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Hydrogen Sulfide: From Brain to Gut

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Abstract

Three hundred years have passed since the first description of the toxicity of hydrogen sulfide (H_2S). Three papers in 1989 and 1990 described relatively high concentrations of sulfide in the brain. In 1996 we demonstrated that cystathionine β-synthase (CBS) is a H_2S producing enzyme in the brain and that H_2S enhances the activity of NMDA receptors and facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory. In the following year, we demonstrated that another H_2S producing enzyme, cystathionine γ-lyase is in the thoracic aorta, portal vein, and the ileum, and that H_2S relaxes these tissues. Based on these observations we proposed H_2S as a neuromodulator as well as a smooth muscle relaxant. We recently demonstrated that the third H_2S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT) produces H_2S in the brain as well as in vascular endothelium. Various functions in many tissues have been proposed. H_2S protects neurons and cardiac muscle from oxidative stress. H_2S has pro- and anti-inflammatory effects, nociceptive effects, the regulatory function of insulin release, and is even involved in longevity. Recent progress in the studies of physiological functions of H_2S in neurons and smooth muscle was described. *Antioxid. Redox Signal.* 12, 1111–1123.

Introduction

Nitric oxide (NO) is the first gaseous molecule that was discovered as a smooth muscle relaxant (24). Acetylcholine released by autonomic nerve in the walls of blood vessels activates its receptors on endothelial cells that release a diffusible substance to relax smooth muscle. It was designated as endothelium-derived relaxing factor (EDRF), later identified as NO, and activates soluble guanylyl cyclase to produce cyclic GMP (33, 57). After NO was proposed as EDRF, questions were raised. NO relaxes nonvascular smooth muscles, while EDRF released from bovine endothelial cells does not (64). The relaxing activity of EDRF but not that of NO is lost on passage through anionic exchange resins. These observations suggest that EDRF consists of more than two components. In addition to the difference in relaxing activity, the effect on membrane potential showed in a different manner between NO and EDRF. NO does not hyperpolarize membrane potential of smooth muscle as effectively as EDRF does. Based on these observations, it was proposed that EDRF contains another factor, designated as endothelium-derived hyperpolarizing factor (EDHF) (13). Although several candidates have been proposed, the identity of EDHF remains elusive.

EDRF or NO was also found to be released from neurons when NMDA receptors are stimulated (26). It was a well-known observation that cGMP is induced by an excitatory neurotransmitter glutamate in the brain, especially in the cerebellum. Garthwaite *et al*. found that EDRF is released from neurons in a Ca^{2+}-dependent manner and its activity accounts for the cGMP responses. This finding led to the identification of NO synthase (NOS), which produces NO from arginine as a substrate (9, 10). In the process of NOS purification with DEAE column, enzymatic activity was not recovered in one fraction, suggesting that NOS and its co-factor were separated in different fractions. Bredt and Snyder discovered that calmodulin is a cofactor for NOS (10). When neurons are excited, glutamate is released from presynapses and activates NMDA receptors on postsynapses. Ca^{2+}, which enters into postsynapses through NMDA receptors, activates NOS. NO produced by NOS passes through postsynaptic membrane and reaches presynapses and activates guanylyl cyclase to modify presynaptic activity. By this mechanism, NO induces hippocampal LTP and is called a retrograde transmitter (52).

Carbon monoxide (CO), which is a well-known toxic gas, competes with oxygen for binding to hemoglobin. The CO–hemoglobin complex, which is tight and slowly reversible, decreases the oxygen carrying capacity of blood and the availability of oxygen to tissues. CO is a second member of toxic gas recognized as a physiological mediator. CO is produced from biliverdin by heme oxygenase (HO) of which the activity is suppressed by tin protoporphyrin. Biliverdin is rapidly reduced to bilirubin by biliverdin reductase. Bilirubin

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was thought to be a toxic by-product; it was recently recognized as a physiological antioxidant that protects cells from oxidative stress caused by lipid peroxidation (49). CO relaxes thoracic aorta smooth muscle by decreasing calcium concentrations in smooth muscle (45). Snyder and his colleagues discovered that CO functions in the brain. HO-2 is highly expressed in the brain and co-localized with soluble guanylyl cyclase (71). CO has also been proposed as a retrograde neurotransmitter in hippocampal LTP (52). Since NO and CO are not stored in vesicles unlike other neurotransmitters, HO-2 has to rapidly produce CO in response to neuronal excitation. HO-2 was recently found to be activated by calcium-calmodulin as with NOS (8). Although it is a slower response than the activation by calcium-calmodulin, HO-2 is also activated by phosphorylation by casein kinase-2 (CK-2) (7). The calcium influx induced by neuronal excitation activates protein kinase C that phosphorylates and activates CK-2, resulting in the activation of HO-2. Since a substrate of HO-2 is heme, which is present in the form of heme proteins, it is still not clear whether heme is supplied enough to produce CO to elicit a physiological function.

Many studies about H_2S have been devoted to its toxicity since the first description in 1713. Memory loses are common in survivors of sulfide poisonings. Acute intoxication with H_2S inhibits monoamine oxidase and changes the levels of neurotransmitters in the brain (72). The toxic effects of H_2S on neurons were also studied by electrophysiological approaches in dorsal raphe serotonergic cells, and it was found that sulfide activates Ca^{2+} channels as well as Ca^{2+}-dependent K^+ channels and suppresses the delayed rectifier (40). In spite of many studies on toxic effects of H_2S, very little attention has been paid to understanding its physiological function. The sulfide levels in the brain have been measured in rats, humans, and bovine (27, 59, 72), suggesting that H_2S may have a physiological function. Although the sulfide levels measured by these studies were recognized as the amounts of H_2S released from acid-labile sulfur (34), it is certain that these studies triggered the exploration of the physiological function of H_2S. H_2S was initially demonstrated as a neuromodulator in the brain. CBS is expressed in the brain and produces H_2S. This production is enhanced by a CBS activator, S-adenosyl methionine, and is suppressed by CBS inhibitors. H_2S facilitates the induction of hippocampal LTP by enhancing the activity of NMDA receptors (1). The involvement of H_2S and CBS has also been shown in the regulation of a hormone release. The release of corticotropine-releasing hormone (CRH) is suppressed by H_2S, and S-adenosyl methionine, an activator of CBS, exhibits a similar effect on CRH release to H_2S (18). We also demonstrated that another H_2S-producing enzyme cystathionine β-synthase (CSE) is localized to the thoracic aorta, portal vein, and the ileum and that H_2S relaxes these tissues (30). H_2S was later demonstrated to activate ATP-dependent K^+ channels (79). These findings led to the recent discovery that H_2S mediates smooth muscle relaxation of human corpus cavernosum (16).

In addition to a function as a signal molecule, H_2S acts as a protectant of cells against oxidative stress. H_2S protects neurons from oxidative stress by increasing the levels of GSH through enhancing the activity of cystine transporters (38). Cardiac muscle is also protected by H_2S from ischemia-reperfusion injury by preserving mitochondrial function (21). H_2S inhibits the release of insulin (4, 35, 76) and is the endogenous modulator of inflammation (44,77). It exerts antinociceptive effects in the gastrointestinal tract, while it also has an effect as a nociceptive messenger in peripheral tissues (19,51).

Endogenous H_2S

Despite the various effects of H_2S in many tissues, the major cellular sources of H_2S and the mechanism of its release are not well understood. H_2S can immediately be released after its production by enzymes such as CBS, CSE, and 3MST (23, 41, 62, 65, 68). In addition, H_2S produced by enzymes can be stored which can then be released in response to physiologic stimuli. Two forms of sulfur stores that can release H_2S have been identified in cells (53, 70). Acid-labile sulfur, which is mainly localized to the iron–sulfur center of enzymes in mitochondria, releases H_2S under acidic conditions. Another form of storage is bound sulfane sulfur, which is localized to the cytoplasm and releases H_2S under reducing conditions (34).

H_2S dissociates to H^+ and HS^- in solution. In physiological saline at 37°C and pH 7.4, less than 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 (20). Although it has not been possible to determine which form of H_2S (H_2S, HS^-, or S^{2-}, the mix of free inorganic sulfides) is active, the term "hydrogen sulfide" has been used.

Free H_2S

The levels of free H_2S in the brain have been measured (25, 34). Leviten *et al*. demonstrated in 2008 that monobromobimane, which binds to thiols, was mixed with brain homogenates. Monobromobimane bound to thiols was extracted and applied to mass spectroscopy. The amounts of monobromobimane specifically bound to H_2S were measured. In this method, the level of free H_2S in the brain showed $\sim 8\,\mu M$.

In another method, brain homogenates were vigorously mixed with phosphate buffer and free H_2S evaporated into the air was measured. The levels of free H_2S were ~ 14 nM in the brain and 17 nM in the liver (25). The evaporation of H_2S was induced at pH 6.0 where more than 95% of H_2S exists in the form of gas. However, exogenously applied H_2S is absorbed and stored as bound sulfane sulfur which releases H_2S in reducing condition but not under acidic conditions (34). Because H_2S produced by enzymes can also be stored as bound sulfane sulfur, endogenous free H_2S may not escape from the storage and evaporate into the air.

In another method, brain homogenates were mixed with powdered silver, which absorbs H_2S as silver sulfide on the surface of silver particles. Because the adhered brain homogenates on the surface of silver particles would release H_2S under acidic conditions, silver particles were completely washed. The powdered silver was transferred into tubes filled with N_2 gas, and thiourea and H_2SO_4 were applied to release H_2S. The minimal detectable level of H_2S in this method is 25 nM/tube, corresponding to 9.2 μM in the brain. Since H_2S was not detected in this method, free H_2S, if any, is $<9.2\,\mu M$ in the brain (34). Like NO, free H_2S is maintained low in the basal condition. For example, a repetitive application of H_2S to astrocytes decreases the responses to H_2S. This suggests the desensitization of the sensitive molecule to H_2S (50). Low levels of H_2S in the basal condition may have an advantage to properly respond to H_2S.

H_2S-Producing Enzymes

Cystathionine β-synthase

H_2S can be produced from cysteine by pyridoxal-5′-phosphate (PLP)-dependent enzymes, including CBS and CSE (1, 30, 65, 79). CBS and CSE are expressed in many tissues, including the liver and the kidney. The brain expresses CBS and produces H_2S from cysteine (1) (Fig. 1). The production is suppressed by CBS inhibitors hydroxyl amine and aminooxyacetate but not by CSE inhibitors propagylglycine and β-cyanoalanine. A CBS activator, S-adenosyl methionine, enhances the production of H_2S. CBS also catalyzes the condensation reaction of homocysteine with cysteine to produce H_2S (14).

Western blot analysis and immunohistochemistry show that CBS is mainly localized to cerebellar Bergmann glia and astrocytes in the brain (22, 32). At early developmental stages, CBS was expressed in neuroepithelial cells in the ventricular zone, but its expression changed to radial glial cells and to astrocytes during the late embryonic and neonatal periods. CBS is upregulated by epidermal growth factor (EGF), transforming growth factor-α (TGF-α), cyclic adenosine monophosphate (cAMP), and dexamethasone in reactive astrocytes. Cultured astrocytes synthesized H_2S at the rate of 15.06 μM/g protein/h, which is 7.57-fold higher than microglial cells (43). Inflammatory activation of astrocytes and microglia decreases the expression of CBS, resulting in the decreased production of H_2S. Cerebellar morphological abnormalities were observed in CBS knockout mice (22). The localization of CBS to cerebellar Purkinje and hippocampal neurons were also reported (58), but the localization of CBS to neurons is controversial (22).

Down syndrome (DS) or trisomy 21 is the most common syndrome clinically associated with mental retardation. CBS is encoded on chromosome 21 (21q22.3), and a polymorphism of CBS allele is significantly underrepresented in children with high IQ, suggesting that CBS may influence cognitive function (6). The levels of CBS in DS brains are approximately three times greater than those in the normal individuals (32). CBS is localized to astrocytes and those surrounding senile plaques in the brains of DS patients with Alzheimer's disease.

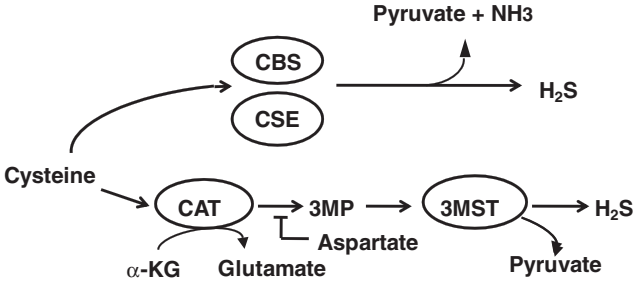

FIG. 1. Schematic representation of the metabolic pathways to produce H_2S from cysteine. Both CBS and CSE produce H_2S with pyruvate and ammonia from cysteine. 3MST produces H_2S and pyruvate from 3MP, which is co-produced with glutamate by CAT from cysteine and α-ketoglutarate. CAT is also known as aspartate aminotransferase, which uses aspartate as a substrate. Aspartate, which competes with cysteine on CAT, is used as an inhibitor for the production of H_2S.

The overexpression of CBS may cause the developmental abnormality in cognition in DS children and that may lead to Alzheimer's disease in DS adults (32).

CBS deficiency causes homocysteinemia. Patients of homocysteinemia have seizures, and homocysteinemia is a graded risk factor for chronic heart failure. Recent observations suggest that the upregulation of hypothalamic NMDA receptors may be involved in chronic heart failure. The antagonist to the NMDA receptors protects against homocysteine-induced oxidative damage in neurons as well as against the increase in heart rate induced by NMDA analog, suggesting that homocysteine is an agonist to NMDA receptors. Homocysteinemia has been proposed to activate mitochondrial matrix metalloproteinase, which induces structural remodeling, and causes myocyte mechanical dysfunction by agonizing NMDA receptors (47).

Cystathionine γ-lyase

In addition to localization in the liver and the kidney, CSE is expressed in the thoracic aorta, ileum, portal vein, and uterus, as well as pancreatic islets and the placenta (30, 35, 56). The ileum, uterus, placenta, and pancreatic islets also express CBS. The expression of CSE is upregulated by the NO donor, S-nitroso-N-acetylpenicillamine (SNAP), and CSE activity is enhanced by another NO donor, sodium nitroprusside (SNP) (79).

CSE catalyzes the reaction to produce H_2S from cysteine or homocysteine. Under normal conditions, ~70% of H_2S is produced from cysteine and the remaining 30% is from homocysteine metabolism. However, under conditions with higher concentrations of homocysteine such as homocysteinemia, homocysteine rather than cysteine becomes the preferred source for CSE-derived H_2S (15).

The activity of NOS and HO-2 is regulated by calcium/calmodulin (8, 10). In basal conditions, intracellular concentrations of Ca^{2+} are maintained at 0.1 μM, and they are increased up to 2–3 μM in the active states. 50% of maximal stimulation of NOS is ~200 nM Ca^{2+} with maximal enhancement of activity at 1 μM Ca^{2+} (10). Similarly, EC50 of HO-2 is 287 nM Ca^{2+} with maximal enhancement of activity at 2 μM Ca^{2+} (8). Although a requirement for Ca^{2+} concentrations is too high (1 mM), a recent study showed that CSE activity is also regulated by calcium-calmodulin (75). CSE directly binds to calmodulin, and the binding is abolished by EGTA and the calmodulin antagonist W7. H_2S formation by endothelial cells is augmented by a calcium ionophore A23187 and is blocked by a calcium chelator BAPTA (75).

Although in *in situ* hybridization CSE is mainly expressed in smooth muscle but not in endothelium of the thoracic aorta in rats (63, 79), recent studies with immunohistochemistry and Western blot analysis shows that CSE is predominantly localized to thoracic endothelium of mice, bovine, and humans with faint staining in smooth muscle (75). This observation suggests that H_2S is produced by CSE localized to vascular endothelium and may function as a component of EDRFs.

3-mercaptopyruvate sulfur transferase

We recently found that H_2S is produced from cysteine in brain homogenates of CBS knockout mice (62). This observation suggests that there is another H_2S producing enzyme

in the brain. The enzymatic activity requires two fractions, a fraction of synaptosome and mitochondria and a substance with molecular weight <3 kDa in a cytosol fraction. 3-Mercaptopyruvate sulfur transferase (3MST) along with cysteine aminotransferase (CAT), which is identical with aspartate aminotransferase (AAT), can produce H_2S from cysteine in the presence of α-ketoglutarate (41). Because the activity of 3MST along with CAT reaches its maximum at alkaline condition (pH 9.7), and because 3-mercaptopyruvate (3MP) is an unstable molecule and has not been identified in tissues, this pathway had not been thought to produce H_2S under the physiological conditions (65). 3MST and CAT are localized to mitochondria and a substance with molecular weight <3 kDa in cytosol fraction is α-ketoglutarate. 3MST with CAT efficiently produces H_2S from cysteine and α-ketoglutarate, and this production is suppressed by competition with aspartate, a preferred substrate for CAT or AAT, in a dose-dependent manner.

3MST produces H_2S more efficiently than does CBS, which was previously believed to be the sole H_2S-producing enzyme in the brain. The striking difference between 3MST and CBS is that 3MST produces bound sulfane sulfur more efficiently than CBS in the cells (62). This is also the first experimental evidence for the hypothesis that enzymes that produce H_2S can also generate bound sulfane sulfur in the cells. A difference between 3MST and CBS in the efficiency of producing bound sulfane sulfur may be the result of the following. Because 3MST has ~66% homology with rhodanese, which has sulfur-carrier activity from bound sulfane sulfur to acid-labile sulfur, it is possible that 3MST has the sulfur-carrier activity from H_2S to bound sulfane sulfur, whereas CBS has only weak sulfur-carrier activity.

The fact that H_2S is produced by 3MST along with CAT in the presence of cysteine and α-ketoglutarate supports the existence of 3MP, which has not been identified. The existence of 3MP has been suggested based on the observation that mercaptolactate-cystene disulfide, a metabolite of 3MP, was found in urine. The observation that the pathway produces H_2S even without the addition of 3MP indicates the existence of 3MP.

In contrast to CBS, which is mainly localized to astrocytes, 3MST is localized to neurons, including hippocampal pyramidal neurons, cerebellar Purkinje cells, and mitral cells in the olfactory bulb in the brain (62). H_2S facilitates the induction of hippocampal LTP, and induces Ca^{2+} waves in astrocytes (1, 50). H_2S may mediate the reciprocal interaction between neurons and astrocytes, resulting in the regulation of synaptic activity.

In the thoracic aorta, our study with immunohistochemistry and Western blot analysis shows that 3MST is localized to both endothelium and smooth muscle, and CAT is localized only to endothelium (63). 3MST along with CAT in rat vascular endothelial cells produces H_2S from cysteine and α-ketoglutarate. Based on these observations, H_2S produced by 3MST along with CAT in vascular endothelium may function as a component of EDRFs.

3MST can also catalyze the reaction to produce thiosulfate and pyruvate from 3MP and SO_3^{2-}. Thiosulfate reacts with glutathione to produce H_2S, H_2SO_3, and an oxidized form of glutathione. This reaction can be catalyzed by rhodanese. The application of Na-thiosulfate to the mouse model of heart failure, which was created by an arteriovenous fistula, prevented the decline in cardiac function. Thiosulfate may modulate cardiac dysfunction by increasing ventricular H_2S generation (61).

Forms of Sulfur That Can Release H_2S

Despite the various effects of H_2S in many tissues, the major cellular sources of H_2S and the mechanism of its release are not well understood. At least two possibilities exist. One possibility is that H_2S is immediately released after its production by enzymes. Another possibility is that H_2S produced by enzymes is stored and is released in response to a physiologic signal. Two forms of sulfur stores in cells have been identified (53, 70) (Fig. 2). Acidic conditions release H_2S from acid-labile sulfur. Acid-labile sulfur in the brain of rats, humans, and bovine has been measured as brain sulfide (27, 59, 72). Another form of storage is called bound sulfane sulfur, which releases H_2S under the reducing conditions (54).

Acid-labile sulfur

Acid-labile sulfur is mainly sulfur atoms in iron–sulfur complexes, which play a critical role in a wide range of redox reactions in enzymes of the respiratory chain in mitochondria. If acid-labile sulfur is a physiological source of H_2S, it should release it at physiological pH. The critical pH at which H_2S is released from acid-labile sulfur is 5.4 (34). Because mitochondrial pH is between 7 and 8 and usually does not become acidic, it may be difficult for H_2S to be released from acid-labile sulfur under physiologic conditions. Iron–sulfur complexes are unstable and readily release H_2S, when they are detached from enzymes by detergents and protein denaturants.

One gram of H_2S is dissolved in 242 ml of H_2O and dissociates to H^+ and HS^-. PK_1 is 6.76 at 37°C, ~85% of H_2S exists as H_2S gas and can evaporate. In order to measure free H_2S as gas, dissolved HS^- and S^{2-} are changed into H_2S gas by shifting the equilibrium into acidic conditions. A method in

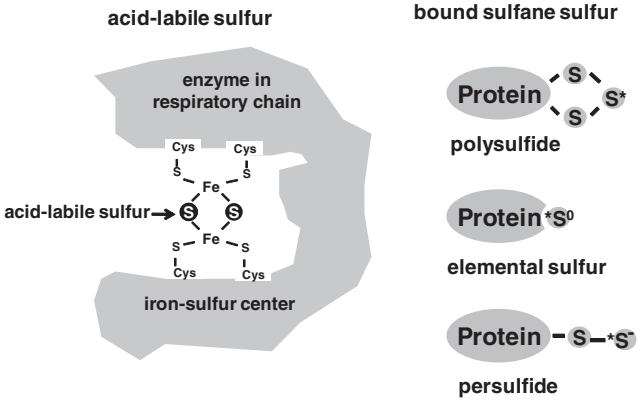

FIG. 2. Two forms of sulfur that can release H_2S. Acid-labile sulfur is a component of the iron–sulfur center, which is localized to the active site of enzymes in the respiratory chain and has a critical role for redox reactions. Acid-labile sulfur is unstable under acidic conditions and readily releases H_2S. Bound sulfane sulfur, which consists of polysulfide, elemental sulfur, and persulfide, releases H_2S under reducing conditions.

which H_2S reacts with N,N-dimethyl-p-phenylenediamine sulfate with $FeCl_3$ in high concentrations of HCl and produced methylene blue is widely used (65). In this method, H_2S released from reaction mixture containing tissue homogenates were trapped with center well filled with zinc acetate solution and the reaction is stopped by trichloroacetic acid. Because the amount of H_2S produced by enzymatic reaction is abundant, this method can be applied. In contrast, this method is not appropriate to measure endogenous levels of free H_2S that are much lower than that released from acid-labile sulfur.

Bound sulfane sulfur

The sulfur of H_2S can be incorporated into proteins as bound sulfane sulfur that is divalent sulfur bound only to other sulfur, such as outer sulfur atoms of the persulfides and inner chain atoms of polysulfides. Bound sulfane sulfur is rapidly labeled when [^{35}S] cysteine is injected into an animal (17), suggesting that cysteine is metabolized to produce sulfide that is incorporated into a pool of bound sulfane sulfur. This pool of sulfur releases H_2S under reducing conditions (53, 54). The pool of bound sulfane sulfur is distinct from acid-labile sulfur (34). When HCl is added to brain homogenates, and all free H_2S removed, the addition of DTT to the resultant supernatant releases almost the same amount of H_2S as without HCl pretreatment. Conversely, DTT released almost the same amount of H_2S after pretreatment with HCl.

H_2S may be released immediately after its production by enzymes, as occurs with the release of NO from NO synthase (9). Alternatively, H_2S can be transiently stored and then released when the cells are stimulated. For example, exogenously applied free H_2S is immediately absorbed in a sulfur store as bound sulfane sulfur (34). DTT released more H_2S from preabsorbed homogenates than from homogenates without preabsorption. In contrast, acids or detergents released H_2S from endogenous acid-labile sulfur but it does not release preabsorbed H_2S, suggesting that H_2S is not absorbed into an acid-labile sulfur pool. H_2S is absorbed in brain homogenates more slowly than in liver and heart homogenates, and the release is also slower from brain homogenates than from those of the liver and the heart. Therefore, once H_2S is released from bound sulfane sulfur or from H_2S-producing enzymes, free H_2S may remain longer in the brain than in the liver and the heart.

Cells expressing 3MST and CAT increase the levels of bound sulfane sulfur twice as much as control cells (62). In contrast, in cells expressing a defective mutant 3MST, in which an active center cysteine 247 is replaced to serine and does not produce H_2S, the levels of bound sulfane sulfur remain at the control levels. These observations suggest that the intracellular levels of bound sulfane sulfur are dependent on the H_2S producing activity of 3MST and that H_2S produced by 3MST is stored as bound sulfane sulfur in the cells. A synaptosome and mitochondrial fraction contains $\sim$60% of bound sulfane sulfur and the remaining is in cytosol and microsome fractions.

Is H_2S released from bound sulfane sulfur under physiologic conditions? In the presence of major cellular reducing substances, glutathione and cysteine at their physiologic concentrations, H_2S is released from lysates of cultured neurons and astrocytes at pH 8.4 (34). Because the reducing activity of thiols is greater in alkaline conditions than at a neutral pH, H_2S release is observed at pH higher than 8.4. The changes in pH described here are not the systemic changes, which are approximately up to 0.2 in either alkalosis or acidosis, but the local changes in the restricted area. When neurons are excited, sodium ions enter and potassium ions exit from cells, resulting in high potassium concentrations in the extracellular environment which depolarizes the membrane of surrounding astrocytes. To recover from the depolarized state to the quiescent condition, Na^+/HCO_3^- cotransporters are activated in astrocytes. Entrance of 1 Na^+ and 2 or 3 HCO_3^- is electrogenic, and HCO_3^- causes the alkalinization of the cells (11). In primary cultures, $\sim$60% of the astrocytes responded to high concentrations of K^+ causing intracellular alkalinization, while the remaining astrocytes were quiescent (34) (Fig. 3). Although H_2S has not been detected, 10% of the astrocytes responded well and shifted their intracellular pH to 8.4 that is able to release H_2S. We explored 31 physiologic stimuli that may shift the intracellular pH to alkaline including neurotransmitters, growth factors, amino acids, and second messengers, but only KCl was effective to alkalinize the intracellular pH (34).

Nervous System

Neuromodulator

Since H_2S is produced from cysteine, H_2S was predicted to have a physiological function in the brain (1). Studies of H_2S toxicity showed that H_2S suppressed synaptic potentials and modified K^+ channels (40). Our observations show that excitatory post synaptic potentials (EPSPs) were suppressed at high toxic concentrations of H_2S but not affected at low concentrations. H_2S induces hippocampal LTP when it is applied associatively with a weak tetanic stimulation, which alone does not induce LTP (1). Occlusion experiments show that the LTP induced in the presence of H_2S shares common mechanisms with LTP induced by a regular stimulation. H_2S activates LTP at active, but not quiescent, synapses, and may be involved in associative learning as defined by Hebb. Although H_2S, like other gaseous messengers, NO and CO, facilitates the induction of LTP, the mechanism of action of H_2S is different from those of NO and CO. It is well known that the activation of NMDA receptors is required for the induction of LTP. NO and CO, retrograde neurotransmitters, do not require NMDA receptor activation (80), while H_2S does not facilitate the LTP induction under the blockade of NMDA receptors. H_2S enhances the currents mediated by NMDA receptors. Another critical difference is that NO and CO activate soluble guanylyl cyclase and increase intracellular cGMP (71), while H_2S does not (1).

Disulfide bonds play a role in modulating the function of many proteins, including NMDA receptors (2). It is possible that H_2S reduces disulfide bonds or makes bound sulfane sulfur with free thiols in NMDA receptors. A high concentration of dithiothreitol (DTT) facilitates the induction of LTP, while even a low concentration of H_2S further facilitates the induction of the greater amplitudes of LTP. DTT does not occlude the effect of H_2S. Only reducing the redox sites may not contribute the potentiating effect of H_2S on the induction of LTP, but rather the formation of bound sulfane sulfur at thiols of NMDA receptors or the activation of other signal cascade may be an alternative mechanism.

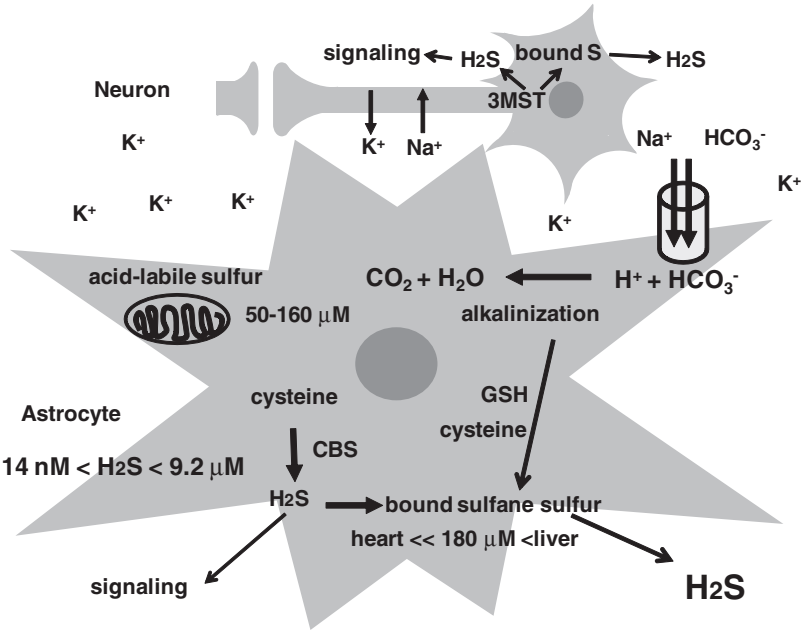

FIG. 3. Sources of H_2S. H_2S is produced by enzymes and functions as a signal molecule. Some H_2S may be stored as bound sulfane sulfur that, in turn, may release H_2S when cells are stimulated. For example, when neurons are excited, Na+ enters and K^+ is released. The extracellular concentrations of K^+ reach 10–12 m*M*, which depolarize the membrane of surrounding astrocytes. When the membrane is repolarized, $NaHCO_3$ transporters are activated and the intracellular concentrations of HCO_3^- are increased that induces the alkalinization. GSH and cysteine reduce bound sulfane sulfur under alkaline conditions and release H_2S. Acid-labile sulfur is contained in enzymes localized to mitochondria. Because the pH of mitochondria is not acidic, acid-labile sulfur may not release H_2S under the physiological conditions. The levels of free H_2S are maintained in low levels in the basal conditions.

Cells with no electrical changes were thought to be glia. Recent studies have shown that glia have neurotransmitter receptors and respond to transmitters. Accumulating evidence shows the reciprocal interactions between neurons and glia. Neural activity evokes glial calcium waves, and glial calcium waves drive neuronal activity. Glia communicate with each other by increasing intracellular concentrations of Ca^{2+} and propagate the signal as Ca^{2+} waves. The Ca^{2+} waves often appear to be initiated at sites of contact with neurons, suggesting that glial Ca^{2+} waves are initiated by neuronal excitation (12).

Astrocytes, a type of glia, respond directly to a neurotransmitter released from neurons. In neurons, H_2S enhances the activity of NMDA receptors activated by a neurotransmitter, glutamate (1), while H_2S increases intracellular concentrations of Ca^{2+} by mainly increasing Ca^{2+} influx in astrocytes (50). Although the sensitive molecule or receptors for H_2S in astrocytes has not been identified, considering the fact that responses to H_2S were suppressed by La^{3+}, Gd^{3+}, and ruthenium red, which are known as blockers of voltage-dependent Ca^{2+} channels or TRP channels, these channels may be activated by H_2S (50). TRP channels have been demonstrated to be involved in the responses to H_2S. Contraction of the detrusor muscle is regulated by capsaicin-sensitive primary afferent neurons in the urinary bladder. Contraction induced by H_2S is suppressed by unselective TRP channel blocker, ruthenium red, but not by specific TRPV1 channel blocker, capsazepine and SB366791, suggesting some type of TRP channels other than TRPV1 channels may be involved in the responses to H_2S (55). H_2S evokes an increase in neuropeptide release in the airways that is significantly attenuated by capsazepine. Contraction induced by H_2S in the airways is also reduced by ruthenium red, capsazepine, and SB366791, suggesting the involvement of TRPV1 channels in contraction induced by H_2S. CHO cells expressing TRPA1 channels respond to NaHS (66). These observations suggest that TRP channels are H_2S -sensitive molecules or receptors.

Neuronal excitation induces Ca^{2+} waves in astrocytes, which are propagated to neighboring astrocytes and reach nearby synapse and may modulate its activity (Fig. 4). The neuronal transmission is fast, while Ca^{2+} waves propagate slowly. The involvement of astrocytes may enable time-delayed signal transmission. For example, an electrical stimulation of mossy fiber induces two distinct types of signal propagation from CA3 to CA1 (60). A fast signal transmits from CA3 directly to CA1, while a delayed signal propagates from CA3 to CA1 mediated through CA2. Ca^{2+} waves in astrocytes may be involved in this type of time-delayed transmission.

Neuroprotectant

There are two forms of glutamate toxicity; ionotropic receptor-initiated excitotoxicity and nonreceptor-mediated oxidative glutamate toxicity (48) (Fig. 5). Oxidative glutamate toxicity is a well-studied programmed cell death pathway that is independent of ionotropic glutamate receptors (48). It has been observed in primary cultures of neuronal cells (38), neuronal cell lines (36, 48), and brain slices. Oxidative glutamate toxicity is initiated by high concentrations of glutamate in cultures of neurons that have not yet expressed ionotropic glutamate receptors and is not suppressed by the antagonists of ionotropic glutamate receptors. Glutamate shares the same amino acid transporter with cystine, and it competes with cystine for transport into cells (5). Therefore, elevated extracellular glutamate inhibits the transport of cystine that is the primary source of intracellular cysteine necessary for glutathione synthesis. Glutathione, which is a tripeptide consisting of cysteine, glutamate, and glycine, is a major antioxidant in the cellular defense against oxidative stress. Since the intracellular concentrations of cysteine are lower than those of glutamate and glycine, the availability of cysteine limits the *de novo* synthesis of glutathione.

H_2S increases the levels of glutathione, which is a cellular major and potent antioxidant (1–8 m*M*) (38). H_2S enhances the activity of cystine/glutamate antiporter to increase the levels of a substrate, cysteine, for glutathione synthesis (38) (Fig. 6). In addition to the cystine transport, cysteine is also efficiently transported into cells by H_2S. It has been thought that cysteine

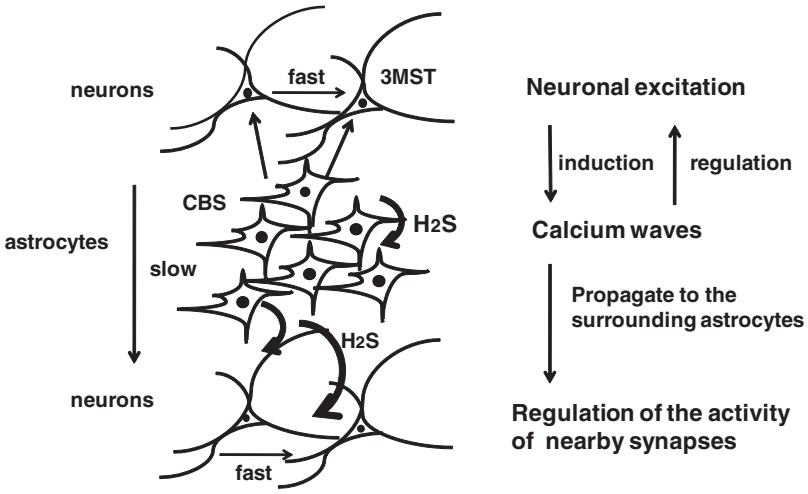

FIG. 4. The interaction between neurons and astrocytes. When neurons are excited, the neurotransmitters are released from the presynapse and reach the postsynapse. The transmitters also stimulate the surrounding astrocytes and induce calcium waves, which in turn modulate the synaptic activities. The calcium waves also propagate to the neighboring astrocytes and reach the nearby synapse to modulate it. The synaptic transmission is fast, while the propagation of calcium waves is slow. The involvement of calcium waves may enable the time delayed modification of synaptic transmission.

normally exists as its oxidized form, cystine, in extracellular space. However, significant amounts of cysteine ($\sim 20\,\mu M$) were measured in plasma and blood. Since H_2S readily passes through the plasma membrane, it is possible that H_2S produced in the cells may be released into extracellular space and reduces cystine into cysteine. Because transporters of cysteine are widely distributed in various types of cells, once extracellular cystine is reduced into cysteine in the presence of H_2S, cysteine can readily be imported into cells and used for glutathione production (37).

Glutathione is produced through sequential metabolism by two enzymes γ-glutamyl cysteine synthetase (γ-GCS) and glutathione synthetase (GS). H_2S enhances the activity of γ-GCS and increases the levels of γ-glutamyl cysteine, resulting in the increase of glutathione levels, especially the reduced form of glutathione (38). The enhancement of the activity of γ-GCS by H_2S is not caused by transcriptional regulation, or by the direct contact of H_2S with γ-GCS. Although the mechanism has not been well understood, the activity of γ-GCS is enhanced only when cells are exposed to H_2S. This observation suggests that H_2S may activate some sensitive molecule or receptor to induce an intracellular signal that may enhance the activity of the enzyme. In contrast to γ-GCS, the activity of GS is not affected by H_2S.

Cellular GSH is mainly localized to the cytoplasm, and most of the remaining GSH is stored in mitochondria. H_2S increases the total cellular concentrations of GSH and also the pool in mitochondria. In addition to the protection from oxidative glutamate toxicity, H_2S protects cells from broader oxidative stress, including that caused by H_2O_2 (37). Another example of protection from cell injury is that H_2S suppresses cell death induced by rotenone, a commonly used toxin to establish Parkinson's disease models. H_2S stabilizes mitochondrial membrane potential and regulates JUK-MAPK pathway (31).

The *in vitro* finding that H_2S protects brain by reinstating GSH levels decreased by oxidative stress is supported by the following observation *in vivo* (37). All fetal brains 24 h after reperfusion from ischemia, which was caused by complete arrest of maternal–fetal blood supply, were macerated, and the GSH levels in the fetal brains were severely decreased. In contrast, brains of only one-fourth of fetuses were macerated by a treatment of NaHS prior to the ischemia-reperfusion. Although the GSH levels of macerated brains, even with a NaHS application before ischemia-reperfusion, were decreased, those of nonmacerated brains were maintained $\sim 90\%$ of a control. H_2S protects fetal brains by reinstating the GSH levels decreased by intra-utero ischemia-reperfusion.

H_2S has another mechanism to protect cells from oxidative stress. Sulfur-containing substances, dimethylsulphoniopropionate (DMSP) and its enzymatic cleavage product dimethylsulphide (DMS), have recently been identified as

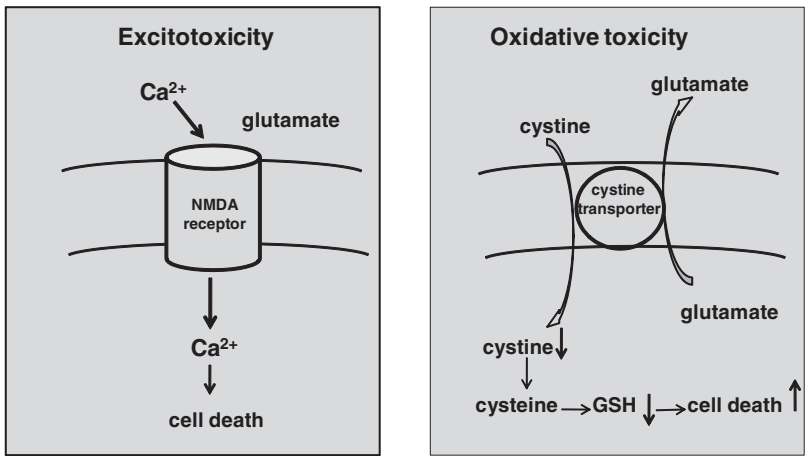

FIG. 5. Two forms of glutamate toxicity. In receptor-initiated excitotoxicity, an excess amount of Ca^{2+} enters through ionotropic glutamate receptors into cells and causes cell death. In oxidative glutamate toxicity, high concentrations of glutamate suppress the cystine/glutamate antiporter and decrease the import of cystine, resulting in the decreased levels of glutathione (GSH), a cellular major antioxidant.

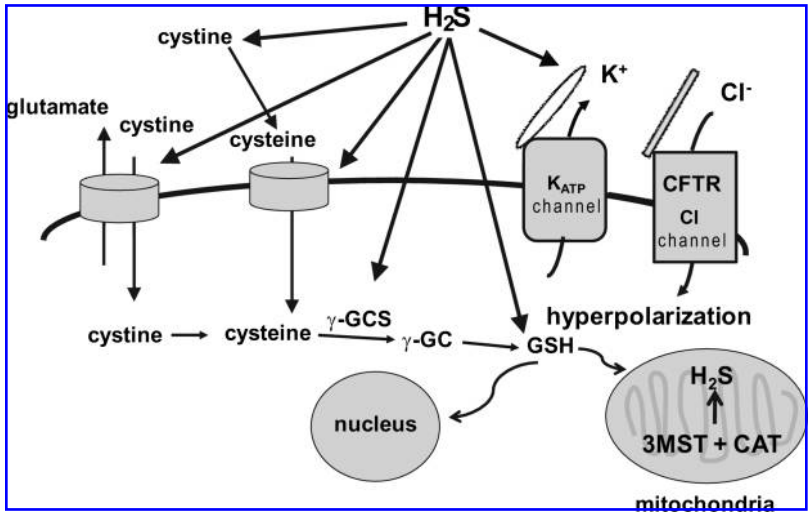

FIG. 6. H_2S protects cells from the oxidative glutamate toxicity. H_2S enhances the activity of cysteine and cystine transporters to increase the levels of substrate for GSH production. It also enhances the activity of γ-GCS to increase the levels of GSH. 3MST and CAT are mainly localized to mitochondria, which is a major organelle to produce reactive oxygen species. H_2S produced by 3MST along with CAT may directly suppress oxidative stress in mitochondria. H_2S enhances the activity of K_{ATP} and CFTR Cl^- channels and stabilizes the membrane potential.

endogenous scavengers for hydroxyl radicals and other reactive oxygen species in marine algae (67). H_2S is a reducing agent that readily reacts with reactive oxygen species and hydrogen peroxide. H_2S-producing enzymes 3MST and CAT are mainly localized to mitochondria. Mitochondria, which are a major source of oxidative stress, play a key role in cell death pathways. Various types of proapoptotic signals start the cell death cascade, and they may all converge on the mitochondria. Perturbation of the mitochondrial function causes loss of the mitochondrial transmembrane potential and the release of apoptogenic factors such as cytochrome C into the cytosol, and ultimately results in cell death. Mitochondrial dysfunction caused by oxidative stress leads to numerous neurodegenerative diseases. Neuronal cells expressing 3MST and CAT show significant resistance against oxidative glutamate toxicity (37), suggesting that H_2S produced by 3MST along with CAT can scavenge oxygen species in mitochondria and protect cells from oxidative stress.

Cells can be rescued from oxidative stress by mechanisms that are either dependent upon or independent of glutathione metabolism. Vitamin E protects cells from oxidative glutamate toxicity by acting directly as antioxidants even when the intracellular glutathione levels are decreased (48). In contrast, dihydroxyphenylglycine, an agonist of group 1 metabotropic glutamate receptors, protects neurons by upregulating glutathione (38). As described above, H_2S is a unique substance that protects cells from oxidative stress by both mechanisms (Fig. 6).

K_{ATP} channels are involved in protection against ischemia and excitotoxicity (28). The K_{ATP} channel openers reduce abnormal excitatory synaptic activity and are protective against neuronal death. These protective effects are suppressed by K_{ATP} channel blockers. K_{ATP} channel blockers, glibenclamide and glipizide, suppress the protection by H_2S from oxidative stress (36). A K_{ATP} channel opener, pinacidil, suppresses glutamate toxicity, and the protection is increased by the simultaneous application of H_2S. A blocker selective to mitochondrial K_{ATP} channels, 5-hydroxydecanate, does not modulate protection by H_2S (36). K_{ATP} channels localized to plasma membrane but not those to mitochondria may mediate H_2S-induced neuroprotection.

CFTR Cl^- channels and sulfonylurea receptors, a component of K_{ATP} channels, belong to the ATP-binding cassette superfamily, and both groups share significant sequence homologies (29). The Cl^- channel blockers NPPB and IAA-94 suppress protection by H_2S, and levamisole, an opener of Cl^- channels, efficiently suppresses glutamate toxicity (36). These observations suggest that CFTR Cl^- channels are also involved in protection by H_2S against oxidative stress. The recent findings that a decrease in transmembrane Cl^- gradients causes cell death in hippocampal pyramidal neurons and that the expression of CFTR gene is reduced in the hypothalamus of patients with Alzheimer's disease (42) suggest that homeostasis of transmembrane Cl^- gradients is required for normal cell survival. In primary cultures of neurons, H_2S protects cells from oxidative glutamate toxicity mainly by increasing glutathione levels in the cells (38), while in a cloned hippocampal neuronal cell line, HT22 cells, H_2S protects cells mainly by enhancing the activity of K_{ATP} and CFTR Cl^- channels (36). K_{ATP} channels are highly expressed in neurons of the hippocampus, and expression of CFTR is greater in the hippocampus than in the cortex. Because the primary cultures are a mixture of cells, H_2S probably increases glutathione levels to protect all neurons but in addition may activate K_{ATP} and CFTR Cl^- channels in a subset group of neurons (Fig. 6).

H_2S also protects astrocytes from oxidative stress caused by hydrogen peroxide. H_2S increases production of glutathione and ATP but decreases the production of reactive oxygen species in astrocytes (46). H_2S inhibits peroxynitrite-induced cytotoxicity, intracellular protein nitration, and oxidation (73).

In addition to the protecting activity in the brain, H_2S protects cardiac muscle from oxidative stress. Application of H_2S at the time of reperfusion limits infarct size and preserves left ventricular function (21). Cardiac-specific transgenic mice with overexpression of CSE increased myocardial levels of H_2S and have a reduction in infarct size after ischemia reperfusion injury, suggesting that H_2S protects against myocardial infarction. H_2S has a cytoprotective effect and either administration of H_2S or the modulation of endogenous production may be of clinical benefit in ischemic disorders.

Smooth Muscle

Interaction of H_2S with NO

The production of H_2S by the thoracic aorta, portal vein, and ileum was suppressed by CSE inhibitors, and the

production by the ileum was partially suppressed by CBS inhibitors, reflecting its expression of CBS. Although the relaxation effect of H_2S alone is weak, there is a synergy between NO and H_2S on smooth muscle relaxation (30). A similar synergistic effect between H_2S and NO was observed in the inhibition of the twitch responses of the ileum to electrical stimulation (69). In addition to the synergistic effect of H_2S with NO, CSE activity is also enhanced by NO. Aortic tissue homogenates exposed to a NO donor, sodium nitroprusside (SNP), increased the production of H_2S, and another NO donor, S-nitroso-N-acetylpenicillamine (SNAP), upregulated the expression of CSE (79). CSE contains 12 cysteine residues that are potential targets for S-nitrosation that may enhance the activity of CSE (39). H_2S-induced vasorelaxation was partially attenuated either by removal of the endothelium, by blockade of NO synthase or by the Ca^{2+}-dependent K^+ channel blockers, suggesting that H_2S might stimulate endothelial cells to release certain factors that facilitate the relaxation of smooth muscle (78). This observation also supports the previous finding that H_2S enhances relaxation activity of NO. In contrast to these observations, H_2S has also been reported to suppress the activity of NO to relax smooth muscle. For example, H_2S may induce vasoconstriction by scavenging endothelial NO and increase the mean arterial pressure in rats (3). Pretreatment of aortic tissues with H_2S inhibits the vasorelaxant effect of SNP (78).

H_2S-mediated vasorelaxation is dependent on the concentrations of O_2. Low concentrations of H_2S are sufficient to relax vessels under physiological O_2 conditions (39). H_2S causes contraction of aortic smooth muscle at high O_2 levels, while H_2S induces rapid relaxation at lower physiological O_2 levels. In peripheral vessels with a smaller diameter and wall thickness and greater proportion of smooth muscle cells than collagen compared with the aorta, the O_2 consumption rate is higher, yet the levels of O_2 are lower. This observation suggests that peripheral arteries normally operate at lower O_2 concentrations than the aorta and that H_2S efficiently relaxes arteries under low O_2 conditions. S-nitrosothiols in vascular tissue may also contribute to vessel tone, when the bound NO is effectively reduced and liberated (39). H_2S catalyzes the reactivation of NO-inhibited GAPDH in cultured cells and the stoichiometric release of NO from S-nitrosoglutathione in an O_2-dependent manner (39). This observation further supports the interaction between H_2S and NO.

H_2S relaxes vascular smooth muscle mostly by opening ATP-dependent K^+ channels in a non-ATP-associated manner (79). This is based on the following observations (78, 79): a) H_2S-induced vasorelaxation was suppressed by high concentrations of tetraethyl ammonium (TEA) that blocks many K^+ channels in vascular smooth muscle cells, including K_{Ca}, voltage-dependent K^+ and K_{ATP} channels. The other inhibitors for K_{Ca} and voltage-dependent K^+ channels did not affect the vascular effects of H_2S; b) Pinacidil, a K_{ATP} channel opener, relaxed aortic tissues; c) Glibenclamide, a K_{ATP} channel blocker, suppressed the H_2S-induced vasorelaxation; d) Both pinacidil and H_2S effectively increased the whole cell K_{ATP} channel currents in single smooth muscle cells. These effects of pinacidil and H_2S were suppressed by glibenclamide; e) H_2S induced a significant membrane hyperpolarization of smooth muscle cells; and f) The hypotensive effect of H_2S *in vivo* is suppressed by glibenclamide.

H_2S and thoracic endothelium

The vasorelaxation effect of H_2S was not affected or slightly enhanced in the presence of endothelium (30, 79). The enhancement is thought to be caused by interaction of exogenously applied H_2S with endothelium-derived relaxing factors, including EDRF and EDHF, of which the release may be induced by H_2S. This possibility is based on the observation that the application of L-NAME and the co-application of charybdotoxin and apamin, which inhibit the effect of EDHF, reduced the H_2S-induced vasorelaxation (79). Because H_2S still significantly relaxed vascular tissues after endothelium removal, the contribution of endothelium to the relaxation effect of exogenously applied H_2S must be low (30, 78).

The possibility that EDRF is not identical to NO has been based on the following observations. NO relaxed nonvascular smooth muscles, but EDRF released from cultured endothelial cells did not (64). The activity of EDRF is lost on passage over anionic exchange resins, but that of NO is not. In addition, endothelial-dependent relaxation of vascular smooth muscle reflected the release of an additional factor to EDRF, which could cause relaxation by hyperpolarizing the membrane of the smooth muscle, designated as EDHF (13). Since CSE is expressed in smooth muscle of the rat thoracic aorta, H_2S has been thought to be a smooth muscle-derived relaxing factor (30, 79). Although species were different, it was recently reported that CSE was found in endothelial cells of mice, bovine, and humans (75). We recently demonstrated that 3MST along with CAT produces H_2S in the brain (62). This finding led us to investigate whether 3MST is expressed in the thoracic aorta. Western blot analysis and immunohistochemistry show that 3MST is localized to both endothelium and smooth muscle (63) (Fig. 7). Although CAT mRNA was reported to be expressed in both endothelial cells and smooth muscle, our immunohistochemistry showed that both cCAT and mCAT were localized only to endothelial cells (63). Lysates of endothelial cells produce H_2S from 3MP as a substrate and also

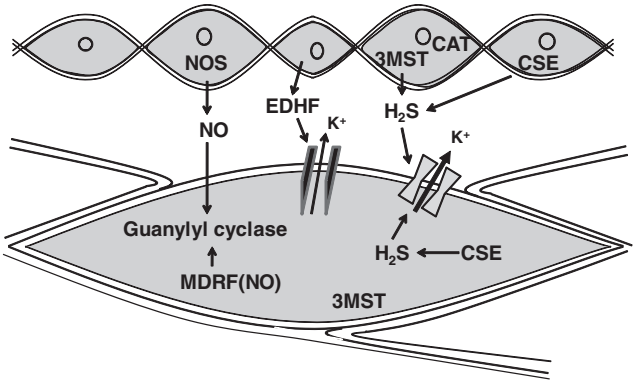

FIG. 7. H_2S relaxes vascular smooth muscle. 3MST and CAT are localized to vascular endothelium and produce H_2S from cysteine in the presence of α-ketoglutarate. 3MST is also localized to smooth muscle, but CATs are not. 3MST in smooth muscle may not be involved in the production of H_2S. CSE mainly produces H_2S in smooth muscle. NO produced by NOS, which is localized to endothelium, activates soluble guanylyl cyclase in smooth muscle. Smooth muscle-derived relaxing factor (MDRF) shows characteristics quite similar to NO.

from cysteine in the presence of α-ketoglutarate. In the absence of α-ketoglutarate, no H_2S was produced from cysteine, suggesting that H_2S production in endothelium is highly dependent on the activity of CATs. This conclusion is also supported by the observation that the production of H_2S from cysteine and α-ketoglutarate was suppressed by aspartate, which competes with cysteine on CAT, in a dose-dependent manner. Although 3MST is also localized to smooth muscle, CATs are not. Therefore, 3MST in smooth muscle may not be involved in the production of H_2S. CSE may mainly produce H_2S in smooth muscle. These observations suggest that H_2S is produced by 3MST along with CAT in vascular endothelium and that H_2S may be a component of EDRFs (63). In mice, bovine, and humans, H_2S is produced by CSE localized to endothelium and its function as being one of EDRFs has been proposed (75). The production of H_2S in both endothelium and smooth muscle is similar to that of NO. The vascular smooth muscle generates a labile relaxing factor that possesses pharmacological and chemical properties that are similar to those of NO (74). It is also similar to the nervous system. CBS is localized mainly to astrocytes and 3MST is to neurons. Neurons and astrocytes reciprocally regulate and H_2S may mediate the regulation.

Corpus cavernosum smooth muscle

Smooth muscle of another tissue recently demonstrated to be regulated by H_2S is corpus cavernosum smooth muscle (16). Penile corpus cavernosum is a highly vascularized tissue and its function depends on an equilibrium between vasodilatory and vasoconstrictory tone. Erectile dysfunction is thought to be predominantly a vascular disease, and it is considered an early sign of cardiovascular disease. Both CBS and CSE are expressed in human penile tissue and homogenates of the tissue produce H_2S. CBS and CSE are localized to the muscular trabeculae and the smooth muscle of the penile artery, and CBS is also localized to peripheral nerves. Exogenously applied H_2S and cysteine causes a concentration-dependent relaxation of strips of human corpus cavernosum. Cysteine-induced relaxation is suppressed by a CBS inhibitor, aminooxyacetate. Electrical field stimulation of the tissue, under resting conditions, causes an increase in tension that is significantly potentiated by either propagylglycine or aminooxyacetate. In rats, H_2S and cysteine promote penile erection, and the response to cysteine is blocked by propargylglycine. A functional cysteine/H_2S pathway may be involved in mediating penile erection in humans and other mammals.

Conclusions and Perspectives

Since the demonstration of endogenous levels of sulfide in the brain, the characteristics of this molecule and its physiological functions have been unveiled. Although the levels of free H_2S were thought to be relatively high, it was recently clarified that free H_2S is maintained in low levels in the basal conditions like NO. The mechanisms for the regulation of H_2S-producing enzymes are to be clarified. CSE was demonstrated to be regulated by Ca^{2+}/calmodulin like NOS and HO-2, but the mechanism for the regulation of CBS and 3MST along with CAT has not been well understood. A possible mechanism has been demonstrated as to the storage and release of H_2S. H_2S may function as a signal molecule immediately after produced by its producing enzymes. Some of it may be stored as bound sulfane sulfur, which in turn releases H_2S when cells receive certain physiological signals. Although the activation of the $NaHCO_3$ transporter in response to neuronal excitation has been demonstrated to be the mechanism for H_2S release from bound sulfane sulfur in astrocytes, the release of H_2S has not successfully measured.

For the studies of the physiological function of H_2S, it is important to accurately measure the levels of H_2S. There are several methods to measure H_2S including the methylene blue method and the silver-sulfide electrode. These methods are initially developed to measure H_2S levels in water, and they are not appropriate to apply to tissue samples and blood without any modification. It is therefore necessary to develop reliable methods with a high sensitivity that can correctly measure the levels of endogenous H_2S without being disturbed by other molecules, including proteins. We are especially interested in a H_2S-specific dye, which enables the visualization of H_2S and detect the production, movement, and the cessation of H_2S action in real time *in situ*.

Until the demonstration of 3MST, CBS and CSE have been the major enzymes. In the vascular system, CSE was thought to be expressed only in smooth muscle, but it was recently found even in vascular endothelium of mice, bovine, and humans, and H_2S was proposed to be a component of EDRFs. Our recent findings show that 3MST along with CAT is localized to vascular endothelium and that endothelial cells produce H_2S from cysteine. The production in endothelial cells is highly dependent on α-ketoglutarate, and in the absence of α-ketoglutarate, the production was below detectable levels. Although the contribution of H_2S production between CSE and 3MST has to be determined, it is certain that their product H_2S is produced in vascular endothelium and is added to a candidate for EDRFs.

Although NMDA receptors, K_{ATP}, CFTR Cl^-, voltage-dependent Ca^{2+}, and TRP channels have been proposed as target molecules of H_2S, the mechanisms of stimulation have not been clarified. Sulfhydration has recently been proposed to mediate the diverse physiologic actions of H_2S and a major post-translational modification (49). By solving these problems and clarifying the mechanisms, H_2S can be established as a physiological mediator, and be applied for therapeutic use.

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Abbreviations Used

3MP = 3-mercaptopyruvate
3MST = 3-mercaptopyruvate sulfur transferase
AAT = aspartate aminotransferase
AMP = adenosine monophosphate
CAT = cysteine aminotransferase
CBS = cystathionine β-synthase
CFTR = cystic fibrosis transmembrane conductance regulator
CK = casein kinase
CSE = cystathionine γ-lyase
CO = carbon monoxide
CRH = corticotropine-releasing hormone
DMS = dimethylsulfide
DMSP = dimethylsulfoniopropionate
DS = Down syndrome
DTT = dithiothreitol
EDHF = endothelium-derived hyperpolarizing factor
EDRF = endothelium-derived relaxing factor
EGF = epidermal growth factor
EPSP = excitatory post synaptic potentials
γ-GCS = γ-glutamyl cysteine synthase
GMP = guanosine monophosphate
GS = glutathione synthase
HO = heme oxygenase
H_2S = hydrogen sulfide
LTP = long-term potentiation
NMDA = N-methyl D-aspartate
NO = nitric oxide
NOS = NO synthase
PLP = pyridoxalo-5′-phosphate
SNAP = S-nitroso-N-acetylpenicillamine
SNP = sodium nitroprusside
TGF-α = transforming growth factor-α
TRP = transient receptor potential

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