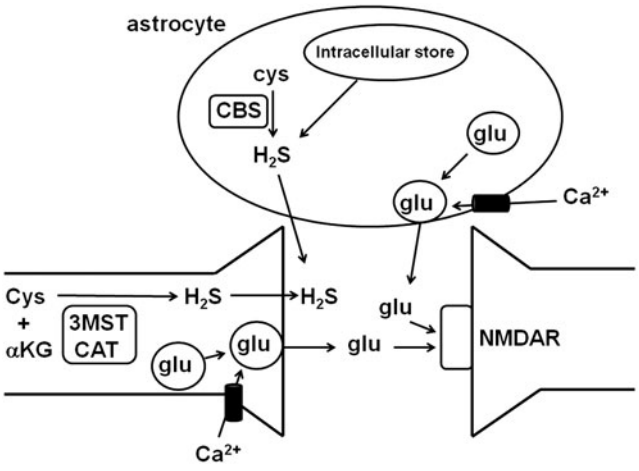


**FIG. 4. Release of $H_2S$ and gliotransmitters from astrocytes.** Gliotransmitters such as glutamate and ATP are released from gliotransmitter vesicles to the extracellular space when $Ca^{2+}$ enters into cells. The release of gliotransmitters from astrocytes is similar to the release of neurotransmitters from neurons. In contrast, $H_2S$, which is produced by enzymes and intracellular stores, readily passes through the plasma membrane without the need for vesicle formation. ATP, adenosine triphosphate; CAT, cysteine aminotransferase; CBS, cystathionine $\beta$-synthase.

These gliotransmitters, in turn, activate pre- or post-synaptic receptors in neurons and modulate synaptic activity.

$H_2S$ increases intracellular $Ca^{2+}$ concentrations in astrocytes that propagate to the surrounding astrocytes as $Ca^{2+}$ waves (57) (Fig. 3). This increase in intracellular $Ca^{2+}$ concentrations is greatly suppressed in the absence of extracellular $Ca^{2+}$ and weakly suppressed by thapsigargin, which depletes intracellular $Ca^{2+}$ stores, suggesting that $H_2S$ mainly increases $Ca^{2+}$ influx, similar to glutamate, and partially increases $Ca^{2+}$ release from intracellular stores. The responses to $H_2S$ are strongly suppressed by $La^{3+}$, $Gd^{3+}$, and ruthenium red, which are known as broad-spectrum inhibitors of transient receptor potential (TRP) channels; these channels are categorized as nonselective cation channels, and some of them are highly selective for $Ca^{2+}$ (14) (Fig. 3). TRP channels are activated by several physiological stimuli such as osmolarity, pH, mechanical force, and ligand interaction. The involvement of TRP channels in response to $H_2S$ has been demonstrated. For example, the contraction of the detrusor muscle by $H_2S$ in the urinary bladder is regulated by TRP channels, the contraction of airway muscle by $H_2S$ is induced by activating TRPV1 channels, and Chinese hamster ovary cells expressing TRPA1 channels respond to $H_2S$ (61).

Gliotransmitters are released by exocytosis (84). Glutamate is synthesized *via* the tricarboxylic acid cycle, and ATP is produced by glycolysis and by oxidative phosphorylation in astrocytes. Both glutamate and ATP are loaded into vesicles by vesicular transporters. Vacuolar-type proton ATPase (V-ATPase) drives protons into vesicles to create the proton concentration gradient required for the transport of glutamate and ATP into the vesicles (84). In contrast to gliotransmitters, $H_2S$, which readily passes through the plasma membrane of astrocytes, is released into the extracellular space between neurons and astrocytes (47) (Fig. 4).

Microglia also transmit $Ca^{2+}$- mediated signaling to astrocytes, and the $Ca^{2+}$ waves induced in astrocytes, in turn, propagate to the neighboring microglia. In microglia, the $Ca^{2+}$ imaging in the presence of specific inhibitors of PKA and PKC showed that $H_2S$ activates $Ca^{2+}$ channels in the plasma membrane as well as $Ca^{2+}$ activated intracellular $Ca^{2+}$ channels by activating cyclic adenosine monophosphate (cAMP)/PKA pathway (44).

### *Suppression of $Ca^{2+}$ influx in retinal photoreceptor cells and cardiomyocytes*

In contrast to its effect of increasing the $Ca^{2+}$ influx in neurons and astrocytes, $H_2S$ suppresses $Ca^{2+}$ influx in the retinal photoreceptor cells to which 3MST and CAT are co-localized (50). The intracellular concentration of $Ca^{2+}$ in cells is $\sim$100 n$M$ when cells are under quiescent conditions, and increases to approximately 3 $\mu M$ when $Ca^{2+}$ channels are opened. The intracellular concentrations of $Ca^{2+}$ are shifted to lower concentrations specifically in the retinal neurons. When retinal photoreceptor cells are exposed to light, cyclic guanosine monophosphate-gated channels are closed and the intracellular concentration of $Ca^{2+}$ is decreased to 10 n$M$. This is increased to approximately 600 n$M$ in darkness (43). The production of $H_2S$ by the 3MST/CAT pathway is dramatically altered in this range of $Ca^{2+}$ concentrations. At low concentrations of intracellular $Ca^{2+}$, the 3MST/CAT pathway is activated to produce $H_2S$ in both horizontal cells and

photoreceptor cells, which make synaptic contact with the horizontal cells. $H_2S$ activates V-ATPase on horizontal cells. V-ATPase has a cysteine disulfide at its active site. When this disulfide is reduced, V-ATPase is in the active state. The activated V-ATPase releases protons, which suppress voltage-gated $Ca^{2+}$ channels on the photoreceptor cell membrane, thereby maintaining intracellular $Ca^{2+}$ concentrations at much lower levels than seen in other cell types (50) (Fig. 3).

In cardiomyocytes, $H_2S$ directly suppresses L-type $Ca^{2+}$ channels (71, 82). The depolarization induced by the electrical stimulation returns to the resting level faster in the presence of $H_2S$ than in its absence in cardiomyocytes, suggesting that $H_2S$ suppresses L-type $Ca^{2+}$ channels. In contrast, $Ca^{2+}$ release from the intracellular $Ca^{2+}$ stores such as sarcoplasmic reticulum is not affected by $H_2S$, judging from the observation that $H_2S$ did not have any effect on caffeine-induced increase in the intracellular $Ca^{2+}$ concentrations (71). Sulfhydration may be involved in the suppression of L-type $Ca^{2+}$ channels by $H_2S$ (82).

## Cytoprotective Effect

### *Reducing cascade by producing glutathione*

There are two forms of glutamate toxicity: oxidative toxicity and ionotropic toxicity. Oxidative glutamate toxicity is caused by high concentrations of glutamate, which suppress the glutamate/cystine antiporter and thereby decrease the import of cystine into cells (52). Cystine is reduced in the cells to cysteine and incorporated into glutathione. Therefore, lack of cysteine causes a decrease in the production of glutathione, the major intracellular antioxidant. $H_2S$ enhances the activity of the cystine/glutamate antiporter, thereby increasing the transport of cystine into the cells (40). In blood or extracellular fluid, cystine is the predominant form, but $\sim 20\ \mu M$ cysteine is also present. $H_2S$ enhances the activity of the cysteine transporter as well as the cystine transporter to contribute to the production of glutathione (39, 40).

Glutathione is a tripeptide that consists of glutamate, cysteine, and glycine. Of these three amino acids, cysteine has the lowest level in the cytosol; therefore, glutamylcysteine synthase, which produces glutamylcysteine, is a limiting enzyme for glutathione synthesis. $H_2S$, which enhances the activity of glutamylcysteine synthase, is required to exist in extracellular spaces to increase the production of glutamylcysteine (39, 40). The exposure of the enzyme to $H_2S$, causing the direct interaction of the enzyme with $H_2S$, does not increase the production of glutathione, suggesting that $H_2S$ activates some receptor localized to the cell surface to induce a signal which leads to the production of glutathione. Oxidative glutamate toxicity has been studied without the effect of excitotoxicity in cultured embryonic neurons, in which ionotropic glutamate receptors are not expressed. The recovery of decreased glutathione levels caused by ischemia-reperfusion injury in embryonic brains was also observed *in vivo* by the application of sodium hydrosulfide (NaHS) (39).

Excitotoxicity is caused by the entry of an excess amount of $Ca^{2+}$ into cells through NMDA receptors or voltage-activated $Ca^{2+}$ channels, which are opened by depolarization induced by the activation of alpha-amino-3-hydroxy-5-methy-4 isoxazole proprionic acid (AMPA) receptors (13). Under physiological conditions, NMDA receptors are not activated by their cognate ligand, glutamate, due to suppression by $Mg^{2+}$. Glutamate initially activates AMPA receptors, causing depolarization of the membrane, which, in turn, releases the $Mg^{2+}$ block and activates NMDA receptors. In brain slices of adult animals, $H_2S$ alone has no effect on neuronal activity at concentrations lower than 130 $\mu M$ NaHS, but the activation of NMDA receptors by their ligand is enhanced by $H_2S$ (1). Even at toxic levels (up to 640 $\mu M$), $H_2S$ completely but reversibly suppresses synaptic transmission, leaving Na channels unaffected (1).

Recently, it was reported that $H_2S$ and NMDA, which cause excitotoxicity, induce the expression of 1649 genes in common, accounting for more than 80% of the genes induced by NMDA (11). The gene families involved include those related to cell death, endoplasmic stress, calcium homeostasis, cell cycle, heat shock proteins, and chaperones. Based on these observations, Chen *et al.* concluded that $H_2S$ causes neuronal cell death by activating NMDA receptors.

### *Scavenging free radical species*

Another mechanism by which $H_2S$ protects neurons is by scavenging free radical species induced by glutamate, hydrogen peroxide, NO, peroxynitrite, and hypochlorous acid (39, 78, 79). Significant amounts of ROS are produced in mitochondria, to which 3MST and CAT are localized. Cells expressing 3MST and CAT are resistant to oxidative stress caused by glutamate and hydrogen peroxide, suggesting that $H_2S$ produced by both enzymes may scavenge ROS in mitochondria (39). Since mitochondria contain $\sim 1$ m$M$ cysteine, which is greater than the level seen in the cytoplasm, appropriate amounts of $H_2S$ can be produced in mitochondria (29, 72, 74). However, compared with glutathione, which exists at several m$M$ in cells and has greater reducing potential than $H_2S$, the levels of $H_2S$ are much lower. Therefore, the glutathione cascade induced by $H_2S$ may contribute to the suppression of oxidative stress more efficiently than the scavenging of free radical species by $H_2S$ itself.

### *Regulation of transcription and translation*

$H_2S$ protects cardiac muscle from ischemia-reperfusion injury by preserving mitochondrial function (21). $H_2S$ reduces cardiomyocyte apoptosis, as demonstrated by a decreased number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei, by decreasing the activity of caspase3 in the presence of $H_2S$. The transcriptional regulation of this cardioprotective effect was recently elucidated (9). $H_2S$ increases the nuclear localization of nuclear factor erythroid 2-related factor 2 (Nrf2), which is a nuclear basic leucine zipper transcription factor that regulates a number of antioxidants and related enzymes, including thioredoxin and heme oxygenase-1. $H_2S$ also increases the phosphorylation of proteins in the protein kinase C$\varepsilon$/signal transducers and activators of transcription 3 (STAT-3) signaling cascade, leading to the up-regulation of the expression of the survival factors Bcl-2 and Bcl-xL and the down-regulation of the apoptotic factor Bad (Fig. 5) (9).

Another instance of transcriptional regulation in the anti-apoptotic action of $H_2S$ was recently revealed in the effect of the multifunctional proinflammatory cytokine tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (65). TNF-$\alpha$ activates the I$\kappa$B kinase complex that phosphorylates I$\kappa$B, leading to I$\kappa$B degradation and the subsequent translocation of NF-$\kappa$B to the nucleus. TNF-$\alpha$ also

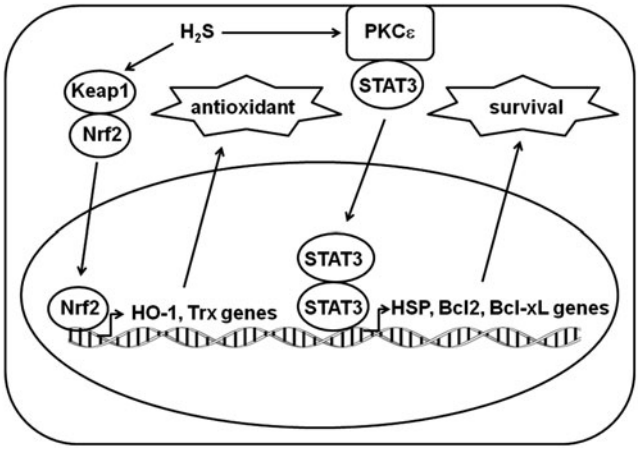


**FIG. 5. $H_2S$ up-regulates antioxidant- and survival-related genes.** $H_2S$ translocates Nrf2 into the nucleus to activate antioxidant-related genes such as *HO-1* and *Trx*. It also activates PKCε and STAT3 to translocate dimerized STAT3 to the nucleus, which then activates survival-related genes such as *HSP*, *Bcl2*, and *Bcl-xL*. *RPS3*, ribosomal protein S3.

stimulates the binding of a transcription factor, SP1, to the CSE promoter to increase the production of $H_2S$ (33, 65). $H_2S$, in turn, sulfhydrates the p65 subunit of NF-κB, which promotes its binding to the coactivator ribosomal protein S3 (RPS3). The NF-κB/RPS3 complex binds to the promoters of several antiapoptotic genes, leading to the suppression of apoptosis (Fig. 6).

The endoplasmic reticulum (ER) is the primary site of protein synthesis, and it controls the folding and modification of newly synthesized proteins. After being properly folded and modified, proteins are trafficked to the Golgi apparatus. However, if proteins are misfolded or improperly modified, a

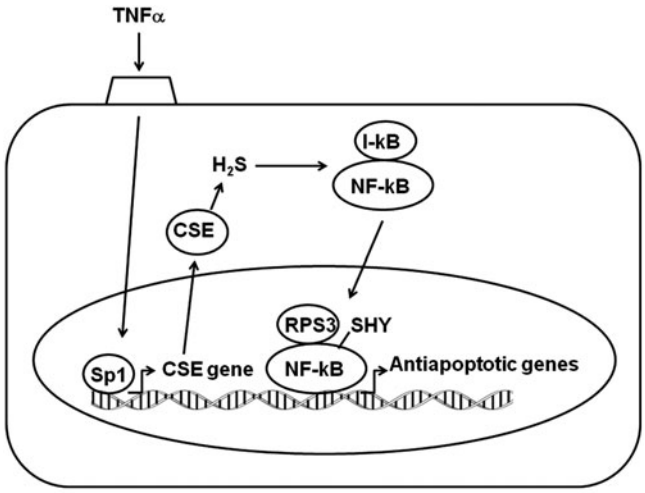


**FIG. 6. Antiapoptotic action of $H_2S$.** TNFα activates the transcription of *CSE* gene to increase the levels of CSE. $H_2S$ produced by CSE sulfhydrates NF-kB, which leads to the translocation of NF-kB into the nucleus to activate the transcription of antiapoptotic genes. CSE, cystathionine γ-lyase; Nrf2, nuclear factor erythroid 2-related factor 2; STAT3, signal transducers and activators of transcription 3;TNF-α, tumor necrosis factor-α.

condition known as ER stress, the unfolded protein response (UPR) is activated (46). UPR is triggered by transmembrane sensor proteins that detect unfolded proteins in the ER and transduce signals to fine tune protein synthesis and folding. If this tuning is not successful, it triggers proapoptotic cascades. ER stress increases the production of $H_2S$ by CSE, which sulfhydrates and thereby inactivates protein tyrosine phosphatase, resulting in the protection of protein kinase-like ER kinase (PERK) from dephosphorylation. Activated PERK inhibits global translation by phosphorylating eukaryotic translational initiation factor 2 alpha (elF2α), resulting in promoting the restoration of ER homeostasis (42).

### *$Ca^{2+}$ homeostasis*

Light-induced photoreceptor cell apoptosis represents an animal model for the study of human retinal degenerations. Apoptosis is the mode of photoreceptor cell death in both human cases and animal models. An increase in intracellular calcium levels occurs during apoptotic cell death, and this may activate calcium-dependent proteases, such as calpain. Voltage-gated $Ca^{2+}$ channel blockers such as diltiazem suppress photoreceptor cell apoptosis, and the lack of the V-ATPase a3 subunit causes retinal degeneration, suggesting that voltage-gated $Ca^{2+}$ channels and V-ATPase are involved in photoreceptor degeneration (19, 38). $H_2S$ activates V-ATPase in horizontal cells to release protons, which suppress voltage-gated $Ca^{2+}$ channels in photoreceptor cells, thereby maintaining intracellular $Ca^{2+}$concentrations at low levels in photoreceptor cells (Fig. 3). $H_2S$ greatly suppresses the intensive light-induced photoreceptor degeneration (50). The number of TUNEL-positive photoreceptor cells and those containing 8-hydroxy-2′-deoxyguanosine, a product of ROS-induced DNA damage, is decreased in animals administered $H_2S$. The retina is susceptible to oxidative stress because of its high consumption of oxygen and daily exposure to light. Under normal conditions, $H_2S$ maintains the intracellular $Ca^{2+}$ concentrations at low levels. However, this regulation by $H_2S$ may fail when photoreceptor cells are exposed to excessive levels of light. Even under such conditions, $H_2S$ supplementation protects cells from degeneration.

### *Universal defense mechanism*

The cytoprotective effect of $H_2S$ produced by CBS, CSE, and 3MST is a phenomenon that is observed not only in mammalian cells but also in prokaryote bacteria, as demonstrated by Nudler and colleagues (66). For centuries, it has been well known that bacteria produce $H_2S$, but it was recognized as a byproduct of metabolic pathways, and not considered as having a particular function. In four clinically relevant and evolutionarily distant pathogenic species of bacteria, all three enzymes were demonstrated to produce $H_2S$. The overexpression of 3MST increases spectinomycin resistance; whereas the chemical inhibition of CBS, CSE, or 3MST renders bacteria more sensitive to antibiotics. NaHS suppresses the antibiotic sensitivity of CBS-, CSE-, and 3MST-deficient bacteria. These observations suggest that endogenously produced $H_2S$ enhances the resistance of bacteria to antibiotics. $H_2S$ protects bacteria from oxidative stress and antibiotics *via* two mechanisms; suppressing DNA breaks and enhancing the activity of catalase and superoxide dismutase. $H_2O_2$ and ampicillin caused linearization of the chromosome

in 3MST-deficient bacteria; while overexpression of 3MST suppressed this linearization, suggesting that $H_2S$ produced by 3MST protects chromosomal DNA.

The cytoprotective effect of $H_2S$ is similar to that of NO in bacteria (66). Bacteria deficient in both bacterial NO synthase and CBS or CSE are unable to establish colonies, suggesting that bacteria cannot survive in the absence of both NO and $H_2S$. The levels of $H_2S$ are increased in NO-deficient bacteria, and vice versa, suggesting that one substance compensates for the lack of the other. There is also a synergistic effect in the protection afforded by $H_2S$ and NO against antibiotics. Nudler also noted that NO synthase is present only in a small number of gram-positive bacteria, while $H_2S$-producing enzymes are essentially universal. $H_2S$ can be a diffusible defense agent in the entire population of bacteria. Since bacterial CBS, CSE, and 3MST have substantially diverged from their mammalian counterparts, designing specific inhibitors of these bacterial enzymes may enhance the effect of antibiotics against a broad range of pathogens (66).

## Concluding Remarks

CBS and CSE have been intensively studied as $H_2S$-producing enzymes. Both enzymes are mainly localized to the cytosol in cells. In contrast, 3MST is localized to mitochondria as well as the cytosol, and CAT is present in two forms: mitochondrial and cytosolic. Therefore, the 3MST/CAT pathway can produce $H_2S$ both in mitochondria and the cytosol. CBS and CSE require only substrates, while 3MST requires a reducing cofactor, as well as a substrate, to produce $H_2S$. Since the endogenous reducing cofactor for 3MST has not been known so far, the 3MST/CAT pathway was not recognized to be involved in $H_2S$ production. Now that the cofactors thioredoxin and DHLA have been identified, the role of the 3MST/CAT pathway in the nervous and vascular systems as well as in other systems will be clarified (49, 50).

$H_2S$ sometimes exerts contrasting effects in different tissues. For example, $Ca^{2+}$ influx is increased by $H_2S$ in neurons and astrocytes by enhancing the activity of NMDA receptors, and by activating TRP channels, respectively (1, 57). In contrast, $Ca^{2+}$ influx is decreased in retinal neurons *via* the activation of V-ATPase, which releases $H^+$ to suppress voltage-gated $Ca^{2+}$ channels (50). $H_2S$ reduces the cysteine disulfide bonds of NMDA receptors and V-ATPase to modulate their function, whereas it may produce persulfide formation at the cysteine residues in the regulatory site of TRP channels.

In addition to the direct activation or modulation of channels, receptors, and enzymes, $H_2S$ modulates enzyme activity through intracellular signaling. For example, the enhancement of $\gamma$-glutamylcysteine synthase by $H_2S$ is observed only when $H_2S$ is applied to the outside of the cells, but not when the enzyme is directly exposed to $H_2S$, suggesting that $H_2S$ activates a cell surface receptor to induce a second messenger, which then activates the enzyme (39, 40).

The cytoprotective effect of $H_2S$ was initially found in the mammalian brain and heart, and subsequently in many other organs (21, 40, 78). This effect is not restricted in mammals. It is well known that bacteria produce $H_2S$, but it has been considered only a byproduct of metabolic pathways. Recently, $H_2S$ was found to protect bacteria from antibiotics (66). The enzymes that produce $H_2S$ in bacteria are the bacterial counterparts of CBS, CSE, and 3MST. The disruption of these enzymes renders bacteria sensitive to antibiotics. Since $H_2S$ is diffusible, it is effective in protecting surrounding bacteria as well. These findings indicate that the cytoprotective effect of $H_2S$ is a universal defense mechanism in organisms from bacteria to mammals. Since mammalian enzymes have diverged from their bacterial counterparts, it is possible to make specific inhibitors of bacterial enzymes that leave mammalian counterparts unaffected, and conjugate them to currently used antibiotics to produce novel antibiotics which do not elicit resistance from bacteria.

The equilibrium between $H_2S$ and $HS^-$ changes depending on the pH. Since the pH varies in each organelle, various ratios of $H_2S/HS^-$ are seen in cells. The pH of extracellular fluids such as blood is slightly alkaline compared with that of the cytosol; therefore, $HS^-$ is more dominant in blood. Since $H_2S$ but not $HS^-$ readily passes though the lipid bilayer membrane, this ratio may limit its efficiency to pass through the membrane (47). The levels of $H_2S$ are also affected by the rate of $H_2S$ absorption in the form of bound sulfane (32). For example, $H_2S$ is immediately absorbed in liver homogenates, but it takes minutes in brain homogenates, suggesting that $H_2S$ may remain longer in some tissues than in others.

It is possible that some of these observed effects which are considered induced by $H_2S$ might be induced by other forms of sulfur compounds (58, 73). It is necessary to address these problems in order to establish $H_2S$ as a physiological mediator and to identify its appropriate therapeutic applications to diseases in which $H_2S$ is involved.

During the revision of this article, we discovered the fourth pathway for $H_2S$ production (67). 3MST produces $H_2S$ from 3MP, which is produced from D-cysteine by D-amino acid oxidase. This pathway operates predominantly in the cerebellum and the kidney. Administration of D-cysteine protects primary cultures of cerebellar neurons from oxidative stress induced by $H_2O_2$ and attenuates ischemia-reperfusion injury in the kidney more than L-cysteine. The novel pathway of $H_2S$ production provides a new therapeutic approach to deliver $H_2S$ to specific tissues.

We also discovered that polysulfides are possible $H_2S$-derived signaling molecules (40a).

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Address correspondence to:
*Dr. Hideo Kimura*
*NCNP*
*National Institute of Neuroscience*
*Kodaira*
*Tokyo 187-8502*
*Japan*

*E-mail:* kimura@ncnp.go.jp

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**Abbreviations Used**

3MP = 3-mercaptopyruvate
3MST = 3-mercaptopyruvate sulfurtransferase
AMPA = alpha-amino-3-hydroxy-5-methy-4 isoxazole proprionic acid
ATP = adenosine triphosphate
CAT = cysteine aminotransferase
CBS = cystathionine $\beta$-synthase
CSE = cystathionine $\gamma$-lyase
DHLA = dihydrolipoic acid
DTT = dithiothreitol
elF2$\alpha$ = eukaryotic translational initiation factor 2 alpha
ER = endoplasmic reticulum
GABA = $\gamma$-aminobutyric acid
$H_2S$ = hydrogen sulfide
NADH (NAD) = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
NaHS = sodium hydrosulfide
NMDA = *N*-methyl-D-aspartate
NO = nitric oxide
Nrf2 = nuclear factor erythroid 2-related factor 2
PERK = protein kinase-like ER kinase
PLP = pyridoxal 5′-phosphate
ROS = reactive oxygen species
RPS3 = ribosomal protein S3
STAT3 = signal transducers and activators of transcription 3
TNF-$\alpha$ = tumor necrosis factor-$\alpha$
TRP = transient receptor potential
UPR = unfolded protein response
V-ATPase = vacuolar-type proton ATPase