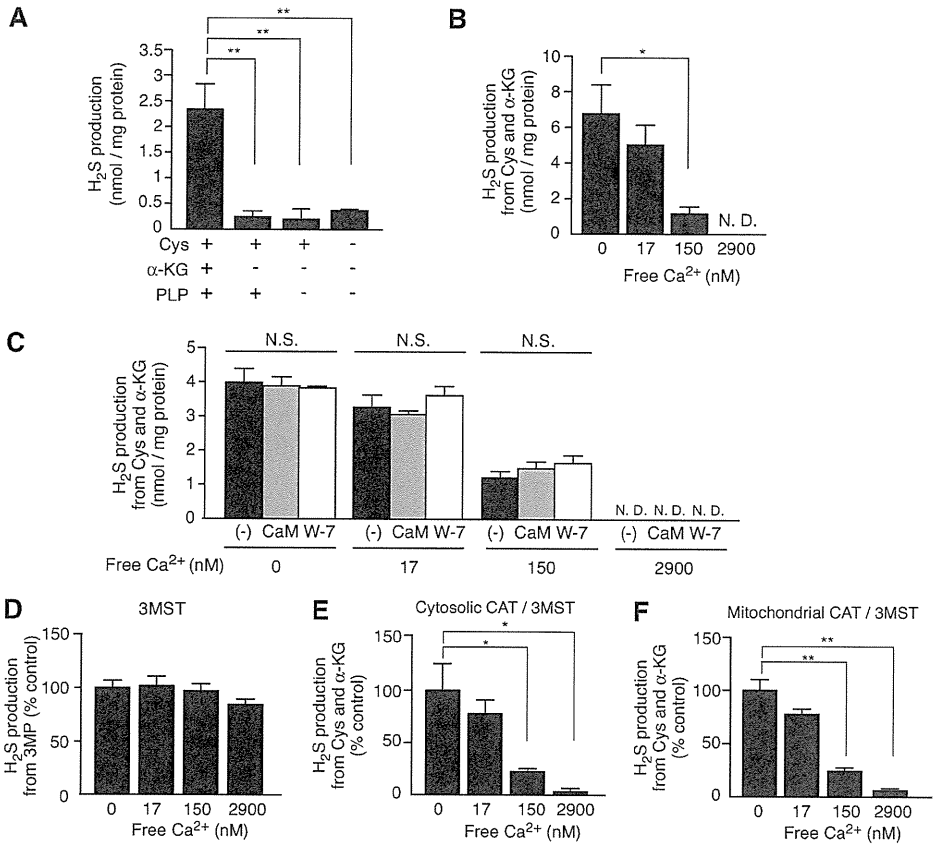

FIGURE 2. **H_2S production by 3MST along with CAT is regulated by Ca^{2+}.** *A*, H_2S production requires cysteine and α-KG in the retina. Lysates of the retina were mixed with 2 mM cysteine, 0.5 mM α-KG, or 0.05 mM PLP and 17 nM Ca^{2+}. **, $p < 0.01$ *versus* the mixture of cysteine, α-KG and PLP. *B*, H_2S production from cysteine and α-KG depends on Ca^{2+}. Lysates of the retina produced H_2S in the presence of cysteine, α-KG and Ca^{2+} with the concentrations indicated. *, $p < 0.05$ *versus* Ca^{2+} free control. *C*, calmodulin is not involved in the regulation of H_2S production from cysteine and α-KG. The production of H_2S from cysteine and α-KG by retinal lysates was not changed in the presence of 1 μM calmodulin or 100 μM W-7, a calmodulin inhibitor. (−): control. *NS*, not statistically significant between indicated groups. *D*, H_2S production by 3MST from 3-mercaptopyruvate (*3MP*) does not depend on Ca^{2+}. The lysates of HEK 293-F cells expressing 3MST were incubated with 0.01 mM 3MP. *E* and *F*, H_2S production by 3MST and CATs depends on Ca^{2+}. The lysates of HEK 293-F cells expressing cytosolic CAT (*E*) or mitochondrial CAT (*F*) and those expressing 3MST were mixed and incubated with 2 mM L-cysteine, 0.5 mM α-KG in the presence of Ca^{2+}. *, $p < 0.05$; **, $p < 0.01$ *versus* Ca^{2+} free control. All results are indicated as means ± S.E. of at least three experiments.

Post hoc multiple comparisons were made using the Bonferroni/Dunn test.

RESULTS

3MST and CAT Are Localized to the Retinal Neurons—Because H_2S-producing enzymes 3MST and CAT are localized to neurons in the brain (4), we examined the localization of both enzymes in the retina by immunohistochemistry (Fig. 1*A*). Both 3MST and CATs were localized to the inner plexiform layer, the outer plexiform layer, the inner nuclear layer, the outer nuclear layer and the outer segments of photoreceptors (Fig. 1, *B--I*). Especially 3MST co-localized with calbindin, a specific marker for horizontal cells (Fig. 1, *J–Q*). Neither CBS nor CSE was found in the retina (Fig. 1, *R–U*).

The Production of H_2S by 3MST and CAT Is Regulated by Ca^{2+}—Because both 3MST and CAT are localized in the retina, it is possible that the retina can produce H_2S. The production of H_2S by lysates of the retina was examined. Lysates produced H_2S in the presence of cysteine, α-KG and pyridoxal 5′-phosphate (PLP) (Fig. 2*A*). In the absence of α-KG, which is required for CAT but not for CBS nor CSE, lysates produced little H_2S (Fig. 2*A*). These observations suggest that H_2S is produced by 3MST along with CAT in the retina.

Because Ca^{2+} regulates the activity of many enzymes, we investigated the effect of Ca^{2+} on the activity of 3MST and CAT by measuring the amount of H_2S produced by retinal lysates. H_2S production was the maximum in the absence of Ca^{2+} and suppressed by Ca^{2+} in a dose-dependent manner (Fig. 2*B*). The production of H_2S was completely suppressed at 2.9 μM Ca^{2+}. Since the intracellular concentrations of Ca^{2+} change from ~600 nM in darkness to less than 10 nM during illumination in mouse retinal photoreceptor cells (28), H_2S is likely to be produced by 3MST and CAT when photoreceptor cells are exposed to light.

Because of its role in Ca^{2+}-dependent regulation, the involvement of calmodulin in the Ca^{2+} regulation of H_2S production was examined. Neither calmodulin nor a calmodulin specific inhibitor, W-7, showed any effect on the production of H_2S in cell lysates (Fig. 2*C*). These observations suggest that calmodulin is not involved in the regulation of H_2S-producing activity of 3MST and CAT.

To determine which enzyme, 3MST or CAT, is regulated by Ca^{2+}, H_2S production from lysates of HEK 293-F cells overexpressing 3MST and CAT was examined. To evaluate Ca^{2+} dependence of 3MST, the amount of H_2S was measured using 3MP as a substrate in the absence of CAT. The production of

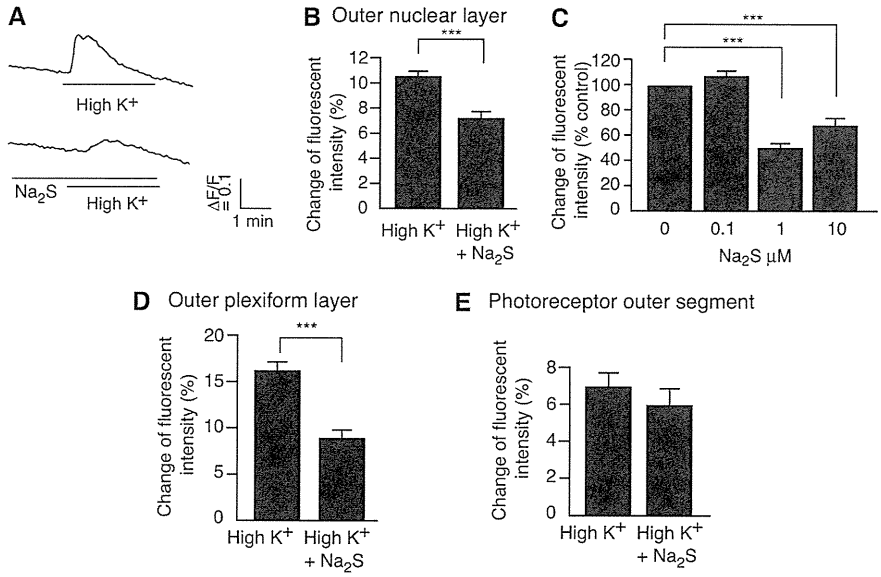

FIGURE 3. **H_2S suppresses the high K^+-evoked Ca^{2+} influx in the outer nuclear layer.** *A*, Ca^{2+} influx was suppressed by H_2S. Na_2S (10 μM) were added 3 min before and during 30 mM K^+ stimulation. The fluorescence signals were recorded from the outer nuclear layer. *B*, calcium Green-1 AM fluorescence was measured on ~100 μm^2 square regions (n = 60–80) from retinal slices (n = 3–4 slices). ***, $p < 0.001$. *C*, suppressing effect of H_2S on Ca^{2+} influx. The sensitivity of Ca^{2+} influx to H_2S is different depending on regions in the retina. ***, $p < 0.001$. *D*, Ca^{2+} influx was suppressed by H_2S in the outer plexiform layer. Calcium Green-1 AM fluorescence was measured on ~100 μm^2 square regions (n = 30–40) from retinal slices (n = 3–4 slices). ***, $p < 0.001$. *E*, Ca^{2+} influx was not suppressed by H_2S in retinal photoreceptor outer segment. Na_2S (10 μM) were added 3 min before and during 30 mM K^+ stimulation. Error bars indicate S.E.

H_2S with 3MP as a substrate was not changed by Ca^{2+} (Fig. 2*D*). This observation suggests that the activity of 3MST is not regulated by Ca^{2+}. Because 3MP is unstable and difficult to measure, the amount of H_2S produced from cysteine and α-KG by combined 3MST and CAT was measured to evaluate the Ca^{2+} dependence of CAT. The production of H_2S was decreased by Ca^{2+} in a concentration-dependent manner (Figs. 2*E* and 2*F*). These observations suggest that the activity of CAT is regulated by Ca^{2+}.

H_2S Suppresses Ca^{2+} Influx by Activating V-ATPase—High concentrations of K^+ evokes Ca^{2+} influx in photoreceptor cells (29). Because H_2S regulates Ca^{2+} channels in astrocytes (11), the effect of H_2S on Ca^{2+} influx evoked by high K^+ was examined. Na_2S, a donor of H_2S, suppressed Ca^{2+} influx in the outer nuclear layer (ONL) (Fig. 3, *A–C*). Na_2S alone did not induce Ca^{2+} influx. A similar observation was made in the outer plexiform layer (OPL) (Fig. 3*D*). In contrast, the photoreceptor outer segment only weakly responded to high-K^+, and the responses were not affected by Na_2S (Fig. 3*E*). These observations suggest that H_2S may regulate voltage-dependent Ca^{2+} influx in cells of ONL and OPL.

The center-surround organization of receptive field is one of the most important characteristics of the retinal neurons. The negative feedback from horizontal cells to photoreceptor cells plays an important role in the center-surround organization (30). Feedback from horizontal cells to photoreceptor cells is mediated by the suppression of L-type Ca^{2+} channels on photoreceptor cells by protons released from V-ATPase on horizontal cells (31–34). V-ATPase has two cysteine residues at the ATP-binding site of catalytic subunit and reversible formation of disulfide bond results in inactivation of the V-ATPase (35). Because H_2S has reducing ability, it can activate the V-ATPase by reducing the disulfide bond in the catalytic site. We therefore examined the type of calcium channels mediating Ca^{2+} influx and the involvement of V-ATPase in H_2S-induced suppression of Ca^{2+} influx. Inhibitors specific to L-type voltage gated calcium channels, nifedipine and diltiazem, greatly suppressed Ca^{2+} influx (Fig. 4*A*), indicating that L-type Ca^{2+} channels are activated by high K^+ in agreement with the previous study (29, 36, 37). Because protons released from V-ATPase cause acidification, whether or not acidification suppresses Ca^{2+} influx was examined. The acidification from pH 7.4 to pH 7.2 suppressed the Ca^{2+} influx (Fig. 4*B*). This observation suggests that V-ATPase mediates H_2S-induced suppression of Ca^{2+} influx.

To confirm the involvement of V-ATPase, the suppressing effect of H_2S on Ca^{2+} influx was investigated in the presence of inhibitors of V-ATPase, bafilomycin A1 and *N*-ethylmaleimide (NEM) (34). 100 nM bafilomycin A1, a specific inhibitor of a V-ATPase, abolished the suppressing effect of H_2S (Fig. 4*C*). Five hundred μM NEM, which masks SH-groups at the active site of V-ATPase (35), also abolished the suppressing effect of H_2S on Ca^{2+} influx (Fig. 4*D*). These observations suggest that H_2S activates V-ATPase on horizontal cells to release protons that suppress L-type Ca^{2+} channels on photoreceptor cells.

It was reported that GABA mediates the feedback signal from horizontal cells to photoreceptor cells (38). We examined whether or not GABA mediates the suppressing effect of H_2S on Ca^{2+} influx using inhibitors specific for GABA receptors, picrotoxin and bicuculline. Neither picrotoxin nor bicuculline changed the suppressing effect of H_2S on Ca^{2+} influx (Fig. 4, *E* and *F*). These observations suggest that GABAergic input is not involved in the suppressing effect of H_2S on Ca^{2+} influx.

H_2S Protects Retinal Photoreceptor Cells from Light-induced Degeneration—The retina is susceptible to oxidative stress because of its high consumption of oxygen and daily exposure

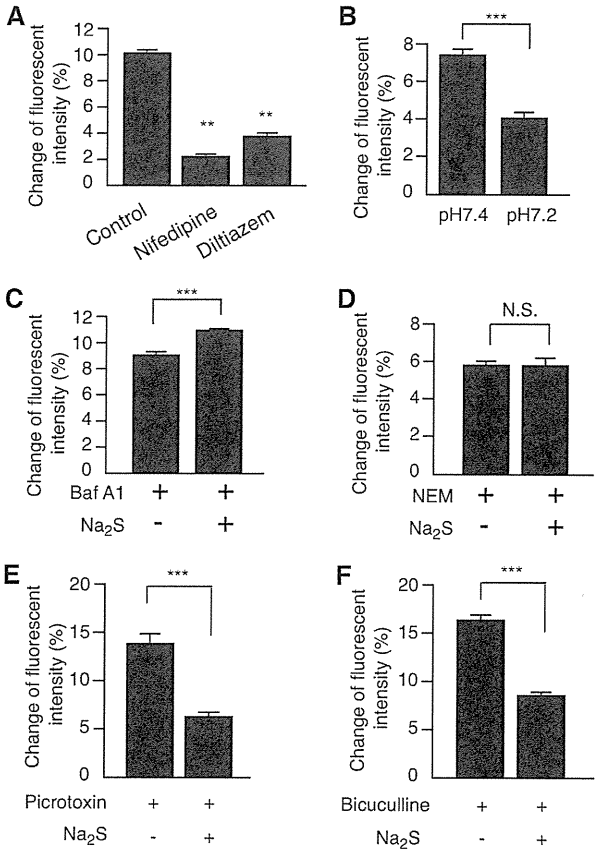

FIGURE 4. **H_2S suppresses Ca^{2+} influx by activating V-ATPase.** *A*, Ca^{2+} influx was suppressed by inhibitors of L-type Ca^{2+} channels. Nifedipine (20 μM) and diltiazem (100 μM) suppressed Ca^{2+} influx. (n = 72 regions from 3 retinal slices). **, $p < 0.01$. *B*, Ca^{2+} influx was suppressed under acidic conditions. High K^+ stimulation was applied with bicarbonate buffer saline (BBS) at pH7.4 and pH7.2. (n = 96 regions from 4 retinal slices). ***, $p < 0.001$. *C*, effect of H_2S on Ca^{2+} influx was abolished by bafilomycin A1 (Baf A1). Baf A1 (100 nM) was added 3 min before and during high K^+ stimulation. (n = 96–120 regions from 4–5 retinal slices). ***, $p < 0.001$. *D*, effect of H_2S on Ca^{2+} influx was abolished by *N*-ethylmaleimide (NEM). NEM (0.5 mM) was added 6 min before high K^+ stimulation. (n = 72 regions from 3 retinal slices). *E* and *F*, suppressing effect of H_2S on Ca^{2+} influx in the outer nuclear layer was not affected by picrotoxin or bicuculline. Picrotoxin (100 μM) (*E*) or bicuculline (20 μM) (*F*) was added 3 min before and during high K^+ stimulation. n = 72 regions from retinal slices (n = 3 slices). ***, $p < 0.001$. The fluorescence signals were recorded from the outer nuclear layer. Error bars indicate S.E.

to light. Excessive light exposure leads to photoreceptor degeneration in the retina (39). Photoreceptor cell death is an irreversible injury that is caused by various factors, such as reactive oxygen species (ROS) and elevated intracellular concentrations of Ca^{2+} (40). Because H_2S protects neurons from oxidative stress (17, 18, 21), it is possible that H_2S protects photoreceptor cells from light-induced retinal degeneration. To examine this possibility, we investigated the effect of H_2S on retinal degeneration caused by exposing the retina to excessive levels of light. The cytoprotective effect of H_2S *in vivo* was investigated with NaHS, and NaHS as well as Na_2S suppressed Ca^{2+} influx in the retina (Fig. 5, *A–C*) (18). For these reasons we administered NaHS to mice and examined its effect on photoreceptor degeneration induced by light exposure. The photoreceptor outer segments were deteriorated (Fig. 5, *E* and *H*). In contrast, the light-induced damage was significantly suppressed in mice administered NaHS (Fig. 5, *F* and *I*). Many cells in the outer nuclear layer, in which rod inner segments are located, became TUNEL-positive following exposure to light (Fig. 5, *K* and *N*). The administration of NaHS decreased the number of TUNEL-positive cells by ~80% relative to a control without any effect on the density of cells (Fig. 5, *L* and *O–Q*).

To confirm the protective effects of H_2S on the retinal neurons from light-induced degeneration, we examined the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is a product of DNA damaged by ROS. We used immunohistochemistry with an antibody against 8-OHdG. Light exposure produced a lot of 8-OHdG positive cells in the outer nuclear layer (Fig. 5*T*). In contrast, the number of cells positive to 8-OHdG was decreased in NaHS-treated mice (Fig. 5*V*). These observations suggest that H_2S protects photoreceptor cells from light-induced retinal degeneration and oxidative stress.

DISCUSSION

The Localization of 3MST and CAT in the Retina—Both 3MST and CAT were localized to the retinal neurons, while CBS and CSE were not found, and H_2S was not produced from cysteine in the absence of α-KG (Figs. 1, *B—U* and 2*A*) (41, 42), indicating that the 3MST along with CAT can produce H_2S in the retina. Although both CBS and CSE were found in salamander retina (42), the 3MST/CAT pathway is a major pathway to produce H_2S in mammalian retina.

Ca^{2+} Regulation on 3MST/CAT Pathway—The range of intracellular Ca^{2+} is shifted to the lower concentrations in the retinal neurons compared with the other types of cells in which the intracellular concentrations of Ca^{2+} are between 100 nM and 1–2 μM (43). In brightness, the voltage-gated Ca^{2+} channels are closed, and the intracellular concentrations of Ca^{2+} in photoreceptor cells are reduced to less than 10 nM. When photoreceptor cells are hyperpolarized in brightness, a release of glutamate from photoreceptor terminals is decreased, resulting in horizontal cells being in a quiescent state. The intracellular concentrations of Ca^{2+} in horizontal cells are maintained ~50–75 nM in the resting state (44). In darkness, Ca^{2+} ions enter the photoreceptor cells, and the intracellular concentrations of Ca^{2+} reach ~600 nM (28). The present study shows that the H_2S-producing activity of 3MST/CAT pathway is increased at low concentrations of Ca^{2+} that is achieved in brightness (Fig. 2*B*).

The present study shows that Ca^{2+} regulates the CAT activity but that calmodulin is not involved in the regulation (Fig. 2, *C*, *E*, and *F*). The regulation by Ca^{2+} without the involvement of calmodulin has been demonstrated for three mitochondrial dehydrogenases; pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase (24). These enzymes do not have any Ca^{2+} binding domain, but they are stimulated by Ca^{2+}. The other example is serine racemase, which is a PLP-dependent enzyme similar to CAT, is activated by Ca^{2+} (45). In this case Ca^{2+} binds to the site formed by two carboxylate-containing residues (glutamate and aspartate) (45, 46). No such Ca^{2+} binding site has been identified in CAT.

H_2S Regulates Ca^{2+} Influx—The center-surround organization is caused by the inhibitory feedback from horizontal cells (30). The proposed mechanisms for the feedback are the changes in pH in the synaptic cleft and the involvement of

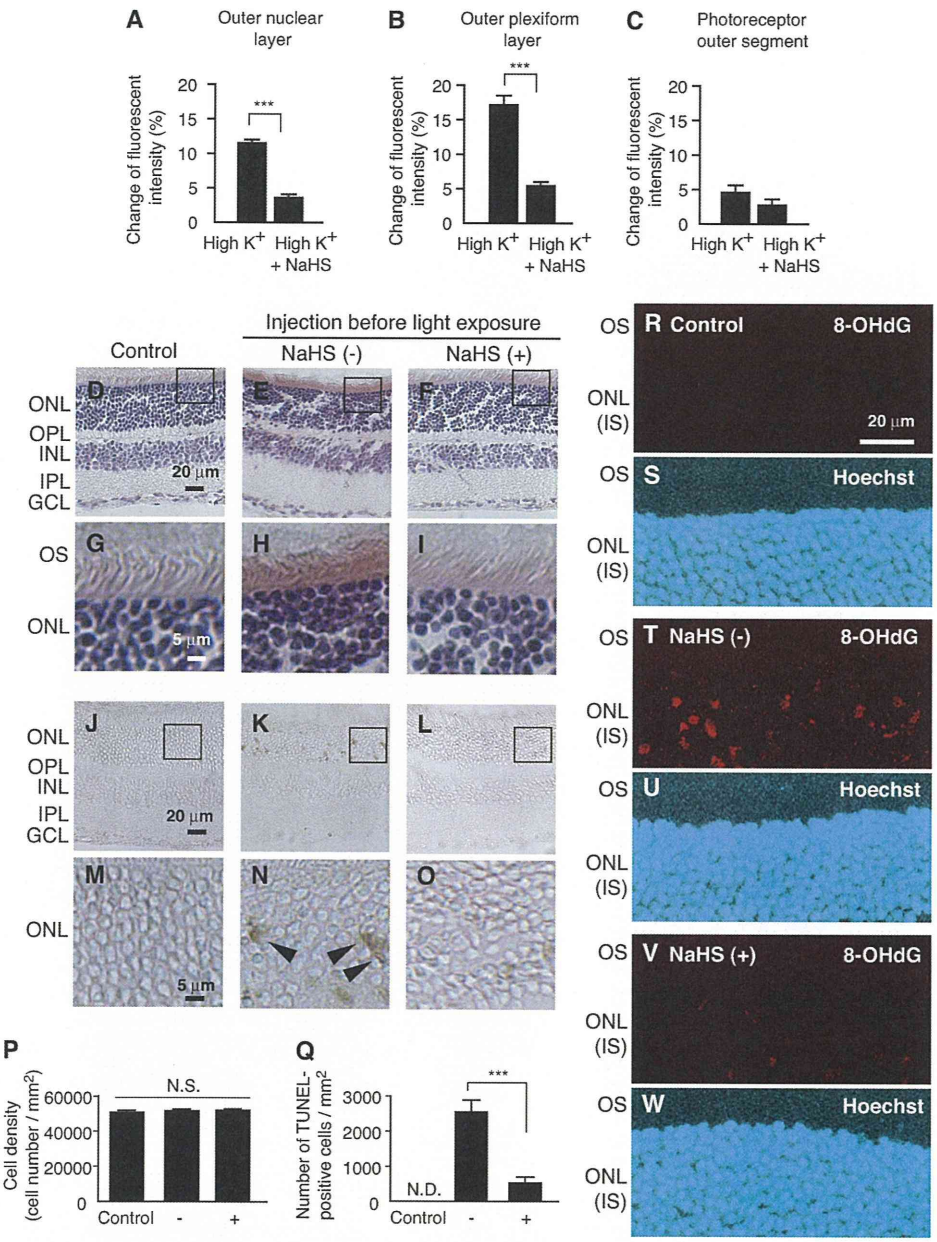

FIGURE 5. **H_2S protects retinal neurons from light-induced degeneration.** *A*, Ca^{2+} influx was suppressed by H_2S. NaHS (10 μM) were added 3 min before and during 30 mM K^+ stimulation. The fluorescence signals were recorded from the outer nuclear layer (*ONL*). Calcium Green-1 AM fluorescence was measured on ~100 μm^2 square regions ($n = 60–80$) from retinal slices ($n = 3–4$ slices). ***, $p < 0.001$. *B*, Ca^{2+} influx was suppressed by H_2S in the outer plexiform layer. ***, $p < 0.001$. *C*, Ca^{2+} influx was not suppressed by H_2S in retinal photoreceptor outer segment. *D*—*I*, H&E staining of the retina exposed to great intensity of light. Cross sections of non-treated- (*D*), light exposure plus vehicle (PBS) (*E*), light exposure plus NaHS treated- (*F*) retina at 24 h after light exposure to mice. *Square areas* in *D*, *E*, and *F* were magnified and shown in *G*, *H*, and *I*, respectively. *J*—*O*, H_2S decreased the number of TUNEL-positive cells generated by the light exposure. Cross sections of non-treated- (*J*), light exposure plus vehicle (*K*), light exposure plus NaHS treated- (*L*) retina at 24 h after light exposure to mice. TUNEL-positive cells (*arrowheads*) are observed in the ONL as *dark brown* precipitates. *Square areas* in *J*, *K*, and *L* were magnified and shown in *M*, *N*, and *O*, respectively. *P*, quantitative analysis of the total cells in the ONL. Data are represented as means ± S.E., $n = 5$ eyes. *Q*, quantitative analysis of the TUNEL-positive cells in the ONL. Data are represented as means ± S.E., $n = 5$ eyes. ***, $p < 0.001$. *R*–*W*, H_2S suppresses the levels of 8-OHdG in the retinal neurons induced by light exposure. The immunohistochemical analysis with an antibody against 8-OHdG in the retina obtained from mice without exposure to light (*R*); PBS-treated mice 24 h after light exposure (*T*); and NaHS-treated mice with light exposure (*V*) are shown. Nuclei were stained by Hoechst 33342 (*S*, *U*, *W*, respectively).

GABAergic neurons (31–34, 38). The present study shows that H_2S activates V-ATPase to suppress high K^+ evoked Ca^{2+} influx mediated by L-type Ca^{2+} channels (Fig. 4, *A*–*D*). The influence of pH changes caused by Na_2S and NaHS on the suppression of Ca^{2+} influx is negligible for the following reasons. 1) Because bicarbonate buffered saline (BBS) contains 22.6 mM $NaHCO_3$ continuously bubbled with 95% (v/v) O_2 and 5% (v/v) CO_2, concentrations less than 10 μM Na_2S or NaHS do not have any effect on pH. 2) Na salts shift pH, if any, to alkaline, while suppression of Ca^{2+} influx occurs by acidification. Because photoreceptor cells and horizontal cells have 3MST and CAT to produce H_2S, it is possible that H_2S activates V-ATPase in

horizontal cells to release protons to the synaptic cleft that suppress Ca^{2+} channels in photoreceptor cells.

GABA receptor inhibitors did not affect the suppression of Ca^{2+} influx induced by H_2S (Fig. 4, *E* and *F*). GABAergic input from horizontal cells to cone photoreceptor cells is present but its contribution is weak and limited, and picrotoxin could not completely suppress the cone receptive field surround (32, 47). These observations suggest that GABA may not mediate H_2S-induced suppression of Ca^{2+} influx.

Ca^{2+} Homeostasis and Photoreceptor Damage—H_2S suppresses the elevation of Ca^{2+} in the photoreceptor cells by activating V-ATPase in horizontal cells and maintains Ca^{2+} homeostasis (Figs. 3 and 4). Intracellular concentrations of Ca^{2+} are increased during photoreceptor apoptosis, but the L-type Ca^{2+} channel blocker diltiazem prevents light-induced photoreceptor cell death (48). The lack of the V-ATPase *a*3 subunit causes retinal degradation in mice (49). These observations suggest that the failure of Ca^{2+} homeostasis, which is regulated by Ca^{2+} channels and V-ATPase, by excessive levels of light may cause retinal cell degeneration. The regulation of Ca^{2+} and cytoprotective effect of endogenous H_2S may fail when photoreceptor cells are exposed to excessive levels of light. Even under such conditions the administration of H_2S protects cells from light-induced degeneration.

CONCLUSION

H_2S is produced by 3MST along with CAT in retinal neurons and may modulate the negative feedback from horizontal cells to photoreceptor cells by regulating Ca^{2+} influx through the activation of V-ATPase. Because H_2S protects retinal neurons from light-induced degeneration, the enhancement of 3MST/CAT pathway or the administration of H_2S may have clinical benefit for diseases with retinal cell degeneration.

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FORUM REVIEW ARTICLE

Hydrogen Sulfide Is a Signaling Molecule and a Cytoprotectant

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Abstract

Significance: Accumulating evidence shows that hydrogen sulfide may function as a signaling molecule in processes such as neuromodulation in the brain and smooth muscle relaxation in the vascular system. It also has a cytoprotective effect, since it can protect neurons and cardiac muscle from oxidative stress and ischemia-reperfusion injury, respectively. Hydrogen sulfide can also modulate inflammation, insulin release, and angiogenesis. ***Recent Advances:*** The regulation of the activity of 3-mercaptopyruvate sulfur transferase (3MST) along with cysteine aminotransferase (CAT), one of the H_2S producing pathways, has been demonstrated. The production of H_2S by the pathway, which is regulated by Ca^{2+} and facilitated by thioredoxin and dihydrolipoic acid, is also involved in H_2S signaling as well as cytoprotection. Sulfur hydration of proteins by H_2S has been proposed to modulate protein functions. H_2S-sensitive fluorescent probes, which enable us to measure the localization of H_2S in real time, have been developed. ***Critical Issues:*** The basal concentrations of H_2S have recently been measured and found to be much lower than those initially reported. However, the concentration of H_2S reached in stimulated cells, as well as the regulation of H_2S producing enzymes is not well understood. It has been proposed that some of the effects of H_2S on the regulation of enzymes and receptors might be explained through the properties of sulfane sulfur (S^0), another form of active sulfur. ***Future Directions:*** The determination of H_2S concentrations in activated cells using new methods including H_2S-sensitive fluorescent probes, as well as the investigation of the effects of H_2S using specific inhibitors, may provide better understanding of the physiological function of this molecule. Clarifying mechanisms of H_2S activity may also facilitate the development of new therapeutic compounds. *Antioxid. Redox Signal.* 17, 45–57.

Introduction

THE ENDOGENOUS CONCENTRATIONS OF SULFIDE in the brains of humans, bovine, and rats are in the range of 50–160 μM, strongly suggesting that endogenous H_2S may have significant physiological functions (20, 56, 72). However, the concentrations of sulfide measured in these studies, which contained H_2S released from acid-labile sulfur in addition to free H_2S, were overestimated (23). Recently, the basal concentrations of H_2S in tissues have been measured and found to be much lower than those previously reported (18, 24, 36). During the preparation of this manuscript, H_2S-sensitive fluorescence probes have been reported from several laboratories (38, 39, 50, 52, 55). These probes may enable us to detect the sites at which H_2S is released in real time and assess the local concentrations of H_2S.

We demonstrated that cystathionine β-synthase (CBS), which produces H_2S from cysteine or cysteine with homocysteine, is localized to astrocytes, a type of glia, and 3-mercaptopyruvate sulfur transferase (3MST), which produces H_2S from 3-mercaptopyruvate, is localized to neurons in the brain (16, 57). The activity of CBS is enhanced by S-adenosyl methionine, and that of 3MST pathway is regulated by Ca^{2+} (1, 43). Cystathionine γ-lyase (CSE) is expressed little in the brain (1, 26).

H_2S facilitates the induction of hippocampal long-term potentiation by enhancing the activity of N-methyl D,L-aspartate (NMDA) receptors (1). In the hypothalamus, H_2S suppresses the KCl-stimulated release of corticotropin-releasing hormone (CRH), while it has no effect on the basal release of CRH. S-Adenosyl methionine inhibits the release of glucocorticoid induced by stress without any effect on hypothalamo-pituitary-adrenal function under resting conditions *in vivo* (13). H_2S induces Ca^{2+} influx in astrocytes that propagates to the surrounding astrocytes as Ca^{2+} waves (16, 47). There is a reciprocal interaction between neurons and astrocytes: neuronal excitation activates surrounding astrocytes, which in turn modulate synaptic activity. H_2S regulates synaptic activity by enhancing the activity of both neurons and astrocytes.

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In addition to a possible function as a signaling molecule, we demonstrated a role of H_2S as a cytoprotectant in the brain (30–32). H_2S protects neurons from oxidative stress by restoring glutathione levels decreased by oxidative stress and suppressing oxidative stress in mitochondria. Philip Moore and colleagues also demonstrated that H_2S protects a neuroblastoma cell line from oxidative stress induced by peroxynitrite and hypochlorous acid (76, 77). These oxidants cause the oxidative modification of proteins and lipids seen *in vivo* in patients with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. H_2S also protects retinal neurons from light-induced degeneration by suppressing the excessive Ca^{2+} influx (43). The cytoprotective effect was also found by David Lefer and colleagues in the cardiovascular system. H_2S protects cardiac muscle from ischemia-reperfusion injury by preserving mitochondrial function (15). The application of an H_2S donor or the regulation of endogenous H_2S concentrations by manipulating the *CSE* gene protected cardiomyocytes from ischemia-reperfusion injury. Based on this study, an H_2S donor is being given to the bypass surgery patients in a Phase II clinical trial (51).

We showed that CSE is expressed in the thoracic aorta, portal veins, and ileum, and that H_2S relaxes these tissues (22). The smooth muscle relaxant effect of H_2S in the thoracic aorta and ileum was further studied by Rui Wang and Philip Moore, and H_2S was found to activate adenosine triphosphate (ATP)-dependent K^+ (K_{ATP}) channels (65, 82). The role of H_2S and CSE has also been studied *in vivo* using CSE-knockout mice (25, 81). The regulation of inflammation, insulin release, and angiogenesis by H_2S have also been intensively studied, and this has led to the development of therapeutic agents. This review focuses on the regulation of endogenous H_2S and the regulation of its enzymatic production, as well as the methods recently developed to measure it in addition to the function as a signaling molecule and a cytoprotectant.

Permeability of H_2S Through the Plasma Membrane

The mechanism of H_2S transport through the plasma membrane has recently been demonstrated. Because of the structural similarity of H_2S with H_2O, it was hypothesized that H_2S may pass through aquaporins or water channels (40). However, although reconstituted aquaporin obtained from a sulfide-reducing bacteria, AfAQP, increased the permeability of water in planar lipid bilayers, the permeability of H_2S remained unchanged. The membrane resistance to H_2S permeation is negligible. Even bilayers containing cholesterol and sphingomyelin, which decrease the diffusiveness of the membrane, had the same H_2S permeability. These observations indicate that the membrane is not the limiting factor for H_2S transport (40) (Fig. 1). The aquaporin homolog AqpM, which is derived from bacteria that rely on H_2S as the terminal electron acceptor for energy production remains a candidate H_2S transporter (34).

H_2S is soluble in water and dissociates into H^+ and HS^-. In physiologic saline at 37°C and pH 7.4, approximately one-fifth of H_2S exists as the undissociated form (H_2S), and the remaining four-fifths exists as HS^- plus a trace of S^{2-} at equilibrium with H_2S (14). However, theoretical calculations indicate that transport of HS^- by Cl^- channels or other anion channels is unlikely to play a physiological role (40). The form of the molecule passing through the membrane is not necessarily the functional form. It may be difficult to determine which form, H_2S or HS^-, has a physiological function. In addition, since HS^- can be oxidized to persulfide, it is possible that some observed effects with NaHS or Na_2S may be even induced by a trace amount of persulfide. As to the possible involvement of other sulfur-containing substances as signaling molecules, see the review by Toohey (68).

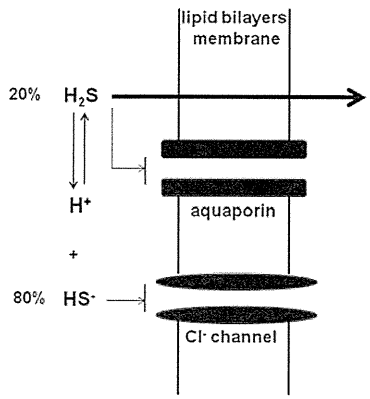

FIG. 1. H_2S passes through the plasma membrane by diffusion. Aquaporin and anion channels are not required for H_2S and HS^- respectively to pass through the plasma membrane.

H_2S Concentrations

In early studies, the concentrations of sulfide in the brain were measured under conditions with high concentrations of acids. Therefore, H_2S released from acid-labile sulfur was measured along with free H_2S, with a combined concentration of between 50–160 μM in the brain (20, 56, 72). Acid-labile sulfur is derived from sulfur atoms in the iron–sulfur center of mitochondria respiratory chain enzymes. A pH less than 5.4 releases H_2S from this source of acid-labile sulfur (24). Since mitochondrial pH is between 7 and 8, H_2S may not be released from acid-labile sulfur under physiologic conditions, and therefore, the reported intracellular concentrations for H_2S are likely overestimated.

The concentrations of free H_2S are much less than those of acid-labile sulfur. Brain homogenates were vigorously mixed in the syringe, and released H_2S was measured by gas chromatography. By this method, the concentration of free H_2S is 14 nM (0.12 μmol/kg protein), while that released from acid-labile sulfur is 916 μmol/kg protein (18, 36). In another method, free H_2S is below detectable levels, while that of H_2S released from acid-labile sulfur is 161 nmol/g protein (24). Although 1 μM Na_2S efficiently activates vacuolar-type H^+ ATPase to suppress Ca^{2+} influx in the retina, the effects of the basal levels of H_2S (10–20 nM) have not been reported. In order to exert its physiological function, μM concentrations of H_2S have to be released from the activated enzymes or the intracellular store, but it is enough in a short time of period in a restricted area. After the induction of responses, H_2S may be re-stored or degraded to be a steady state to cease the reaction. The basal concentrations of H_2S are maintained in low levels in cells to properly respond to H_2S. For example, the application of high concentrations of H_2S (200 μM NaHS) to astrocytes suppresses Ca^{2+} influx induced by the second

application, suggesting that high concentrations of H_2S cause desensitization to H_2S (47).

More recent measurements of the basal concentrations of H_2S in blood still exhibit significant variation. For example, free H_2S in blood measured by gas chromatography, was 0.07 μmol/kg protein (approximately 8 nM) (36). In this method, the efficiency of recovering H_2S from a control sodium sulfide solution was estimated as a blank value in the absence of tissue homogenates. However, tissue homogenates efficiently absorb H_2S (24), and it is therefore possible that the blank value may be overestimated compared to that without tissues, resulting in the relatively lower estimate of free H_2S values in tissue homogenates. Another method mixed blood with monobromobimane, the thiol-specific derivatization agent, and recovered thiol-bound monobromobimane. The concentrations of H_2S-bound monobromobimane were specifically measured by mass spectrometry (79). Free H_2S in blood with this method was in the range of 0.4–0.9 μM. Depending on the method used, the concentration of free H_2S vary up to 100-fold, but a consistent finding is that the basal free H_2S in blood is maintained at a low concentration.

A low basal concentration of H_2S does not necessarily indicate that these are the biologically functional concentrations of this molecule. Because the physiological stimuli of H_2S release have not been identified, it is difficult to estimate the local concentrations of H_2S reached when cells are activated. Additionally, the regulation of the activity of H_2S-producing enzymes is also not well understood. The production of H_2S by CBS is enhanced by S-adenosyl methionine two-fold compared to control samples (1). Activity of the 3MST/CAT pathway is regulated by Ca^{2+}. The activity is highest in the absence of Ca^{2+} and suppressed by Ca^{2+} in a concentration-dependent manner. The production of H_2S by this pathway is dramatically changed in the range of intracellular Ca^{2+} (43). Although the regulation of CSE by Ca^{2+}/calmodulin was reported, the concentrations of Ca^{2+} applied in the study were supraphysiological (1–2 mM), as the intracellular concentrations of Ca^{2+} (100 nM–3 μM) (81).

Another source of H_2S is bound sulfane sulfur, which may function as an intracellular store of H_2S. Cells expressing 3MST and CAT contain twice as much bound sulfane sulfur as control cells. The concentrations of bound sulfane sulfur are low in cells expressing a mutant 3MST that lacks H_2S-producing activity (57). Lysates of neurons and astrocytes release H_2S in the presence of endogenous levels of glutathione and cysteine at pH 8.4, and brain homogenates release it in the presence of dihydrolipoic acid (DHLA) at pH 8.0 (24, 42). H_2S can be released from astrocytes by high K^+, which is induced by neuronal excitation (24). The concentration of bound sulfane sulfur in the brain is 1500 nmol/g protein, which is sufficient to release H_2S to stimulate target molecules (24). Considering the fact that the Km values of H_2S-producing enzymes for substrates are greater than the endogenous concentrations, the endogenous production rate of H_2S must be very low. Therefore, it is possible that H_2S may be stored after it is produced by enzymes, and released from its intracellular stores when cells are stimulated. The percentage of bound sulfane sulfur that releases H_2S in response to cell activation has not been determined (Fig. 2). It is critical that the local concentrations of H_2S reached upon cell stimulation are now re-quantified using methods including H_2S sensitive probes (38, 39, 50, 52, 55).

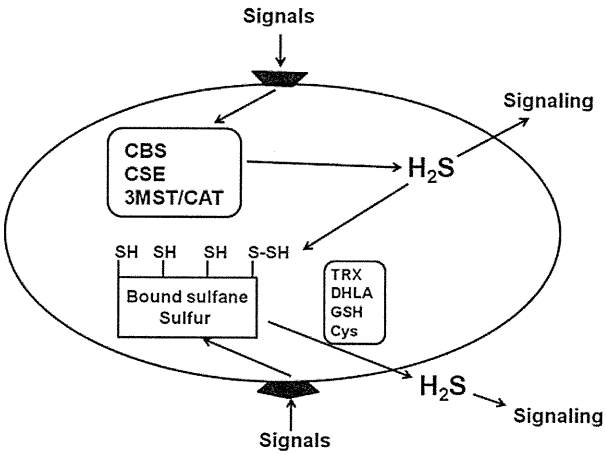

FIG. 2. H_2S is released from the producing enzymes and bound sulfane sulfur. H_2S produced by enzymes may function as a signaling molecule and may also be stored in bound sulfane sulfur as intracellular stores. When cells are stimulated, H_2S can be released from the stores. Although lysates of neurons and astrocytes release H_2S in the presence of endogenous reducing substances, H_2S release from intact cells with physiological stimulations has not been fully elucidated. Which of the enzymatic production or the intracellular stores mainly contributes to the physiological release of H_2S has not been determined.

H_2S-Sensitive Probes

The current methods to detect H_2S, such as colorimetric assays, gas chromatograpy, and polarographic analysis, require its extraction from cells and tissues. Because the catabolism and extinction of H_2S are fast, it has been difficult to measure the concentrations of this molecule accurately. Although a thiol detective imaging probe had been made, it was unable to differentiate sulfide from thiols. Recently, several fluorescent probes specifically detect H_2S have been developed (38, 39, 50, 52, 55).

SF1 and SF2

Based on the reaction that azides can be reduced by H_2S to amine, the azide-caged rhodamine analogues, SF1 and SF2, which generate highly fluorescent rhodamine products, were prepared (38). In a 30-min reaction with H_2S, SF1 and SF2 produce 7- and 9-fold responses, respectively. Although both probes slightly respond to •NO, they do not respond to reactive oxygen species, reactive nitrogen species, and thiols such as glutathione and cysteine. SF1 shows greater responses to H_2S in cells compared to SF2, probably due to the higher lipophilicity and cellular retention of SF1 relative to SF2. The improvement in shorter response time may help applying these probes to the studies of physiological and pathological processes.

DNS-Az

Dansyl is well-known for its strong fluorescence and long emission wavelength. DNS-Az is dansyl azide which emits fluorescence when H_2S reduces sulfonyl azide into sulfonamide (50). The reaction is similar to that of SF1 and SF2 (38).

Twenty five μM H_2S induces a 40-fold fluorescence enhancement. Although the detection is based on the reducing properties of H_2S, other possible reducing anions such as iodide, bromide, fluoride, bisulfate, and thiosulfate did not induce any responses. Although the selectivity of DNS-Az to H_2S compared to the other endogenous thiols, such as glutathione and cysteine, is not shown, its reaction with H_2S is fast, saturated in 5 min in 20 mM phosphate buffer in the presence of 0.05% Tween-20.

Probe 1

To selectively detect nucleophilic H_2S in the biological system, it is important to differentiate H_2S from other nucleophiles, especially thiols such as glutathione and cysteine. H_2S, which is a nonsubstituted thiol, can undergo nucleophilic reaction twice, while other thiols such as glutathione and cysteine are monosubstituted thiols that can only undergo nucleophilic reaction once. Probe 1 consists of a fluorophore, which is quenched by an attached structure containing an electrophilic component (39). H_2S reacts with the electrophilic component to form a free SH containing intermediate, which reacts with intrastructural ester group and causes cyclization to release the fluorophore.

Probe 1 reacts with 50 μM NaHS and the fluorescence intensity was increased by 55 to 70-fold, and the maximum intensity is reached in 1 h. Although probe 1 did not emit fluorescence in the presence of 50 μM glutathione and cysteine, its response to the endogenous levels of glutathione (mM order) was not shown. COS7 cells pre-incubated probe 1 emits strong fluorescence in the cells after the treatment of 250 μM NaHS.

SFP-1 and SFP-2

SFP-1 and SFP-2 consist of fluorophore and an aromatic framework with acrylate methyl ester and aldehyde ortho to each other (52). The aldehyde group reacts with sulfide to form a hemithioacetal intermediate with an exposed thiol, which reacts with the acrylate to yield a thioacetal. The reaction is similar to that of probe 1 (39). This reaction tunes photo-induced electron transfer of the aromatic structure, leading to the activation of fluorophore.

SFP-1 responds to 10–50 μM Na_2S and increases the intensity of fluorescence more than 10 times in 60 min in PBS buffer (pH 7.4). Although it also responds to the most abundant thiols in mammalian cells, glutathione and cysteine, the interference with H_2S can be excluded by selecting emission wave length. SFP-2, which has another fluorophore, showed more than 13-fold increase in the fluorescence intensity and more selective to Na_2S than to cysteine or glutathione. Although both probes slightly emit fluorescence, even without exogenously applied Na_2S in cultured HeLa cells, SFP-1 emits strong fluorescence in the presence of 100 μM Na_2S and SFP-1 with 200 μM Na_2S.

Hydrogen sulfide imaging probe-1

Azamacrocyclic rings form stable metal complexes with Cu^{2+} and the paramagnetic Cu^{2+} center has a pronounced quenching effect on fluorophores. Based on these characteristics of azamacrocyclic rings, hydrogen sulfide imaging probe-1 (HSip-1) was produced (55). H_2S binds to the Cu^{2+} center of HSip-1 to enhance fluorescence, while glutathione does not cause any conformational change in the Cu^{2+} center or the increase in fluorescence intensity.

The fluorescence intensity is increased by 50-fold by 10 μM H_2S in HEPES buffer (pH 7.4) with the maximal intensity at 30 min, while no increase in fluorescence intensity is induced by 10 mM glutathione. HSip-1 has a high selectivity over other thiols such as 1 mM cysteine, homocysteine, and 2-mercaptoethanol, and 100 μM dithiothreitol (DTT), inorganic sulfur compounds, and a reducing compound. It does not respond to reactive oxygen species or reactive nitrogen species. HSip-1 DA, diacetylated HSip-1, is membrane permeable. HeLa cells emit fluorescence upon addition to 200 μM Na_2S to DMEM medium.

Production of H_2S

CBS

H_2S can be produced by three enzymes, CBS, CSE, and 3MST, along with cysteine aminotransferase (CAT), which is identical to aspartate aminotransferase (4, 7, 41). CBS produces H_2S from cysteine via a β-elimination reaction, and more efficiently via a β-replacement reaction in which cysteine is condensed with homocysteine (8). The plasma concentrations of homocysteine in Alzheimer's disease, heart attack, and stroke are elevated. The β-replacement reaction provides an explanation for the finding that pharmacological doses of N-acetylcysteine can lower plasma homocysteine concentrations, for N-acetylcysteine passes through plasma membrane to cytoplasm and is converted to cysteine, which is condensed by CBS with homocysteine to produce H_2S (8).

In the brain, astrocytes express CBS, which is intensively localized to cerebellar molecular layer and hippocampal dentate gyrus (16, 23) (Fig. 3). The specificity of our affinity-purified antibody against CBS was examined using Western blot analysis and immunohistochemistry by comparing CBS knockout and wild-type mice. The expression of CBS changes during development: it is expressed in neuroepithelial cells in

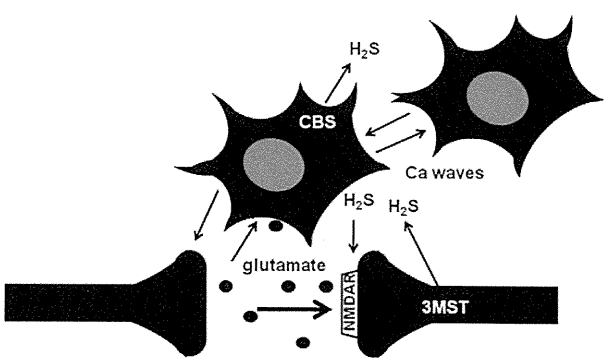

FIG. 3. CBS is localized to astrocytes and 3MST to neurons in the brain. There is a reciprocal interaction between neurons and astrocytes. Neuronal excitation induces Ca^{2+} influx in astrocytes, which in turn regulate synaptic activity. H_2S produced by CBS in astrocytes may be involved in the induction of Ca^{2+} waves, which propagate to surrounding astrocytes and contribute to the signaling between astrocytes. H_2S generated by CBS in astrocytes and by 3MST in neurons may be involved in enhancing the activity of NMDA receptors and the synaptic activity.

the ventricular zone at early developmental stages and in radial cells and astrocytes in the late embryonic and neonatal periods (16). The level of CBS is increased in reactive astrocytes, which mediate neuronal recovery in the injured brain, and is induced by epidermal growth factor (EGF), transforming growth factor (TGF)-α, and cyclic adenosine monophosphate (cAMP). Its levels are also increased in the hippocampus after kainic acid-induced seizures.

The cerebellum of CBS-knockout mice is remarkably smaller than that of wild-type animals (16). The abnormalities are not observed at birth but become apparent around postnatal day 8. Although the structural organization is normal, there was a striking reduction in the thickness of the molecular- and granular-layers with stunted Purkinje dendrites.

CBS is localized to human chromosome 21 (80). Because of trisomy 21 in Down syndrome (DS), CBS levels in patients are predicted to be 1.5-fold greater than those in normal individuals. However, the expression of CBS mRNA is 12 times greater in myeloblasts of DS children than in those of normal individuals, and the levels of CBS in DS patients are 2.4–2.9 times greater than those in age-matched normal individuals (23, 64). Individuals with DS almost invariably develop an Alzheimer type of dementia (AD). CBS is localized to astrocytes and the surrounding senile plaques in the brains of DS patients with AD. The overexpression of CBS may cause a developmental abnormality in cognition in DS children and that may lead to AD in DS adults (23). Overproduction of H_2S may cause the mental disturbance in DS. Another example of brain damage caused by elevated concentrations of H_2S is ethylmalonic encephalopathy, in which the gene that encodes sulfur dioxygenase (ETHE1) is mutated. Defects in sulfur dioxygenase, which normally metabolizes H_2S, lead to elevated concentrations of H_2S (66).

CSE

CSE can facilitate the production of H_2S from cysteine and homocysteine. Under normal conditions, α,β-elimination of cysteine generates H_2S. However, with high concentrations of homocysteine because of homocysteinemia, the α,γ-elimination and γ-replacement reactions of homocysteine become dominant for H_2S production by CSE (10). We could not detect CSE in the brain, and the activity of CSE is 100 times less than that in the liver (1, 17, 26). Although there are reports that show the existence of CSE activity in the brain, this may be due to the presence of a structurally unrelated protein that nonetheless exhibits CSE activity (72).

CSE is proposed to play a major role in vascular smooth muscle, and is mainly localized to smooth muscle in rodents (22, 82). However, CSE localization was recently reported in mouse, human, and bovine endothelium and cultures of human and bovine endothelium (81), although this finding is controversial (49, 57, 81, 82). The lysates of rat endothelium produce H_2S in the presence of cysteine and α-ketoglutarate, but there is no production of H_2S in the absence of α-ketoglutarate (57). Since CSE does not require α-ketoglutarate, this suggests that the other two H_2S-producing enzymes, 3MST and CAT, produce H_2S in rat endothelium. In bovine species, 3MST and CBS are localized to the endothelium, but CSE is not found (49).

Independent studies with CSE-knockout mice show that the involvement of CSE in the regulation of blood pressure is also controversial (25, 81). The observation that blood pressure is unaffected by loss of CSE could be explained by at least two reasons. First, it is possible that 3MST and CAT may themselves be involved in the regulation of blood pressure. Alternatively, increased generation of H_2S by 3MST and CAT may compensate for loss of CSE in the knockout mice.

3MST with CAT

The production of H_2S from 3-mercaptopyruvate (3MP) by 3MST was initially measured at pH 7.4 (41). However, maximum production was found to occur under alkaline conditions (60, 62). CBS and CSE are localized to the cytoplasm, while 3MST is mainly localized to the mitochondrial matrix, with a pH of approximately 8 (45). 3MST is localized to neurons in the brain and retina (43, 58). Neither CBS nor CSE was found in the retina.

3MP is produced by CAT from cysteine and α-ketoglutarate (11, 70) (Fig. 4). The concentrations of cysteine in the cytosol are 0.15–0.25 mM and those in the mitochondria are 0.7–0.99 mM, which may be enough to produce the basal concentrations of H_2S (21, 63). In addition, glutathione, which is an intracellular store of cysteine, is present in 10–100 times greater concentrations in mammalian tissues than is cysteine, and can supply cysteine when the cells need it (63).

A reducing substance such as DTT is required for 3MST to produce H_2S. However, an endogenous substance corresponding to DTT has not been identified. We recently found that thioredoxin and DHLA associate with 3MST to release H_2S (42) (Fig. 4). Approximately 20 μM thioredoxin exists in mitochondria, and it is 4 times more potent than DTT in enhancing H_2S production. The concentration of DHLA is approximately 40 μM in the brain, and DHLA enhances the H_2S production as effectively as DTT. The redox potentials of dithiols such as DTT, DHLA, and 2 cysteine residues in the active site of thioredoxin are in the range of -0.26 to -0.33V, while those of monothiols such as glutathione, cysteine, and

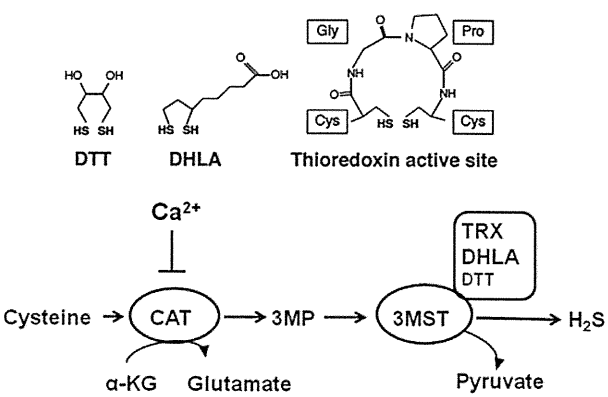

FIG. 4. Regulation of H_2S production by 3MST. Disulfides, such as thioredoxin (TRX) and dihydrolipoic acid (DHLA), are endogenous reducing substances, which can release H_2S from 3MST. The activity of CAT is regulated by Ca^{2+}. In the absence of Ca^{2+}, the production of H_2S by 3MST/CAT pathway from cysteine and α-ketoglutarate is the maximum but is suppressed by Ca^{2+} in a concentration-dependent manner.

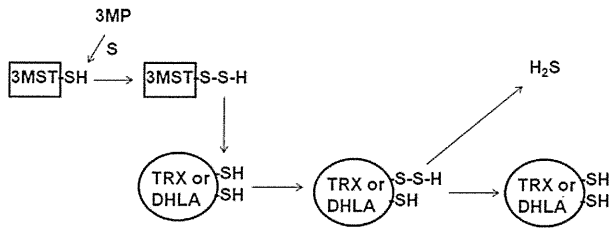

FIG. 5. A mechanism of releasing H_2S from 3MST in the presence of thioredoxin or DHLA. Thioredoxin and DHLA receive persulfide from 3MST. Thiol in thioredoxin and DHLA reduces persulfide to release H_2S.

CoA are from −0.22 to −0.35V (27). Those of nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are around −0.32 V. Because DTT, DHLA, and thioredoxin, whose two cysteine residues at the active site may function as dithiol, are able to release H_2S from 3MST, the dithiol structure but not the redox potential is a critical factor for H_2S release. A possible mechanism is that persulfide, which is produced at the active site cysteine of 3MST by receiving sulfur from 3-MP, is transferred to one of the thiols in thioredoxin or DHLA. The transferred persulfide, in turn, is reduced by another thiol in thioredoxin or DHLA to release H_2S (42, 75) (Fig. 5).

We recently found that production of H_2S from cysteine by the 3MST/CAT pathway is regulated by Ca^{2+} (43). The activity of CAT is suppressed by Ca^{2+} in a concentration-dependent manner. The production of H_2S is maximal in the absence of Ca^{2+}, and becomes completely suppressed at 2.9 μM Ca^{2+}. The range of intracellular Ca^{2+} shifts to the lower concentrations in retinal photoreceptor cells (10 nM–600 nM) compared to other types of cells in which the intracellular concentrations are between 100 nM and 1–2 μM. When retinal photoreceptor cells are exposed to light, the intracellular concentrations of Ca^{2+} are decreased to 10 nM. Intracellular Ca^{2+} concentrations are increased to 600 nM in darkness. In this range of Ca^{2+} concentration, production of H_2S by the 3MST/CAT pathway is dramatically changed. Calmodulin or its inhibitor, W-7, does not change the effect of Ca^{2+} on H_2S production, suggesting that calmodulin is not involved in the regulation of H_2S production by the 3MST/CAT pathway (43).

Both H_2S and DHLA induce nuclear factor erythroid 2-related factor 2 (Nrf 2), which increases the expression of thioredoxin and thioredoxin reductase (6, 59). Since thioredoxin and DHLA enhances the activity of 3MST to produce H_2S, both substances may also promote H_2S production through Nrf2 induction.

Modification of the Activity by Sulfhydration

Modification of the activity of enzymes and receptors is brought about by reversible covalent reactions, including phosphorylation and adenosine diphosphate (ADP)-ribosylation. The reversible incorporation of sulfur into proteins has been considered as a further example of regulation by covalent modification (Fig. 6). Kato et al. demonstrated that serine dehydratase was inhibited by CSE in the presence of cysteine (29). The suppression of the enzyme activity is reversible by glutathione or DTT, suggesting the formation of a

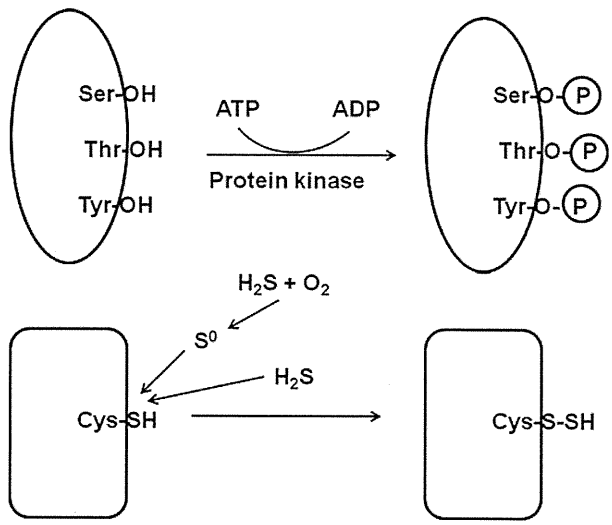

FIG. 6. Modulation of enzyme activity by phosphorylation and sulfurhydration. The reversible incorporation of sulfur into proteins may modulate protein function as phosphorylation does on proteins. H_2S may directly induce sulfurhydration. Alternatively, H_2S may react with oxygen to form S^0 that adds to sulfhydryl groups of proteins.

hydropersulfide group (sulfurhydration) in the enzyme. Enzymes inactivated by the same mechanism include 3-hydroxybutyrate dehydrogenase, alcohol dehydrogenase, and ornithine decarboxylase (67). Solomon Snyder and his colleagues recently demonstrated that H_2S induces sulfurhydration to enhance the activity of proteins, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-tubulin, and actin (19, 44). H_2S increases the activity of GAPDH by 7-fold, and DTT reverses GAPDH activity induced by H_2S. GAPDH is activated in human embryonic kidney (HEK) 293 cells expressing CSE, suggesting that endogenously produced H_2S induces sulfurhydration of GAPDH. However, further studies are required to determine the function of sulfurhydrated GAPDH.

John Toohey proposed another mechanism for sulfhydration. Some of H_2S reacts with oxygen to form S^0 that adds to sulfhydryl groups of proteins, forming a persulfide, and to disulfide groups, forming a trisulfide, though it is a generalized chemical mechanism and not site-specific (68).

Signaling Molecule

Synaptic modulation

The victims of H_2S poisoning suffer coordination and psychiatric disturbances (53). Based on these observations, the toxic effect of H_2S on the nervous system has been intensively studied. H_2S increases the levels of neurotransmitters γ-amino butyric acid (GABA), glutamate, serotonin, dopamine, epinephrine, and norepinephrine (33). The levels of serotonin and catecholamine are increased by inhibition of monoamine oxidase (MAO) (74). H_2S also enhances the effect of acetylcholine by suppressing the activity of its degradation enzyme, acetylcholinesterase. Excitatory postsynaptic potentials are suppressed by high concentrations of H_2S, indicating the suppression of synaptic transmission by H_2S (1). The effect

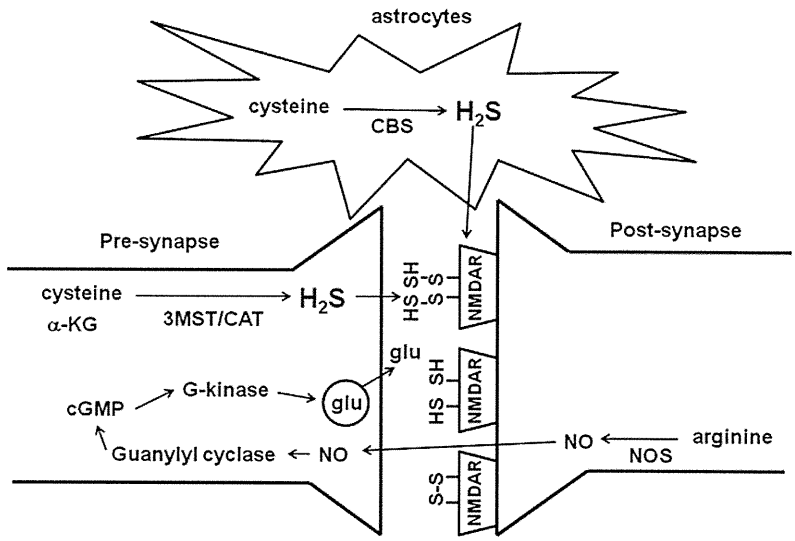

FIG. 7. The mechanisms for the induction of LTP. H_2S and nitric oxide (•NO) facilitate the induction of hippocampal long-term potentiation (LTP) with different mechanisms. •NO, which is produced from arginine, diffuses to presynapse to activate guanylyl cyclase, leading to the production of cyclic GMP that activates G-kinase to increase the release of a neurotransmitter glutamate. Although H_2S does not have any effect on guanylyl cyclase, it enhances the activity of NMDA receptors.

may explain the temporary memory loss occurred following H_2S exposure.

H_2S facilitates the induction of hippocampal long-term potentiation (LTP) similarly to nitric oxide (•NO), albeit by a different mechanism (1, 48) (Fig. 7). •NO, which is produced from arginine by •NO synthetase at postsynapses, diffuses to presynapses to activate guanylyl cyclase. This leads to production of cyclic GMP, which in turn activates G-kinase to increase the release of a neurotransmitter glutamate. Carbon monoxide (CO) facilitates the induction of LTP in a similar fashion to •NO, and both •NO and CO are called retrograde neurotransmitters (48). In contrast H_2S does not have any effect on guanylyl cyclase (1). H_2S enhances the activity of NMDA receptors, which is known to be required for the induction of LTP. Disulfide bonds play a role in modulating the function of NMDA receptors (2). DTT reduces disulfide bonds to generate thiols in NMDA receptors and facilitates the induction of LTP. H_2S further enhances the induction of LTP even after the facilitation induced by DTT (1). These observations suggest that H_2S may be involved at two steps in the modulation of NMDA receptor activity. First, as observed for DTT, H_2S reduces disulfide bonds to generate thiols and enhance the activity of NMDA receptors. Second, H_2S potentiates the activity even further by binding to thiols of receptors and generating persulfide, as suggested by Snyder and Toohey (19, 24, 68).

Another possible mechanism for the modulation of synaptic activity observed in the facilitation of LTP induction may involve astrocytes. Astrocytes were thought to be quiescent cells whose primary role was to support neurons. However, they were recently found to have neurotransmitter-responsive receptors themselves. Neuronal excitation activates astrocytes, and these cells in turn modulate synaptic activity (12). In neurons, H_2S enhances the responses of NMDA receptors to glutamate, while H_2S induces Ca^{2+} influx in astrocytes, which enhances the synaptic activity (1, 47). The effects of H_2S on both neurons and astrocytes may therefore explain its role in the induction of LTP. Astrocyte responses are suppressed by La^{3+}, Gd^{3+}, and ruthenium red, which are known as transient receptor potential (TRP) channel blockers, although the affected channel has not been identified.

Glial fibrillary acidic protein (GFAP) negative-premature astrocytes do not respond to H_2S (69). GFAP positive-mature astrocytes and those induced to mature by treatment with leukemia inhibitory factor (LIF) respond well to H_2S. In contrast, reactive astrocytes induced by EGF, TGF-α, cAMP, and interleukin-1β (IL-1β) do not respond to H_2S. Reactive astrocytes were induced by brain injuries, including trauma, neurodegenerative diseases, and viral infections. EGF is secreted by microglia and TGF-α is synthesized by injured neurons (28). EGF receptors are upregulated in reactive astrocytes after brain injury, and IL-1β is produced by reactive astrocytes. Astrocytes may exchange signals with neurons under normal conditions, but once reactive astrocytes are induced by brain injury, they may stop responding to H_2S to prevent transmitting inappropriate signals (Fig. 8).

Regulation of the activity of retinal neurons

The center-surround organization of the receptive field is one of the most important characteristics of retinal neurons. The negative feedback from horizontal cells to photoreceptor cells plays a key role in the center-surround organization. The negative feedback has been proposed to occur when Ca^{2+} channels of photoreceptor cells are suppressed by H^+ released by vacuolar-type H^+-ATPase present on horizontal cells. When retinal photoreceptor cells are exposed to light, cyclic GMP-gated channels are closed and the intracellular concentrations of Ca^{2+} are decreased. This results in a decrease in the release of the neurotransmitter glutamate to horizontal cells. Without the activation of glutamate receptors, the intracellular concentration of Ca^{2+} is maintained at a low level. Low Ca^{2+} concentrations in both photoreceptor and horizontal cells enhances the activity of 3MST/CAT to produce H_2S, which activates vacuolar-type H^+-ATPase on horizontal cells (43). H^+ suppresses Ca^{2+} channels on photoreceptor cell membrane. H_2S suppresses Ca^{2+} influx and regulates the negative feedback from horizontal cells to photoreceptor cells.

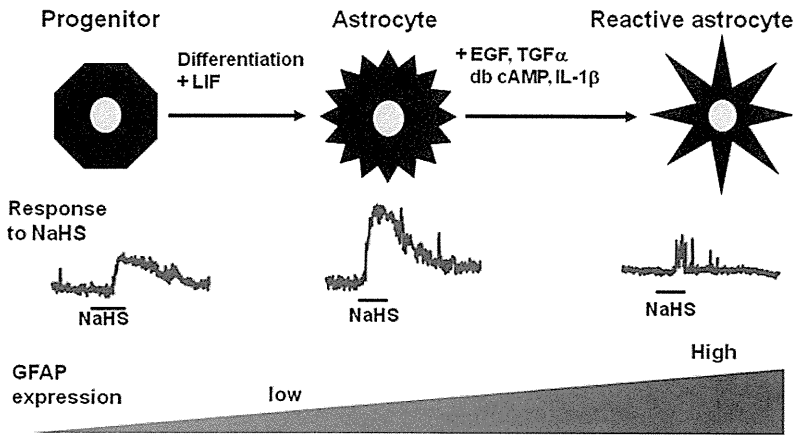

FIG. 8. The sensitivity of astrocytes to H_2S depends on the state of maturation and reactivity. GFAP positive-mature astrocytes and those maturation-induced by leukemia inhibitory factor (LIF) respond well to H_2S. In contrast, premature astrocytes as well as reactive astrocytes induced by EGF, TGF-α, cAMP, and interleukin-1β do not respond to H_2S.

Cytoprotection

Because H_2S is a well-known toxic gas, the concept that it may also have a cytoprotective effect is paradoxical. We and others found that H_2S protects neurons from oxidative stress by reinstating glutathione levels decreased by oxidative stress and by scavenging reactive oxygen species (30–32, 76, 77). Primary cultures of immature neurons, which express cystine/cysteine transporters but do not express ionotropic glutamate receptors, are widely used as a model for oxidative stress (61). The model enabled us to study oxidative stress specifically without the involvement of excitotoxicity. Similarly, even 1 m*M* NaHS is not toxic to a neuroblastoma cell line, SH-SY5Y, which does not express NMDA receptors (76, 77). In contrast, primary cortical neurons, which were cultured for several days and express ionotropic glutamate receptors, were killed by NaHS, and cell death was inhibited by glutamate receptor antagonists, MK801, APV, and CNQX (9). Together, these results suggest the neuroprotective effect of H_2S is specific to oxidative stress, but not to excitotoxicity.

Under physiological conditions, H_2S dissociates to H^+ and HS^- and at equilibrium approximately 20% remains as undissociated H_2S gas, which can evaporate from the medium. Approximately one-third of total H_2S, which includes undissociated H_2S gas and dissociated HS-, remains after 15 min of application, and more than 90% is lost from the medium of culture dishes by 30 min (30). After 1 h of application of NaHS, almost all of $H_2S + HS^-$ has evaporated out of culture dishes. Therefore, the effect of H_2S observed in culture dishes following a single application must be rapid and sustainable. It is possible that H_2S is bound to proteins as sulfane sulfur and in this manner can regulate their function (24, 68). Under oxidative stress caused by high concentrations of glutamate, neuronal cells started dying 8 h after exposure and only around 10% of cells survived after 16 h. Simultaneous application of glutamate and NaHS delayed the onset of cell death to 12 h and a rate of cell survival is much greater than that without NaHS. Because H_2S evaporates in a short time period, this means that the prolonged H_2S-dependent resistance to oxidative stress is induced by only a transient exposure.

H_2S can also protect cells against oxidative stress when given after the original insult. For example, the application of NaHS 4 h after induction of oxidative stress, still provides significant protection compared to control. This is true despite the initial activation of a cell death cascade. This property of H_2S is important for its therapeutic application (51).

Increase in the levels of glutathione

H_2S increases the transport of cystine and cysteine by enhancing the activity of X_{CT}- cystine/glutamate antiporters and $X_{A,G}$- excitatory amino acid transporters to increase the substrate concentrations for glutathione production. H_2S also enhances the activity of γ-glutamayl cysteine synthetase, a limiting enzyme for glutathione production. By these integrated effects H_2S increases the levels of glutathione in cells, and the effects are more profound when cells are under the conditions of oxidative stress (31, 32).

Various types of proapoptotic signals converge on mitochondria, and mitochondrial dysfunction causes numerous neurodegenerative diseases (37). 3MST and CAT are localized to mitochondria, which are the major organelles implicated in production of reactive oxygen species. H_2S produced in mitochondria may directly suppress oxidative stress in this organelle. For example, Neuro2a cells expressing 3MST and CAT show significant resistance to oxidative stress (31).

H_2S protects the embryonic brain from ischemia-reperfusion injury (31). Reperfusion after ischemia caused by stopping the blood flow from mother to fetus caused maceration of all the fetal brains, which contained only 24% glutathione of a control, but by the administration of sodium hydrosulfide (NaHS) to mother at the ischemia, 75% of fetal brains were not macerated and contained 90% of the normal glutathione level. The H_2S-producing enzymes that are also involved in the increase in the levels of glutathione have not been identified. The involvement of CSE in this process is controversial in two studies with CSE-knockout mice (25, 81).

Suppression of Ca^{2+} influx

The retina is susceptible to oxidative stress because of its high consumption of oxygen and daily exposure to light. Excessive light exposure leads to photoreceptor degeneration in the retina. Photoreceptor cell death is an irreversible injury that is caused by various factors such as reactive oxygen species (ROS) and elevated intracellular concentrations of Ca^{2+}. H_2S suppresses the elevation of Ca^{2+} in the photoreceptor cells by activating vacuolar-type H^+ ATPase, which

suppresses L-type Ca^{2+} channels, and maintains Ca^{2+} homeostasis (43). Intracellular concentrations of Ca^{2+} are increased during photoreceptor apoptosis, but the L-type Ca^{2+} channel blocker diltiazem prevents light-induced photoreceptor cell death. The lack of the vacuolar-type H^+ ATPase a3 subunit causes retinal degeneration. The failure of Ca^{2+} homeostasis due to excessive light may cause retinal cell degeneration. The regulation of Ca^{2+} and cytoprotective effect of endogenous H_2S may fail when photoreceptor cells are exposed to excessive light. Even under such conditions, the administration of H_2S protects cells from light-induced degeneration. Many cells in the outer nuclear layer, in which rod inner segments are located, became TUNEL-positive following exposure to light. The administration of 0.4375 μmol NaHS/kg weight of mice decreased the number of TUNEL-positive cells by approximately 80% relative to a control. Light exposure also produces a lot of cells containing 8-hydroxy-2-deoxyguanosine (8-OHdG), which is a product of DNA damaged by ROS, in the outer nuclear layer. In contrast, the number of cells positive to 8-OHdG is decreased in NaHS-treated mice. H_2S protects photoreceptor cells from light-induced retinal degeneration and oxidative stress.

Trophic effect

A treatment of primary cultures of neurons with NaHS increases the reduction of tetrazolium salts, which are reduced by cellular activity to form intensely colored formazans and have often been used to quantitate the number of living cells (71). A similar effect is observed in neuroblastoma Neuro2a cells, but it is not observed in other cell types such as primary cultures of astrocytes, COS-7 and NIH3T3 fibroblast cell lines. H_2S enhances metabolic activity specifically in neurons and the neuronal cell line. Other reducing agents, glutathione, DTT, α-tocopherol, and 2-mercaptoethanol do not enhance metabolic activity of neurons. The effect is increased in 60 min, and the maximal effect is maintained at least for 24 h after the application of NaHS. The increase of tetrazolium salt reduction in the absence of cells, even without washing out NaHS, was much less than that in the presence of cells with washing out of NaHS. This excludes the possibility that the reduction of tetrazolium salts is directly caused by reducing activity of H_2S, and suggests that H_2S stimulates a reductive intracellular environment.

It is well-known that H_2S inhibits cytochrome c oxidase and suppresses respiratory chain metabolic activity (71). The concentrations of H_2S, which reduce tetrazolium salts, do not inhibit cytochrome c oxidase. In contrast, azide, another inhibitor of cytochrome c oxidase, greatly inhibited cytochrome c oxidase but did not enhance the reduction of tetrazolium salts. NaHS increases the metabolic activity without affecting NADH/NAD+ ratio. The effects of NaHS are suppressed by the tyrosine kinase inhibitors, genistein and tyrphostin A23, suggesting that the effect may be mediated by phosphorylation of tyrosine and that H_2S may have a trophic effect in the brain. Proliferation is induced by H_2S in other cell types, such as in lung cells, endothelial cells, cardiac myocytes, and umbilical vein endothelial cells, while apoptosis is induced in smooth muscle cells, lung primary fibroblast cells, and periodontal ligament cells (3). The induction of proliferation or apoptosis by H_2S in these cells is regulated by transcription of genes of kinases and cell cycle proteins.

H_2S-Based Therapeutics

A tremendous amount of effort has been devoted into the development of H_2S based therapeutics. Na_2S is being tested in bypass surgery patients in Phase II clinical trial, and for kidney injury in Phase I clinical trial (51). Since Na_2S and NaHS release H_2S immediately after they are applied *in vivo*, compounds slowly release H_2S in a physiological manner have been developed. For example, GYY4137 inhibits LPS-induced lung and liver injury by decreasing neutrophil accumulation as well as the levels of the anti-inflammatory chemokine IL-10 (78). GYY4137 also protects human articular chondrocytes and trabecular bone-derived mesenchymal progenitor cells from oxidative stress.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used medications but have side effects, mainly gastric ulceration and bleeding. An H_2S-releasing derivative of naproxen (ATB-346) significantly accelerates the ulcer healing process, probably due to increasing the expression of cyclooxygenase (COX)-2 and promoting angiogenesis (5). ATB-346 does not cause gastric damage in rats even at exceedingly high doses. Another H_2S-releasing derivative of mesalamine (ATB-429) has greater anti-inflammatory activity in animal model of colitis compared to parental mesalamine. ACS14 and ACS21 are derivatives of aspirin and salicylic acid, respectively. Both compounds reduce hypertension and lower plasma levels of thromboxane B2 and insulin induced by glutathione depletion (54). S-diclofenac (ACS-15) efficiently suppresses LPS-induced inflammation with less gastric toxicity than parental diclofenac. This drug protects the heart from the development of ischemia-reperfusion injury and suppresses progression of vascular injury, leading to the prevention of arterial thrombosis.

Levodopa (L-DOPA) is the most widely used therapy for Parkinson's disease. The H_2S-releasing derivatives of L-DOPA (ACS5, ACS48, ACS50, and ACS81) were developed that showed significant anti-inflammatory, antioxidant, and neuroprotective properties (35). H_2S released from these compounds suppresses monoamine oxidase and maintains the effective concentrations of dopamine at the synaptic cleft. Released H_2S also suppresses the release of neurotoxins such as tumor necrosis factor-α, IL-6, and •NO from stimulated microglia. These compounds may be candidates for future treatment of Parkinson's disease.

Conclusion and Perspective

The basal concentrations of H_2S have been found to be much lower than those reported in earlier studies (18, 24, 79). However, the concentrations of H_2S achieved following cell stimulation are not well understood. To address this problem, it is necessary to clarify the regulation of the activity of H_2S-producing enzymes. For example, the activity of CBS is enhanced by S-adenosyl methionine and the 3MST/CAT pathway is regulated by Ca^{2+}. The physiological stimulations, which regulate the levels of S-adenosyl methionine and Ca^{2+}, may help determine the mechanism underlying the physiological regulation of CBS and 3MST/CAT.

In addition to the release of H_2S from the producing enzymes, bound sulfane sulfur, which can be an intracellular store of H_2S, releases H_2S under physiological conditions. The mechanism for the release of H_2S from bound sulfane sulfur may also help understand the endogenous local

concentrations of H_2S in the activated cells. The release of H_2S from activated astrocytes has not been successfully measured in the current method (24). Recently, fluorescent probes for imaging H_2S have been developed (38, 39, 50, 52, 55). The probes are able to selectively measure H_2S in aqueous environments at physiological pH. Although the sensitivity and intensity of the probes needs further improvement, their responses are selective for H_2S and can be applied to live cells for imaging. This represents a significant breakthrough for the detection of H_2S and is an improvement over the currently applied methods.

H_2S has the potential to be developed into a therapeutic agent (51). Na_2S is already in a Phase II clinical trial designed to determine whether it can protect cardiac muscle from ischemia-reperfusion injury following bypass surgery. NSAIDs attached to the H_2S-releasing group do not have side effects, such as bleeding or ulcer of gastrointestinal tract, that the parental NSAIDs cause (5). L-Dopa attached to an H_2S-releasing group has additional therapeutic effects that L-Dopa does not have (35). It suppresses the release of inflammatory cytokines produced by microglia, a key cause of neurodegeneration observed in Parkinson's disease. These are only a few examples of many H_2S-based therapeutic applications. Under physiological conditions, H_2S may be locally released to function in a restricted area. In contrast, the administration of H_2S systemically increases its concentrations. Monitoring for unexpected side effects will clearly be required. However, once these issues are overcome, the promise of H_2S-based therapeutic compounds will be realized.

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Author Disclosure Statement

There is no conflict of interest for any of the authors.

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Abbreviations Used

AD = Alzheimer type of dementia
ADP = adenosine diphosphate
ATP = adenosine triphosphate
cAMP = cyclic adenosine monophosphate
CAT = cysteine aminotransferase
CBS = cystathionine β-synthase
cGMP = cyclic guanosine monophosphate
CO = carbon monoxide
CRH = corticotropin-releasing hormone
CSE = cystathionine γ-lyase
DHLA = dihydrolipoic acid
DS = Down's syndrome
DTT = dithiothreitol
EDHF = endothelium-dependent hyperpolarizing factor
EDRF = endothelium-dependent relaxing factor
EGF = epidermal growth factor
GABA = γ-amino butyric acid
GAPDH = glyceraldehydes 3-phosphate dehydrogenase
GFAP = glial fibrillary acidic protein
HEK = human embryonic kidney
H_2S = hydrogen sulfide
IL = interleukin
K_{ATP} = ATP-dependent K^+
L-Dopa = levodopa
LIF = leukemia inhibitory factor
LTP = long-term potentiation
MAO = monoamine oxidase
3MP = 3-mercaptopyruvate
3MST = 3-mercaptopyruvate sulfurtransferase
NADH = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
NaHS = sodium hydrosulfide
NMDA = N-methyl D,L-aspartate
•NO = nitric oxide
Nrf2 = nuclear factor erythroid 2-related factor 2
NSAID = nonsteroidal anti-inflammatory drug
RNA = ribonucleic acid
TGF = transforming growth factor
TRP = transient receptor potential
TRX = thioredoxin