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HIGH IMPACT REVIEW IN NEUROSCIENCE, NEUROLOGY OR PSYCHIATRY - REVIEW ARTICLE

Hydrogen polysulfide (H_2S_n) signaling along with hydrogen sulfide (H_2S) and nitric oxide (NO)

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Abstract Hydrogen sulfide (H_2S) is a physiological mediator with various roles, including neuro-modulation, vascular tone regulation, and cytoprotection against ischemia–reperfusion injury, angiogenesis, and oxygen sensing. Hydrogen polysulfide (H_2S_n), which possesses a higher number of sulfur atoms than H_2S, recently emerged as a potential signaling molecule that regulates the activity of ion channels, a tumor suppressor, transcription factors, and protein kinases. Some of the previously reported effects of H_2S are now attributed to the more potent H_2S_n. H_2S_n is produced by 3-mercaptopyruvate sulfurtransferase (3MST) from 3-mercaptopyruvate (3MP) and is generated by the chemical interaction of H_2S with nitric oxide (NO). H_2S_n sulfhydrates (sulfurates) cysteine residues of target proteins and modifies their activity, whereas H_2S sulfurates oxidized cysteine residues as well as reduces cysteine disulfide bonds. This review focuses on the recent progress made in studies concerning the production and physiological roles of H_2S_n and H_2S.

Keywords H_2S_n · H_2S · NO · Signaling · 3MST · Rhodanese

Abbreviations

AAT	Aspartate aminotransferase
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CO	Carbon monoxide
CSE	Cystathionine γ-lyase
DAO	D-Amino acid oxidase
DTT	Dithiothreitol
eNOS	Endothelial nitric oxide synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSSH	Glutathione persulfide
GSSSG	Glutathione trisulfide
HNO	Nitroxyl
H_2S	Hydrogen sulfide
H_2S_n	Hydrogen polysulfide
HSSNO	Nitrosopersulfide
Keap1	Kelch ECH associating protein 1
LTP	Long-term potentiation
3MP	3-Mercaptopyruvate
3MST	3-Mercaptopyruvate sulfurtransferase
NF-κB	Nuclear factor κB
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PTEN	Phosphatase and tensin homolog
SNAP	*S*-nitroso-*N*-acetyl-D,L-penicillamine
–SNO	*S*-nitrosothiol
SNP	Sodium nitroprusside
SOD1	Superoxide dismutase 1
–SOH	Sulfenic acid
SQR	Sulfur quinone oxidoreductase
TNFα	Tumor necrosis factor α
TRPA1	Transient receptor potential ankyrin 1

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Introduction

Hydrogen sulfide (H_2S) facilitates the induction of hippocampal long-term potentiation (LTP) by enhancing the activity of *N*-methyl D-aspartate (NMDA) receptors (Abe

and Kimura 1996). It relaxes vascular smooth muscle in synergy with nitric oxide (NO) (Hosoki et al. 1997; Zhao et al. 2001). H_2S protects various tissues/organs, including neurons, the retina, heart, and the kidney from oxidative stress or ischemia–reperfusion injury (Kimura and Kimura 2004; Elrod et al. 2007; Tripatara et al. 2008; Mikami et al. 2011b). This molecule also exerts anti-inflammatory effects, induces angiogenesis, and functions as an oxygen sensor (Zanardo et al. 2006; Olson et al. 2006; Cai et al. 2007; Papapetropoulos et al. 2009; Peng et al. 2010).

There are four known pathways through which H_2S is produced: three from L-cysteine and one from D-cysteine, respectively. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), both of which are cytosolic enzymes, metabolize L-cysteine along with L-homocysteine to produce H_2S (Cavallini et al. 1962; Braunstein et al. 1971; Stipanuk and Beck 1982; Abe and Kimura 1996; Hosoki et al. 1997, Zhao et al. 2001; Chiku et al. 2009; Singh et al. 2009). Cysteine aminotransferase (CAT), which is identical to aspartate aminotransferase (AAT), metabolizes L-cysteine along with α-ketoglutarate to 3-mercaptopyruvate (3MP), which is further metabolized by 3-mercaptopyruvate sulfurtransferase (3MST) to H_2S in the presence of thioredoxin (Meister et al. 1954; Stipanuk and Beck 1982; Cooper 1983; Kuo et al. 1983; Nagahara et al. 1995; Shibuya et al. 2009a, b; Mikami et al. 2011a; Yadav et al. 2013). An achiral 3MP is also produced from D-cysteine by D-amino acid oxidase (DAO), which constitutes a pathway with 3MST to produce H_2S from D-cysteine (Fig. 1) (Cooper 1983; Shibuya et al. 2013).

Hydrogen polysulfides (H_2S_n) are recently emerged potential signaling molecules, which activate transient receptor potential ankyrin 1 (TRPA1) channels (Nagai et al. 2006; Oosumi et al. 2010; Nagy and Winterbourn 2010; Kimura et al. 2013; Hatakeyama et al. 2015), regulate the activity of a tumor suppressor, namely, phosphatase and tensin homolog (PTEN) (Greiner et al. 2013), facilitate the translocation of Nrf2 to the nucleus to upregulate the transcription of anti-oxidant genes (Koike et al. 2013), and relax vascular smooth muscle by activating protein kinase G1α (Stubbert et al. 2014). These targets of H_2S_n have generally two sensitive cysteine residues at their active sites, either of which can be sulfurated by H_2S_n and subsequently generate a cysteine disulfide bond by reacting with each other (Fig. 2).

Proteins, such as superoxide dismutase I (SODI) and human growth hormone, have one or more polysulfide bridges between two cysteine residues (Nielsen et al. 2011). Protein-bound form of persulfide and polysulfides was reported to be produced from 3MP, a substrate of 3MST (Hylin and Wood 1959), and cells overexpressing 3MST contain greater amounts of bound sulfane sulfur than that in control cells (Fig. 3) (Shibuya et al. 2009b). These observations suggest that 3MST can produce bound-form persulfide and polysulfides from 3MP. However, the existence of a diffusible form of polysulfide such as H_2S_n in cells is not well understood. We recently found that H_2S_3, H_2S_2, and H_2S_5 as well as H_2S are produced by 3MST from 3MP in the brain (Kimura et al. 2013, 2015).

A crosstalk between H_2S and NO plays an important role in cellular signaling. A synergistic effect between H_2S and NO on vascular smooth muscle relaxation was initially reported (Hosoki et al. 1997). It was subsequently demonstrated that H_2S stimulates endothelial NO synthetase (eNOS) to facilitate the production of NO that protects the heart and liver against ischemia–reperfusion injury (King et al. 2014). Recently, Eberhardt et al. (2014) reported that the chemical interaction of H_2S with NO produces nitrosopersulfide (HSSNO), polysulfides, and dinitrososulfite, whereas another study showed that this interaction results in the production of nitroxyl (HNO) and polysulfides (Cortese-Krott et al. 2015). Although $SSNO^-$ and HNO are unique products in each study, polysulfides are common to both. It is possible that the polysulfides produced by the interaction of H_2S and NO are a result of a synergistic effect of both molecules on vascular smooth muscle relaxation (Hosoki et al. 1997). A recent report that demonstrates a more efficient relaxation of vasculature and reduction of blood pressure by H_2S_n than by H_2S supports the above possibility (Stubbert et al. 2014).

Sulfhydration (sulfuration) was proposed as a mode of H_2S action, wherein the active thiol (-SH) of cysteine residues in the target proteins receives an additional sulfur atom causing conformational changes in the proteins (Mustafa et al. 2009). However, this mechanism is theoretically flawed, because the oxidation state of sulfur in thiol and that in H_2S is the same, i.e., −2. Atoms of the same oxidation state do not react with each other; therefore, H_2S cannot sulfurate cysteine residues. In contrast, the oxidation state of the inner sulfur of H_2S_n is 0, and it readily reacts with thiol (Kimura 2015). Under oxidative conditions, a thiol is oxidized to cysteine sulfenic acid (–SOH), and upon NO signaling, it is oxidized to cysteine S-nitrosothiol (–SNO). These oxidized thiols are sulfurated by H_2S rather than by H_2S_n (Kabil and Banerjee 2014; Kimura 2015). The balance between H_2S and H_2S_n as well as the normal or oxidative state of cysteine residues plays an important role in regulating cellular activity (Fig. 2).

This review will focus on the physiological roles of H_2S and those of H_2S_n produced by 3MST as well as by the chemical interaction of H_2S with NO.

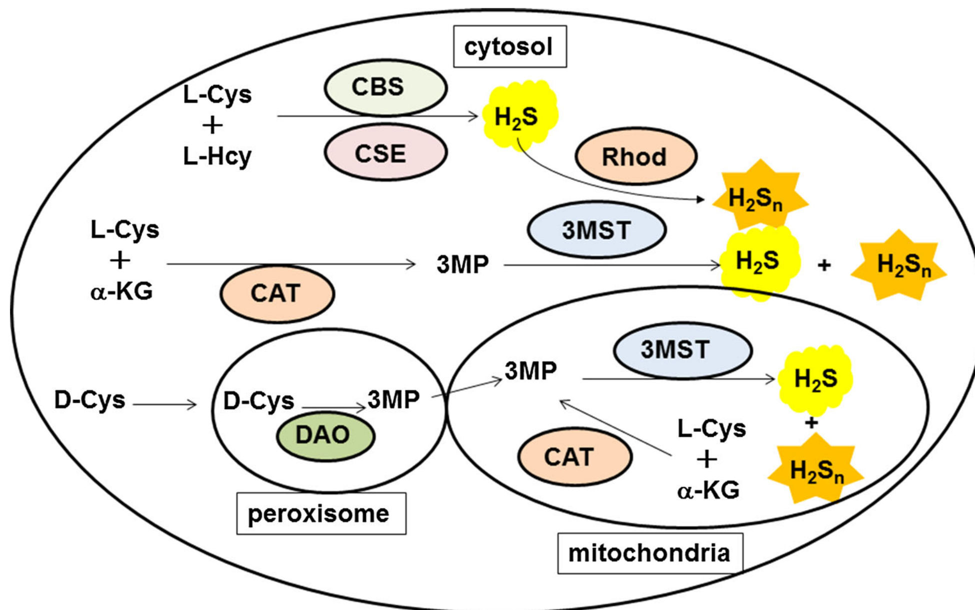

Fig. 1 H_2S- and H_2S_n-producing pathways. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), which localize in the cytosol, metabolize L-cysteine (L-Cys) along with L-homocysteine (L-Hcy) to H_2S. Cysteine aminotransferase (CAT) metabolizes L-cysteine along with α-ketoglutarate (α-KG) to 3-mercaptopyruvate (3MP), which is further metabolized to H_2S and H_2S_n by 3-mercaptopyruvate sulfurtransferase (3MST). Both CAT and 3MST localize to mitochondria as well as in cytosol. D-amino acid oxidase (DAO) metabolizes D-cysteine to 3MP, which is a substrate of 3MST to produce H_2S. H_2S_n is also produced from H_2S that is accelerated by 3MST and rhodanese

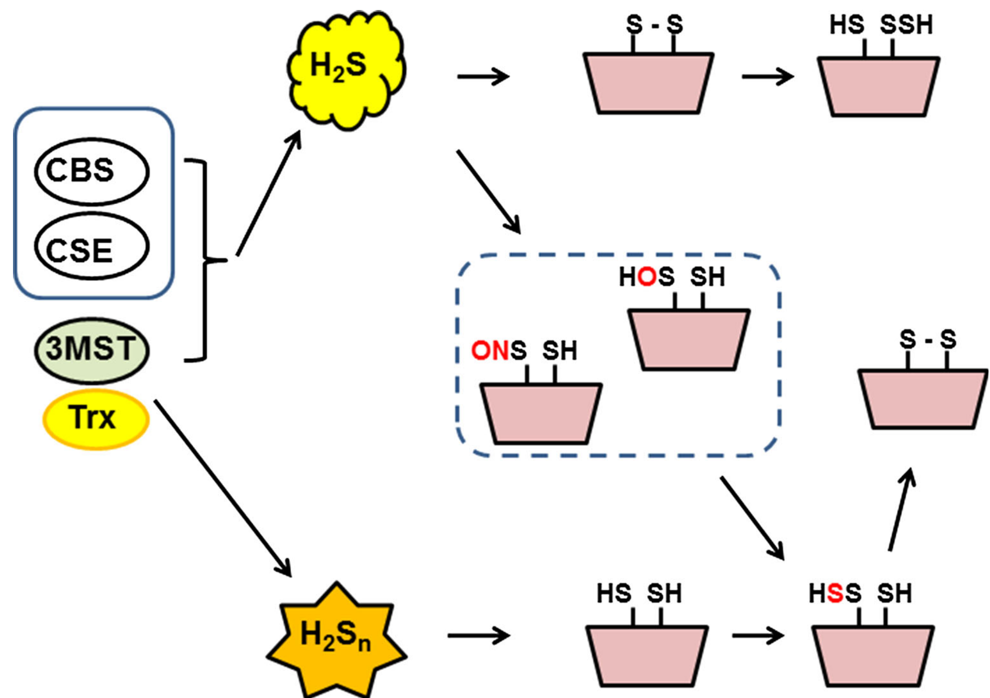

Fig. 2 Sulfhydration (sulfuration) induced by H_2S and H_2S_n. H_2S reduces cysteine disulfide bonds of target proteins to cause conformational changes resulting in the modification of the activity of target proteins. H_2S sulfurates oxidized cysteine residues such as cysteine sulfenic acid (–SOH) and cysteine *S*-nitrosothiol (–SNO) upon signaling by NO. In contrast, H_2S_n sulfurates the cysteine residues of target proteins resulting in the formation of a cysteine disulfide bond

Reduction of cysteine disulfide bond by H_2S

Compared to other gaseous signaling molecules such as NO (5.6 mg/100 ml at 20 °C) and carbon monoxide (CO) (2.8 mg/100 ml), H_2S is readily soluble in water (413 mg/100 ml) and dissociates into H^+ and HS^-. Under physiological conditions, approximately 80 % exists as HS^- and the remaining 20 % as H_2S gas with trace amounts of S^{2-}. H_2S is also easily soluble in lipids and freely passes through the plasma membrane, whereas HS^- travels through AE-1 channels in exchange with Cl^- in mammals, and through HS^- channels in bacteria (Fig. 4)

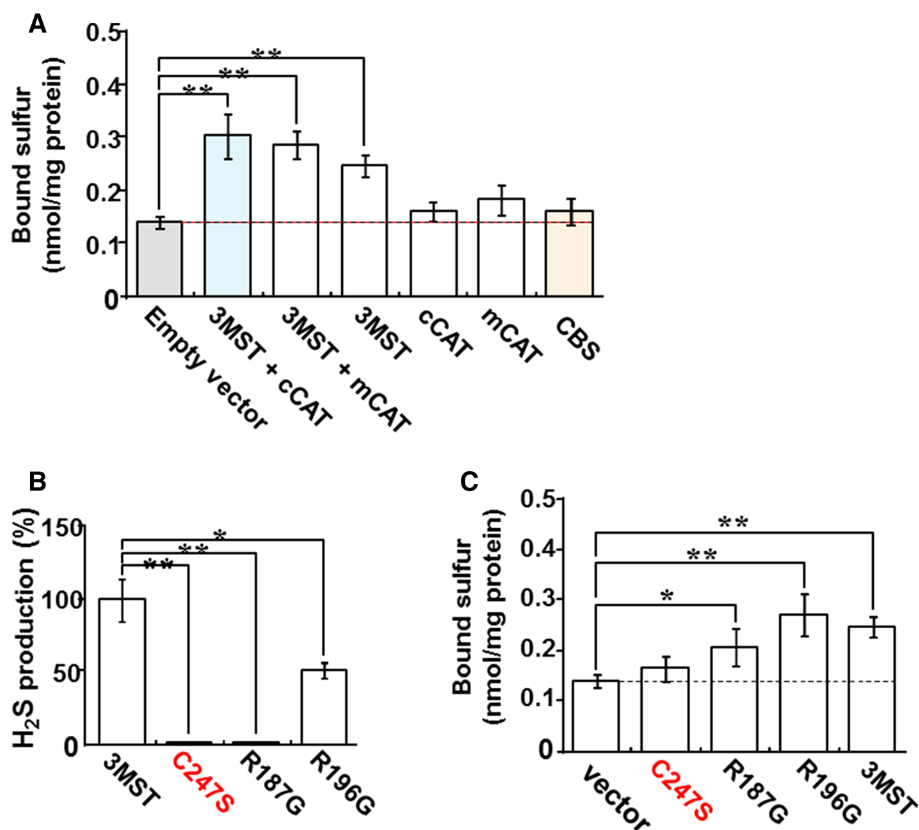

Fig. 3 Production of bound sulfane sulfur by 3MST. **a** 3MST increases the levels of bound sulfane sulfur. 3MST produces bound sulfane sulfur, whereas CBS does not. **b** A 3MST defective mutant in which the catalytic site cysteine 247 is replaced by serine loses the ability to produce H_2S and H_2S_n. **c** Cells expressing a 3MST defective mutant contain bound sulfane sulfur levels that are not significantly different from those of control cells. Reproduced from Shibuya et al. 2009b

(Mathai et al. 2009; Czyzewski and Wang 2012; Jennings 2013). In this review, the term 'H_2S' includes H_2S, HS^-, and S^{2-}.

Aizenman et al. (1989) discovered that the reduction of cysteine disulfide bond at the hinge of the ligand binding domain of *N*-methyl D-aspartate (NMDA) receptors by a reducing substance, dithiothreitol (DTT), enhances the activity of the receptors. The sodium salt of H_2S or NaHS, a reducing substance, also enhances the activity of NMDA receptors (Fig. 5) (Abe and Kimura 1996). Based on basal H_2S concentrations in cells where glutathione and cysteine are major reducing substances, the reducing potential and the intracellular concentrations of H_2S are claimed to be too low to reduce cysteine disulfide bonds (Kabil and Banerjee 2010; Toohey 2011). However, the ligand-binding site of NMDA receptors is located on the extracellular side of the plasma membrane. Moreover, the concentrations of H_2S were measured under basal conditions of bulk tissues. Vasas et al. (2015) reported that the reduction of disulfide bond by H_2S is kinetically and thermodynamically a highly system-specific process, and this process is plausible in an oxidizing environment such as endoplasmic reticulum and the extracellular environment. An example of disulfide bond reduction by H_2S is observed in the first step of H_2S metabolism in mitochondria where H_2S reduces a disulfide bond of sulfide quinone oxidoreductase (Hildebrandt and Grieshaber 2008).

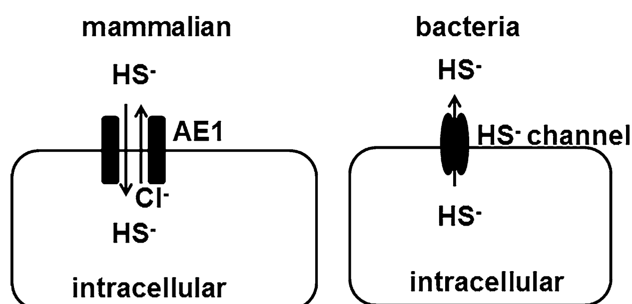

Fig. 4 HS^- channels in bacteria and mammals. H_2S is readily soluble in water and dissociates into H^+ and HS^-; approximately 80 % exists as HS^-, whereas the remaining 20 % exists as H_2S gas with trace amounts of S^{2-} under physiological conditions. H_2S is also soluble in lipids and passes through plasma membrane. HS^- passes though AE1 channels in exchange with Cl^- in mammals and HS^- channels in bacteria

Sulfhydration (sulfuration) by H_2S and H_2S_n

Sulfhydration (sulfuration) has been proposed as a mode of action of H_2S wherein sulfur is added to the thiol of cysteine residues of target proteins causing structural changes and modifying their activity (Mustafa et al. 2009). It is also

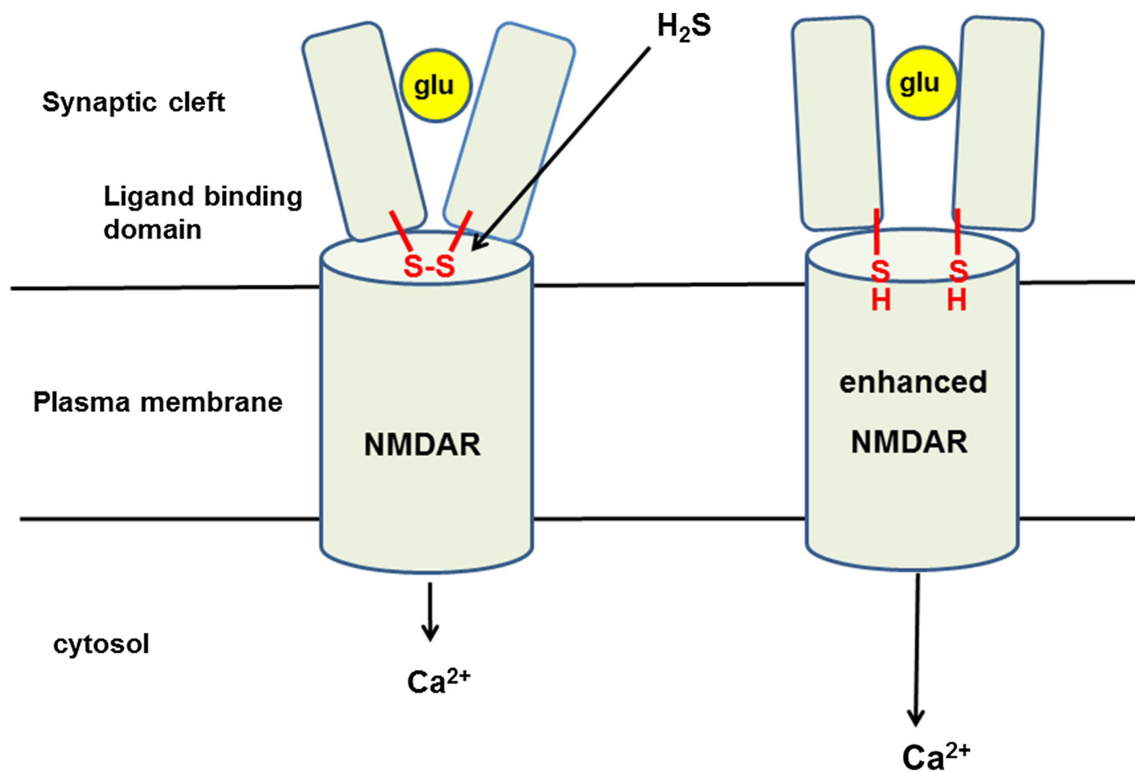

Fig. 5 Reduction of NMDA receptors enhances their activity. The reduction of cysteine disulfide bond at the hinge of the ligand binding domain of NMDA receptors enhances the receptor activity

defined as the production of bound sulfane sulfur, which consists of cysteine persulfides and polysulfides in proteins. Sulfurated cysteine residues or bound sulfane sulfur release H_2S under reducing conditions such as those in the presence of DTT (Ishigami et al. 2009). Although sulfuration of cysteine residues was initially proposed to be induced by H_2S, it subsequently turned out to be by H_2S_n. H_2S only sulfurates oxidized cysteine residues.

Following are examples of activity modification of target proteins that have mistakenly been reported to be sulfurated by H_2S: (1) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) increases its catalytic activity in the presence of H_2S, and the effect is abolished in the presence of DTT. This activation is not observed in CSE knockout mice, suggesting that H_2S produced by CSE sulfurates GAPDH. (2) The cytoprotective effect of H_2S on cardiac myocytes from ischemia–reperfusion injury is induced by the translocation of Nrf2 released from a Kelch ECH associating protein 1 (Keap1)/Nrf2 complex to the nucleus to upregulate the transcription of anti-oxidant genes (Calvert et al. 2009). A detailed mechanism proposed later by Yang et al. (2013) states that sulfuration of Keap1 by H_2S releases Nrf2 from the (Keap1)/Nrf2 complex. (3) ATP-dependent K^+ channels are sulfurated at cysteine 43 on the Kir6.1 subunit by H_2S that hyperpolarizes the membrane potential and relaxes vascular smooth muscle (Mustafa et al. 2011). (4) H_2S produced by CSE, which is stimulated by tumor necrosis factor α (TNFα), sulfurates the p65 subunit of nuclear factor κB (NF-κB) to upregulate the transcription of anti-apoptotic genes (Sen et al. 2012).

These observations indicate that the sulfuration of cysteine residues of target proteins by H_2S plays an important role in regulating their activity. However, as described previously, atoms with the same oxidation state do not undergo a redox reaction. The following are recently reported observations that indicate that H_2S_n rather than H_2S sulfurates target proteins:

(1) We previously found that H_2S induces Ca^{2+} influx in astrocytes with $EC_{50} = 116$ μM. However, in a latter study, we identified H_2S_n in the brain and found that they activate TRPA1 channels with $EC_{50} = 91$ nM (Nagai et al. 2004; Nagai et al. 2006; Oosumi et al. 2010; Kimura et al. 2013). Two cysteine residues at the amino termini of TRPA1 channels are the targets of H_2S_n, as the mutant channels in which two cysteine residues are replaced with serine lose their sensitivity to H_2S_n (Hatakeyama et al. 2015). (2) Greiner et al. (2013) reported that H_2S_n sulfurates and subsequently forms a disulfide bridge between two cysteine residues of the tumor suppressor, PTEN, to repress its activity. (3) We showed that H_2S_n sulfurates cysteine residues of Keap1 to release Nrf2 to the nucleus

(Koike et al. 2013). (4) Sulfuration induced by H_2S_n and the subsequent disulfide bridge formation in protein kinase G1α relaxes vascular smooth muscle (Stubbert et al. 2014). (5) Sulfuration of GAPDH by H_2S_n suppresses its activity. This observation contradicts a previous finding, wherein GAPDH was activated through sulfuration by H_2S (Mustafa et al. 2009; Jarosz et al. 2015). It is possible that sulfuration and the subsequent formation of cysteine disulfide bonds by H_2S_n suppress GAPDH activity, whereas reduction of disulfide bond by H_2S activates GAPDH. Alternatively, sulfuration of oxidized cysteine residues in GAPDH by H_2S may activate it. These observations clearly indicate that unoxidized cysteine residues of target proteins are efficiently sulfurated by H_2S_n rather than by H_2S.

Production of H_2S_n by 3MST

Despite the important physiological roles carried out by H_2S_n, neither the number of its sulfur atoms nor its producing enzyme was known. We previously showed that tissue homogenates absorb Na_2S, which contains trace amounts of H_2S_n in addition to H_2S, and produces bound sulfane sulfur that consists of polysulfides and persulfide bound to proteins (Ogasawara et al. 1993; Ishigami et al. 2009). Not only exogenously applied H_2S and H_2S_n, but also those endogenously produced by enzymes are absorbed or stored as bound sulfane sulfur (Ishigami et al. 2009; Shibuya et al. 2009b). Cells overexpressing 3MST contain more than twice of the amount of bound sulfane sulfur as that in control cells, whereas those expressing a defective 3MST enzyme that does not produce H_2S or H_2S_n did not exhibit increased levels of bound sulfane sulfur (Fig. 3) (Shibuya et al. 2009a, b; Kimura et al. 2015). In contrast, cells expressing CBS did not show an increase in the levels of bound sulfane sulfur (Fig. 3a). These observations suggest that 3MST produces bound sulfane sulfur.

Proteins, such as human growth hormone and superoxide dismutase 1 (SOD1), have a polysulfide bridge between cysteine residues, and these bound forms of polysulfides (bound sulfane sulfur) can be produced from 3MP, a substrate of 3MST (Nielsen et al. 2011; Hylin and Wood 1959). In addition to these bound forms of polysulfides, we found that a diffusible form of polysulfide such as H_2S_n is also produced by 3MST (Kimura et al. 2015).

Our LC/MS–MS analysis showed that 3MST produces H_2S_3, H_2S_2, and H_2S_5 as well as H_2S from 3MP (Kimura et al. 2015). A 3MST defective mutant with the catalytic site cysteine 247 is replaced by serine, which does not produce H_2S_n species (Kimura et al. 2015). Brain cells prepared from wild-type mice produced these H_2S_n species, whereas those prepared from 3MST knockout mice did not. In contrast, rhodanese, which is homologous to 3MST, did not produce H_2S_n species from 3MP.

A possible mechanism of H_2S_n production by 3MST is that 3MST receives sulfur from 3MP to elongate a polysulfide chain at its catalytic site cysteine residue. This polysulfide chain is reduced by thioredoxin to release H_2S_n species such as H_2S_3. When the interaction of thioredoxin and 3MST is stronger or the concentrations of thioredoxin are higher, the shorter forms of H_2S_n such as H_2S_2 and H_2S are produced (Fig. 6) (Nagahara et al. 2007; Mikami et al. 2011a). Moreover, both 3MST and rhodanese can accelerate the generation of H_2S_n from H_2S (Fig. 1) (Kimura et al. 2015).

Crosstalk between H_2S and NO

NO release from products generated by the chemical interaction between H_2S and NO

NO augments the relaxation effect of H_2S on vascular smooth muscle, which suggests a synergy between the two molecules (Hosoki et al. 1997). A similar synergistic effect induced by both molecules was observed in the twitch responses in the ilium (Teague et al. 2002). These observations suggest two possibilities: (1) H_2S and NO activate different target proteins (receptors) and their outputs have more than just additive effects. (2) The chemical interaction between H_2S and NO produces new substances that have a more potent effect than their parental molecules.

Several products have been reported to be generated from the chemical interaction of H_2S with NO: For example, nitrosothiol or thionitrous acid (HSNO), HNO, HSSNO, and H_2S_n (Whiteman et al. 2006; Miljkovic et al. 2013; Eberhardt et al. 2014; Cortese-Krott et al. 2015). In addition, HSSNO has been proposed as a donor of NO. Considering the fact that the synergistic effect of H_2S and NO is greater than that of NO alone, it is possible that HSSNO has its own effect rather than only releasing NO.

Production of H_2S_n by the interaction of H_2S with NO

Cacanviova et al. (2012) reported that the relaxation effect exhibited by H_2S and NO interaction products on vascular smooth muscle is greater and longer lasting compared with that produced by NO alone. Eberhardt et al. (2014) and Cortese-Krott et al. (2015) recently reported that the major products of H_2S and NO interaction are HNO and HSSNO, respectively. Although the two reports differ with respect to the production and functions of HNO and HSSNO, they commonly show that H_2S_n is produced from the interaction of H_2S and NO. Eberhardt et al. (2014) reported that HNO

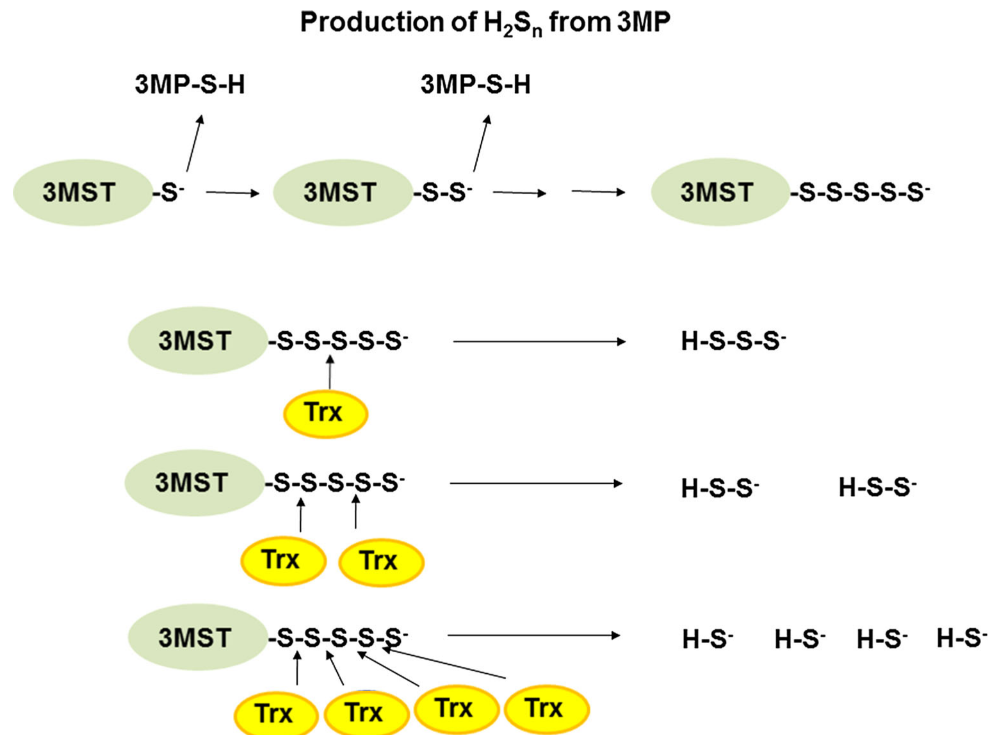

Fig. 6 A possible mechanism for H_2S_n-production from 3MP by 3MST. 3MST receives sulfur from 3MP to elongate a poly sulfur chain at its catalytic site. Thioredoxin (Trx) reduces the chain to release H_2S_n. When the interaction of 3MST and Trx is stronger or the concentrations of Trx are higher, the shorter forms of H_2S_n, such as H_2S_2 and H_2S, can be produced

is an effective molecule to activate TRPA1 channels, and H_2S_n elicits a similar effect (Kimura et al. 2013). The H_2S_n concentrations for maximal activation of TRPA1 channels in dorsal ganglion cells range from 10 to 30 μM, whereas 400 μM of Angeli's salt, a HNO donor, is required to achieve the same effect (Eberhardt et al. 2014; Hatakeyama et al. 2015).

HSSNO, which has the role of an NO carrier, releases NO to relax vascular smooth muscle, whereas H_2S_n relaxes vascular smooth muscle by activating protein kinase G1α (Stubbert et al. 2014). There is a synergy between H_2S and NO on vascular relaxation, and its effect is more potent than that with NO alone (Hosoki et al. 1997; Stubbert et al. 2014). These observations suggest the possibility that the effective molecule is H_2S_n. However, further studies are awaited to determine the identity of the effective molecule(s) produced by the interaction of H_2S with NO.

H_2S and NO mutually regulate their synthesizing enzymes

The NO donors, sodium nitroprusside (SNP), and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNA) enhance the production of H_2S and upregulate the transcription of CSE, respectively (Zhao et al. 2001).

Minamishima et al. (2009) reported that H_2S activates eNOS by facilitating its phosphorylation and attenuates sudden cardiac arrest-induced mitochondrial injury as well as cell death, and that this effect of H_2S was not induced in eNOS knockout mice. King et al. (2014) further clarified the mechanism for this protective effect of H_2S: H_2S activates eNOS through the dephosphorylation of its inhibition site as well as phosphorylation of its activation site to protect the heart from ischemia–reperfusion injury. The protective effect of H_2S is diminished in CSE knockout mice, whereas it is recovered by the administration of H_2S. These observations suggest that H_2S produced by CSE activates eNOS resulting in the increased production of NO, which protects the heart from ischemic and oxidative insults (Minamishima et al. 2009; King et al. 2014).

Glutathione persulfide as a reducing molecule

Massey et al. (1971) initially reported that glutathione persulfide (GSSH or GSS^-), which is produced from glutathione trisulfide (GSSSG), reduces cytochrome c more efficiently than glutathione (GSH), and that cysteine trisulfide has a similar effect. A potent reducing activity and scavenging effect of GSSH was also shown on papain and H_2O_2, respectively (Francoleon et al. 2011; Ida et al. 2014).

Sulfur quinone oxidoreductase (SQR), which catalyzes the first step of the sulfide oxidation pathway in mitochondria, takes GSH as a thiophilic acceptor to produce GSSH (Hildebrandt and Grieshaber 2008; Mishanina et al. 2015). H_2S_n produced by 3MST may also readily generate CysSSH and GSSH by reacting with cysteine or GSH,

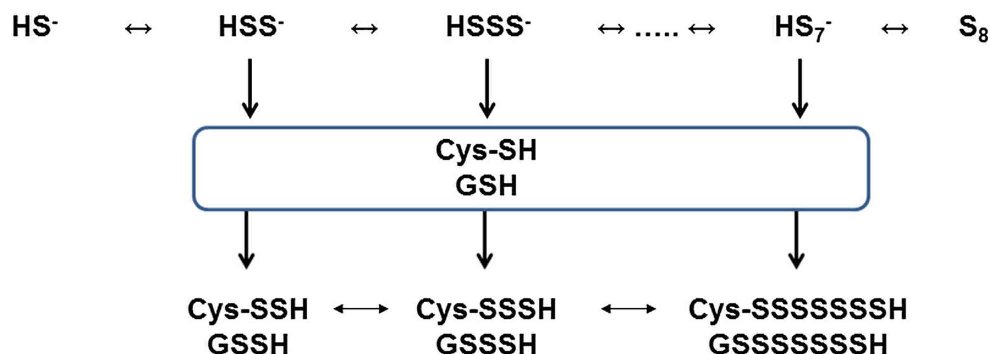

Fig. 7 Production of cysteine and glutathione persulfides. Once H_2S_n is produced, it can react with the intracellular main thiols such as cysteine and glutathione to produce cysteine- and glutathione persulfides as well as cysteine and glutathione polysulfides

which exist at intracellular concentrations of 100 µM and mM, respectively, and are good targets of H_2S_n (Fig. 7) (Kimura et al. 2015).

GSSH has been proposed to be produced via the exchange reaction between GSH and cysteine persulfide (CysSSH), which, in turn, was reported to be produced from cystine by CBS and CSE (Ida et al. 2014). Cavallini et al. (1960) initially proposed this pathway involving CSE. However, the physiological relevance of this pathway needs to be re-evaluated. Although CSE has a high affinity for cystine with a K_m value of 30–70 µM (Stipanuk 1986), CSE as well as CBS are localized to the cytosol where cysteine, but not its oxidized form, is the dominant form. The liver contains 0.2-µM cystine, the lungs, 0.05 µM, whereas no cystine is detected in the heart and brain (Ida et al. 2014). Notably, in the extracellular milieu, blood contains only 40µM cystine (Brigham et al. 1960). Yadav et al. (2016) re-evaluated the pathways and concluded that CysSSH is unlikely to be produced by CSE and CBS. Moreover, considering the observation that cells expressing CBS do not have increased levels of bound sulfane sulfur, CBS may not be involved in the production of CysSSH, which readily reacts with cysteine residues to produce bound sulfane sulfur (Fig. 3a) (Ishigami et al. 2009; Shibuya et al. 2009b).

Conclusion

Twenty years have passed since the first demonstration of H_2S as a neuromodulator and the subsequent finding that it can act as a smooth muscle relaxant in synergy with NO (Abe and Kimura 1996; Hosoki et al. 1997). Since then, various roles for this molecule have been unveiled, including the protection of tissues/organs from oxidative stress or ischemia–reperfusion injury, angiogenesis, anti-inflammation, oxygen sensing, and so on (Kimura and Kimura 2004; Zanardo et al. 2006; Olson et al. 2006; Elrod et al. 2007; Cai et al. 2007; Papapetropoulos et al. 2009; Peng et al. 2010). Sulfhydration (sulfuration) has been proposed as a mode of action of H_2S that causes conformational changes in target proteins to modify their activity (Mustafa et al. 2009). Under oxidative conditions, cysteine residues are oxidized to cysteine sulfenic acid (Cys-SOH), and when NO transmits the signal, they are oxidized to cysteine S-nitrosothiol (Cys-SNO). These oxidized cysteine residues are sulfurated by H_2S, whereas unoxidized residues are not (Kabil and Banerjee 2014; Mishanina et al. 2015). Instead, unoxidized cysteine residues are sulfurated by sulfane sulfur such as H_2S_n.

H_2S_3, H_2S_2, and H_2S_5 have been detected in the brain, and 3MST has been identified as the H_2S_n-synthesizing enzyme (Kimura et al. 2013; Kimura et al. 2015). Sulfuration of two cysteine residues by H_2S_n causes a conformational change in target proteins such as TRPA1 channels, PTEN, Keap1/Nrf2 complex, and PKG1α, all of which have two sensitive cysteine residues (Kimura et al. 2013; Greiner et al. 2013; Koike et al. 2013; Stubbert et al. 2014). The sulfurated cysteine residue reacts with the nearby unsulfurated one to generate a cysteine disulfide bond that induces further conformational changes in the target proteins. H_2S can also modify the activity of target proteins by reducing their cysteine disulfide bonds at active sites. However, this depends on the local concentration of H_2S (Vasas et al. 2015).

Some of the crosstalk between H_2S and NO may also be attributed to the effect of H_2S_n. The interaction of both molecules generates H_2S_n as well as HNO and HSSNO, which activate TRPA1 channels and induce vascular relaxation. Though it is debatable whether HNO or HSSNO is the effective molecule, H_2S_n has been shown to activate TRPA1 channels and relax vascular smooth muscle (Kimura et al. 2013; Eberhardt et al. 2014; Stubbert et al. 2014; Cortese-Knott et al. 2015). The production of H_2S_n by the interaction of H_2S with NO may provide a mechanism for the synergistic effect of H_2S and NO on vascular relaxation (Hosoki et al. 1997).

There are other forms of crosstalk between H_2S and NO: (1) H_2S facilitates the production and release of NO. Some cytoprotective effect of H_2S against ischemia–reperfusion injury of the heart was found to be mediated by NO released from eNOS activated by H_2S (Minamishima et al.

2009; King et al. 2014). (2) NO enhances the production of H_2S. An NO donor, nitroprusside increases H_2S production in the thoracic aorta (Zhao et al. 2001). (3) NO upregulates the transcription of H_2S-producing enzyme. An NO donor, *S*-nitroso-*N*-acetyl-DL-penicillamine enhances the transcription of CSE (Zhao et al. 2001).

Since its identification, H_2S_n, which is produced by 3MST and also generated by the interaction between H_2S and NO, is thought to be a potential signaling molecule that activates channels, relaxes smooth muscle, and regulates the activity of a tumor suppressor and transcription factors (Kimura et al. 2013, 2015; Greiner et al. 2013; Koike et al. 2013; Eberhardt et al. 2014; Stubbert et al. 2014; Cortese-Knott et al.2015). Although the physiological stimulations that regulate the activity of 3MST have not been identified, it is known that the activity of CAT is regulated by Ca^{2+} (Mikami et al. 2011b). It would indeed be intriguing to know how the levels of H_2S_n are regulated.

The regulation of H_2S_n production by the interaction between H_2S and NO is even more complicated. H_2S production is regulated by the activity of its producing enzymes. CBS activity is enhanced by *S*-adenosylmethionine and glutathionylation (Abe and Kimura 1996; Chen et al. 2004; Niu et al. 2015), whereas it is suppressed by NO and CO (Taoka and Banerjee 2001; Morikawa et al. 2012). The activity of CSE and CAT is suppressed by Ca^{2+}, whereas NO production is activated by Ca^{2+}/-calmodulin (Bredt and Snyder 1990; Mikami et al. 2011b, 2013). Notably, responses previously thought to be induced by NO alone can also be induced by H_2S together with NO. It is necessary to understand how the interaction between H_2S and NO is regulated under physiological conditions. By addressing these problems, physiological roles and therapeutic potential of H_2S, H_2S_n, and NO will be clarified.

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