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Functional and molecular insights of hydrogen sulfide signaling and protein sulfhydration

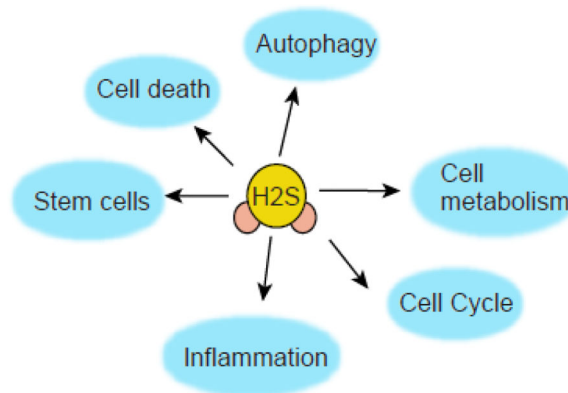
Nilkantha Sen¹

¹Department of Neuroscience and Regenerative Medicine, Augusta University, 1120 15th Street, CA 2018, Augusta, GA, 30907, nsen@augusta.edu

Abstract

Hydrogen sulfide (H₂S) a novel gasotransmitter is endogenously synthesized by multiple enzymes that are differentially expressed in the peripheral tissues and central nervous systems. H₂S regulates a wide range of physiological processes ranging from cardiovascular, neuronal, immune, respiratory, gastrointestinal, liver, and endocrine systems by influencing cellular signaling pathways and sulfhydration of target proteins. This review focuses on the recent progress made in H₂S signaling that affects mechanistic and functional aspects of several biological processes such as autophagy, inflammation, proliferation and differentiation of stem cell, cell survival/death, and cellular metabolism under both physiological and pathological conditions. Moreover we highlighted the crosstalk between nitric oxide (NO) and H₂S in several biological contexts.

Graphical Abstract



Introduction

Unlike neurotransmitters or hormones, gasotransmitter such as hydrogen sulfide (H₂S) can be primarily synthesized by an enzymatic activity of cystathionine gamma-lyase (CSE) or cystathionine beta synthase (CBS). The CSE essentially predominates in peripheral tissues

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to generate H₂S in the liver, kidney, and heart, however, in the brain, CBS is mostly responsible for synthesizing H₂S [1–4]. Recently, a third enzyme known as 3-mercaptopyruvate sulfurtransferase (MST) has been identified to synthesize H₂S [2, 3] (Figure 1), but the evidence for its involvement in the physiological biosynthesis of H₂S in mammals is weak compared to other enzymes [5, 6]. In general, CBS generates H₂S using Cys and homocysteine as its substrate [7]; however, the regulation of the determining the substrate for CBS are unclear. Other than, producing H₂S, CBS is one of the major regulator of homocysteine levels in mammalian. A compelling number of evidence indicates that impairment of CBS activity leads to markedly increase homocysteine levels that can affect cardiovascular function in vivo [7–10]. On the other hand, a reduction in the level of H₂S due to an inactivation of CBS contributes to the vascular contribution in patients [11–14]. This was further supported by the fact that mice lacking CBS (CBS KO) cause death by 4 weeks of age along with growth retardation and developmental defects [15]. CBS generally exists in two states; the ferrous form of CBS is inactive by binding with carbon monoxide in the presence of NADPH or methionine synthase reductase, however, the ferric state of CBS is active both in vitro and in vivo [16–18]. On the other hand, CBS can be allosterically activated by S-adenosylmethionine (SAM) which can bind to the carboxy-terminal domain of CBS and produce H₂S [19, 20]. Similar to CBS, the other enzyme CSE uses homocysteine and Cys as a substrate to produce H₂S along with pyruvate and ammonia as a byproduct. The disulfide form of Cys, cystine can also serve as a substrate for rat CSE and produce H₂S [8, 13, 14].

Obviously, the question arises how the level of H₂S is regulated inside the cells. H₂S can be metabolized by oxygen dependent catabolic processes in the mitochondria. The bound form of H₂S, sulfane sulfur can generate H₂S in the presence of a reducing agent but the role of sulfane sulfur in the signaling pathway is not well studied [21, 22]. H₂S can also interact with haem proteins, such as hemoglobin, and cytochrome c oxidase, which may serve as a store for this gasotransmitter. In addition, H₂S can be methylated in the cytosol by thiol S-methyltransferase to generate methanethiol, which can be further methylated to the less toxic compound dimethyl sulfide [23, 24].

H₂S signaling via protein sulfhydration

H₂S signals inside cells through post-translational modification of proteins, known as sulfhydration [25, 26]. In this modification, H₂S modifies a free Cys residue (–SH) of a target protein to –SSH. The modified biotin switch assay [25] was initially identified to detect sulfhydration of protein. But due to its limitation about specificity, another method of detection of sulfhydrated proteins have been identified which is known as Maleimide assay [27, 28]. In this procedure, a fluorescent conjugated maleimide interacts with both –SH and –SSH residues of a protein, but in the presence of a strong reducing agent such as DTT, the S–S bond is cleaved off and as a result, the fluorescent signal of sulfhydrated species is lost. The details of the procedure are highlighted in the diagram present in Figure 2. The sulfhydration of proteins determined by the acid dissociation constant (pK_a) of Cys residues that can be governed by the local protein environment and protein architecture. Briefly, the thiolate anions (S[–]), produced from Cys residues with low pK_a, functions as a strong nucleophile which is oxidized by hydrogen peroxide to form either sulfenic acid (SOH),

sulfinic acid (SO₂H) or sulfonic acid (SO₃H) forms. The sulfenic form can react with HS– or H₂S to form a persulfide bond [29–31]. Sulfhydrylation of proteins can affect its function, localization inside cells, stability in cells and resistance to oxidative stress [3]. We talked about the importance of H₂S signaling and sulfhydrylation of several proteins in the following biological processes mostly autophagy, cellular metabolism, stem cells, inflammation, cell cycle and cell death under physiological and pathological conditions.

Autophagy and H₂S signaling

Autophagy is a cellular protective and quality control process in cells and is an intracellular catabolic process in which the cytoplasmic constituents, such as aggregated proteins and dysfunctional organelles, are surrounded by a double membrane, termed the autophagosome, and are transported to lysosomes for degradation and recycling [32, 33]. In response to a variety of extracellular and intracellular stresses including nutrients deprivation, hormonal therapy, pathogenic infection, misfolded proteins and damaged organelles it serves as a salvage mechanism for recycling cytoplasmic materials and preserving energy *via* lysosome-driven degradations [34, 35]. The execution of autophagy involves a set of evolutionarily conserved gene products, known as Atg proteins. Among these usually correlates well with the accumulation of autophagosomes [36]. It has been suggested that autophagy malfunction can induce the pathogenesis of diverse human diseases and we will discuss how H₂S influences autophagy in several disease conditions.

Pathological perspectives

Ischemia—Autophagy-related proteins and autophagy complex levels in the ischemic hemisphere results in neurological deficits and an increase in cerebral infarct volume in middle cerebral artery occlusion (MCAO) mice [37, 38]. It was shown that administration of H₂S ameliorated infarct size and preserved LV function during development of myocardial infarction in mice, which suggest that H₂S is cytoprotective and angioprotective during the evolution of myocardial infarction [39] along with an improvement in neurological deficits in a rapamycin-dependent manner [40]. Moreover, this effect is associated with its influence in down-regulating autophagosome accumulation [41]. Autophagy also plays a significant role in myocardial ischemia-reperfusion (IR) injury. H₂S plays a myocardial protective role against IR injury by regulating autophagy via mTOR activation [42]. However, excessive autophagy aggravates myocardial ischemia/reperfusion (IR) injury [43]. It was shown that H₂S can inhibit autophagy in neonatal rat cardiomyocytes exposed to H/R and exert a cardioprotective effect at least partly by regulating PI3K/SGK1/GSK3 β signaling pathway [44]. Other groups have shown that H₂S protected against myocardial I/R injury by activating AMPK and restoring I/R-impaired autophagic flux [45]. H₂S also protects the spinal cord and induces autophagy via miR-30c microRNA in a rat model of spinal cord hernia-reperfusion injury [46]. However, the mechanism is not well elucidated. Interestingly, it was shown that treatment with an antioxidant, Tetrahydrocurcumin (THC) ameliorates autophagy during ischemia/reperfusion injury by reducing homocysteinylolation of cyto-c in cystathionine-beta-synthase heterozygote knockout (CBS^{+/-}) mice (*via* activation of by MMP-9). This study suggests a potential therapeutic role of dietary THC in cerebral ischemia [47].

Ischemic post-conditioning (PC) lost cardioprotection in the aged hearts and cardiomyocytes. NaHS (an H₂S donor) significantly restored cardioprotection of PC through decreasing myocardial damage, infarct size, and apoptosis, improving cardiac function, increasing cell viability and autophagy in the aged hearts and cardiomyocytes [48]. In addition, in the aged cardiomyocytes, NaHS up-regulated AMPK/mTOR pathway, and the effect of NaHS on PC was similar to the overexpression of Atg 5, which suggests that exogenous H₂S restores cardioprotection from PC by up-regulation of autophagy via activation of AMPK/mTOR pathway in the aged hearts and cardiomyocytes [49]. Myocardial I/R induced autophagosome accumulation is evidenced by the increased ratios of LC3-II/LC3-I, upregulation of beclin-1 and P62 and reduction in LAMP-2 [45, 50]. Another H₂S donor, ADT reduced these autophagic changes induced by I/R, indicating that H₂S restored I/R-impaired autophagic flux [45]. Inhibition of AMPK blocked the effects of H₂S on restoring I/R-impaired autophagy flux. Thus AMPK activation and subsequent restoration of I/R impaired autophagic flux are unrecognized mechanisms underlying cardioprotective effects conferred by H₂S donors [45, 51]. During acute pancreatitis (AP) impairment of autophagy and accumulation of autophagic vacuoles and premature activation of trypsinogen are common phenotypes. It was shown that treatment with NaHS exacerbated both processes and attributed to over-activation of autophagy rather than hampered autophagosome-lysosome fusion in an AMPK and mTOR-dependent manner [52].

Myocardial fibrosis—Excessive autophagy induced by extravagant oxidative stress is the main reason for diabetes-induced vascular endothelial cells dysfunction. Exogenous H₂S protect arterial endothelial cells by suppressing excessive autophagy induced by oxidative stress through the Nrf2-ROS/AMPK signaling pathway [53]. Myocardial fibrosis is the predominant pathological characteristic of diabetic myocardial damage. Treatment with NaHS, myocardial fibrosis was ameliorated, myocardial autophagy was decreased and the PI3K/AKT1 pathway suppression was reversed [54]. Moreover, the protective effect of H₂S against diabetes-induced myocardial fibrosis is associated with the attenuation of autophagy via the upregulation of the PI3K/AKT1 signaling pathway [54].

Metabolic disorders—H₂S is effective in protection against neurodegeneration and neurovascular dysfunction [55]. Hyperglycemia (HG) reduces AMPK activation leading to impaired autophagy and matrix accumulation. H₂S treatment improves HG-induced renovascular remodeling, however, its mechanism remains unclear [56]. HG decreased the expression of H₂S regulating enzymes CBS and CSE, and autophagy markers Atg5, Atg7, Atg3 and LC3B/A ratio [56] and H₂S plays a key role in renovasculopathy during HHcy and is mediated through Akt/FoxO3 pathways [57]. HG increased galectin-3 and periostin, markers of matrix accumulation. H₂S mitigates adverse remodeling in HG by induction of autophagy and regulation of matrix metabolism through LKB1/STRAD/MO25 dependent pathway [56]. Diabetes and particularly high blood glucose levels are implicated in neurodegeneration. One of the hallmarks of neurodegeneration is protein aggregation. H₂S treatment increased protein synthesis and aggregation in the diabetic ZDF rat brain and provided a novel strategy against protein aggregation in the diabetic brain [58].

Autophagy plays an important role in liver triglyceride (TG) metabolism. Inhibition of autophagy could reduce the clearance of TG in the liver. H₂S is a potent stimulator of autophagic flux. Recent studies showed H₂S is protective against hypertriglyceridemia (HTG) and nonalcoholic fatty liver disease (NAFLD), while the mechanism remains to be explored. H₂S treatment reduces serum TG level and ameliorates NAFLD by activating liver autophagy via the AMPK-mTOR pathway [59]. Apoptosis and autophagy play important roles in concanavalin A (Con A)-induced acute hepatitis. Aspartate aminotransferase, alanine aminotransferase, and pathological damage were significantly ameliorated by NaHS pretreatment. NaHS pretreatment significantly reduced the levels of interleukin-6 and tumor necrosis factor- α (TNF α) compared with those of the Con A group [60]. The expression of Bcl-2, Bax, Beclin-1, and LC3-2, which play important roles in the apoptosis and autophagy pathways, were also clearly affected by NaHS. H₂S attenuates Con A-induced acute hepatitis by inhibiting apoptosis and autophagy, in part, through activation of the PtdIns3K-AKT1 signaling pathway [60]. Other groups have shown that H₂S attenuates hepatic I/R injury, at least in part, by regulating apoptosis through inhibiting JNK1 signaling [61]. The autophagy agonist rapamycin potentiated this hepatoprotective effect by reversing the inhibition of autophagy by H₂S.

Metabolite accumulation in lysosomal storage disorders (LSDs) results in impaired cell function and multi-systemic disease [62]. Although substrate reduction and lysosomal overload-decreasing therapies can ameliorate disease progression, the significance of lysosomal overload-independent mechanisms in the development of cellular dysfunction is unknown for most LSDs. It was shown that impaired chaperone-mediated autophagy (CMA) in cystinosis, an LSD caused by defects in the cystine transporter cystinosis (CTNS) and characterized by cysteine lysosomal accumulation [62].

TBI—Traumatic Brain Injury (TBI) is one of the major reason for morbidity and mortality and it affects more than 1.7 million Americans each year. 3-mercaptopyruvate sulfurtransferase (3-MST) was a novel hydrogen sulfide (H₂S)-synthesizing enzyme that may be involved in cyanide degradation and in thiosulfate biosynthesis. 3-MST was mainly located in living neurons and TBI leads to an augmentation in the level of 3-MST. Importantly, immunohistochemistry analysis revealed that injury-induced expression of 3-MST was partly co-labeled by LC3 [63]. However, there was no co-localization of 3-MST with propidium iodide (cell death marker) and LC3 positive cells were partly co-localized with propidium iodide. These data suggested that H₂S may be implicated in the autophagy of neuron and involved in the pathophysiology of the brain after TBI [63, 64]. Other groups have shown that H₂S pretreatment had reduced brain edema, improved motor performance and ameliorated performance in Morris water maze test after TBI [64]. Immunoblotting results showed that H₂S pretreatment reversed TBI-induced cleavage of caspase-3 and decline of Bcl-2, suppressed LC3-II, Beclin-1, and Vps34 activation and maintained the p62 level in injured cortex and hippocampus post-TBI [64]. The results suggest a protective effect and therapeutic potential of H₂S in the treatment of brain injury and the protective effect against TBI may be associated with regulating apoptosis and autophagy.

Cellular Metabolism and H₂S signaling

Physiological perspectives

Cellular metabolism mostly depends on cellular energy production and it is known that H₂S reduce cell energy production by inhibiting oxidative phosphorylation, ATP biosynthesis and cytochrome C oxidase in mitochondria [65, 66]. On the other hand, in hyperglycemia H₂S protects the vascular endothelium by preserving mitochondrial function to improve endothelial metabolic state and maintain endothelial function [67]. H₂S protects against vascular remodeling from endothelial damage by normalizing the levels of redox stress, MMPs, and TIMPs [68]. It was shown that CBS^{-/-} mice exhibited thicker neointima and a higher percentage of luminal narrowing in vein grafts which is associated with vascular remodeling in vein graph in mice [69]. Moreover, Other groups have shown that in the liver of CBS^{-/-} mice, mitochondrial oxidative stress is reduced along with a decrease in the level of a serine/threonine kinase, DYRK1A to counteract pro-apoptotic signals [70], [71].

H₂S also affects cell proliferation or apoptosis by altering cell fate during the course of the cell cycle [72]. As a part of the mechanism, it was shown that H₂S affects DNA synthesis, decreases Rb phosphorylation, and increases cell cycle arrest via augmenting p21Cip1 level [72]. H₂S is a reducing agent and possesses antioxidant effects to offer cytoprotection via reducing the cytotoxic effects of hydrogen peroxide and oxidized low-density lipoprotein (oxLDL) on cultured human umbilical vein endothelial cells (HUVECs). H₂S attenuates high-fat diet-induced cardiac dysfunction via suppression of ER stress which suggest that H₂S controls cellular metabolic processes via different processes [73]. Elevated retinal homocysteine in CBS^{+/-} mice alters expression of genes involved in endoplasmic reticular stress, N-methyl-d-aspartate (NMDA) receptor activation, cell cycle, and apoptosis [74].

To identify the cellular targets of H₂S, it was shown that H₂S sulfhydrates KATP channel and sulfhydration of KATP channel prevents the association of KATP with ATP and promotes binding of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) [75] (Figure 3, box 8). This event results in the opening of the channel and vasodilation of smooth muscle cells. On the other hand, it was shown that in CBS^{+/-} mice, where intracellular H₂S was reduced significantly, there is no significant changes in muscle morphology except a reduction in large muscle fiber number in CBS^{+/-} mice [76]. Thus the balance of selecting target determine the overall effects of H₂S. However, greater insights into the mechanistic details are needed to understand the precise role of H₂S on biological effects and diseases related to defective H₂S metabolism.

Pathological perspectives

Imbalance in cellular bioenergetics is responsible for tumor growth, invasion, and metastasis and Tumor cells generate ATP mainly through mitochondrial oxidative phosphorylation and increased aerobic glycolysis. Recent studies have shown that H₂S treatment influences mitochondrial phosphodiesterases and subsequently affect mitochondrial function and electron transfer [77]. Interestingly, CBS also present in mitochondria and depletion of CBS results in attenuation in mitochondrial oxygen consumption with a concomitant increase in the generation of reactive oxygen species in ovarian cancer cells [78]. Similarly, knockdown

of CBS results in a decrease in the basal oxygen consumption rate, ATP production in colon cancer cells suggesting that H₂S supports tumor growth and proliferation [78]. Mitochondrial transcription factor A (TFAM) encodes a key mitochondrial transcription factor containing two high mobility group motifs. The encoded protein also functions in mitochondrial DNA replication and repair. Recently, it was shown that CSE KO (CSE^{-/-}) has lower mtDNA content and copy number due to decreased expression of TFAM [79]. Under physiological conditions, H₂S sulfhydrates the transcriptional repressor interferon regulatory factor 1 (IRF-1) (Figure 3, box 7) and promotes its binding to the promoter of DNA methyltransferase 3a (Dnmt-3a) to inhibit its expression. The inhibition of Dnmt-3a results in lowering the level of DNA methylation and facilitates transcription of TFAM [80]. Metabolic complications may also lead to mitochondrial remodeling by interfering with fusion and fission, therefore, leading to mitochondrial mitophagy and skeletal muscle myopathy. Mitochondrial protection by H₂S may mitigate deterioration of muscle function during metabolic syndromes [81].

Angiogenesis—Angiogenesis is a biological process where new blood vessels are formed from the existing one and it involves proliferation and migration of endothelial cells. Angiogenesis plays a pivotal role in the growth, invasion, and metastasis of solid tumors, which could supply oxygen and nutrients and dispose of metabolic wastes. Several factors including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and angiogenin are known to play an important role in angiogenesis. A compelling number of studies have shown that H₂S can increase VEGF expression and stimulates angiogenesis in several tissues. H₂S also promote tumor growth by promoting angiogenesis and vasorelaxation [82]. Depletion of CBS can reverse H₂S-mediated angiogenesis in colon cancer [83]. Taken together, these results indicate that CBS plays an important role in promoting angiogenesis and tumor growth. Thus, CBS could be a promising molecular target for cancer therapy.

Recently it was demonstrated that lung alveolarization can be reduced to the extent of 50% in CBS^{-/-} and CSE^{-/-} mouse pups. Exogenous administration of H₂S with GYY4137 promoted endothelial tube formation. These data confirm a key role for the H₂S-generating enzymes CBS and CSE in pulmonary vascular development and homeostasis and in lung alveolarization [84]. The blood-brain barrier (BBB) integrity can be maintained by the cross-talk of endothelial cells, junction proteins, and neuroglial network and a recent study has shown that miR29b regulates BBB dysfunction by regulating BBB permeability through DNMT3b. In CBS^{+/-} mice miR29b microRNA levels were high as compared with wild-type (WT) mice along with an increase in global hypermethylation and DNA methyltransferase-1 and -3a. Thus treatment with a DNA methylation inhibitor, 5'-aza can improve BBB permeability by decreasing the expression of miR29b [85] and normalized blood pressure and reversed renal injury similar to The CBS^{+/-} mice [86]. A mouse model of carotid artery air-dry endothelium denudation and re-endothelialization was established and used to evaluate post-injury endothelial repair in mice with the gene deletion of cystathionine-beta-synthase (CBS). Post-injury re-endothelialization was impaired in severe CBS^{-/-} mice [87].

Stem cell and H2S signaling

Physiological perspectives

Tissue homeostasis can be maintained by the orchestration of several cell types such as osteoblasts, chondrocytes, myocytes and adipocytes. Mesenchymal stem cells (MSC) can serve as progenitor cells which have the capacity for self-renewal and multilineage differentiation to generate these cells. It was shown that endogenous H₂S regulates the fate of MSC through several possible mechanisms. Endogenous H₂S controls proliferation and differentiation of neural stem cells through activation of Erk1/2 and augments the proliferation and survival of human-induced pluripotent stem cell-derived MSCs by activation of the PI3K/Akt pathway [88]. A recent study showed that MSCs produce H₂S and that endogenous H₂S plays a crucial role in the maintenance of MSC function to ensure bone homeostasis [89]. Depletion of H₂S in CBS KO mice, osteogenic differentiation, and proliferation in bone marrow MSCs were impaired [90, 91]. As a part of the mechanism, it was shown that reduction in sulfhydrylation of Ca²⁺ transient receptor potential (TRP) channels (TRPV3, TRPV6, and TRPM4) TRP causes aberrant intracellular Ca²⁺ influx (Figure 3, box 5). As a result, Wnt/β-catenin signaling is impaired and causes attenuation of osteogenic differentiation of BMMSCs [91]. NaHS dose-dependently decreased human osteoclast differentiation at concentrations which did not induce toxicity. The inhibition of human osteoclast differentiation was associated with a down-regulation in RANKL-dependent intracellular ROS levels in human pre-osteoclasts cells [92]. Furthermore, NaHS up-regulated NRF2 protein expression, its nuclear translocation, and the transcription of the two key downstream antioxidant genes Peroxiredoxin-1 and NAD(P)H dehydrogenase quinone 1, suggesting that NRF2 activation may inhibit human osteoclast differentiation by activating a sustained antioxidant response in osteoclast progenitors [92]. NaHS also downregulated the RANKL/OPG mRNA ratio in human mesenchymal stem cells, the key osteoclast-supporting cells [92]. These results suggest that NaHS shows a potential therapeutical role in erosive diseases of bone by regulating both direct and indirect mechanisms controlling the differentiation of circulating osteoclast precursors. Recently, it has been shown that S-sulfhydrylation of Kelch-like ECH-associated protein 1 (KEAP1) is a negative regulator of the activity of nuclear factor erythroid 2-related factor 2 (NRF2). H₂S-induced S-sulfhydrylation of KEAP1 at Cys151 (Figure 3, box 1) facilitates the dissociation of KEAP1 from NRF2, thereby resulting in an enhancement of NRF2-mediated antioxidant responses [93].

H₂S treatment augments MSC proliferation and survival and attenuates hypoxia-, oxidants, or serum deprivation-induced apoptosis. Activation of several signaling pathways including Erk1/2 and GSK-3β and an increase in an anti-apoptotic gene such as Bcl-2 are important for hypoxia-induced MSC apoptosis. Under ischemic conditions, H₂S has a protective effect on mesenchymal stem cells and mature adipocytes, and this effect is mediated by the elevation of anti-apoptotic gene expression such as Bcl2 [94]. Thus modulation of H₂S metabolism may serve as a therapeutic approach to promote the viability of transplanted MSCs and facilitate MSC-based regeneration. MSCs have the potential to facilitate cardiac repair following acute myocardial infarction. However, MSC therapy is limited by apoptosis of the stem cells following transplantation. It was demonstrated that hypoxia and serum

deprivation is able to significantly induce apoptosis in MSCs. CSE overexpression, which enhances the endogenous H₂S level, protects MSCs from H/SD-induced apoptosis via attenuation of the mitochondrial injury pathway, inhibition of endoplasmic reticulum stress and activation of the PI3K/Akt signaling pathway [95]. These findings suggest that modulation of, the CSE/H₂S system may be a therapeutic approach with which can promote the viability of transplanted MSCs.

Other groups have shown that H₂S promoted the differentiation of certain hematopoietic stem/progenitor cells in the bone marrow [96]. This gave rise to an idea that H₂S might promote hematopoiesis. In fact, H₂S promoted the generation of megakaryocytes, increased platelet levels, ameliorate menorrhagia, and improved survival. H₂S is a novel promoter for megakaryopoiesis by acting on the TPO receptors but not TPO to generate megakaryocytes/platelets [96]. Moreover, it was shown that in several cell types, including human inducible pluripotent stem cell (hiPSC)-derived neurons, H₂S inhibited complex IV of the mitochondrial respiratory chain and induced apoptosis [97]. H₂S increased hydroxyl radical production in isolated mouse heart mitochondria and F₂-isoprostanes in brains and hearts of mice [98]. H₂S plays an important role in the proliferation and differentiation of human periodontal ligament stem cells (PDLSCs) [99, 100]. Human PDLSCs express both CBS and CSE and produce H₂S. Blocking the generation of endogenous H₂S with CBS inhibitor hydroxylamine significantly attenuated PDLSC proliferation and reduced the osteogenic and adipogenic differentiation capacity of PDLSCs [101]. In contrast, CSE inhibitor DL-propargylglycine had no effect on PDLSC function [101]. Exogenous H₂S could inhibit the production of endogenous H₂S and impair PDLSC function in a dose-dependent manner. Thus, physiologic levels of endogenous H₂S maintain the proliferation and differentiation capacity of PDLSCs, and CBS may be the main source of endogenous H₂S in PDLSCs [101]. NaHS pretreatment can increase the survival of therapeutically used human adipose tissue-derived stem cells via increased antioxidant defense [102]. The underlying mechanisms involve enhanced ERK-phosphorylation and decreased AKT-phosphorylation [102]. Pretreatment with NaHS may represent a simple pharmacological step that may enhance the efficacy of cell-based therapies.

Accumulating evidence has suggested that H₂S acts as a novel neuron-modulator and neuroprotective agent; however, it remains to be investigated whether H₂S has a direct effect on neural stem cells (NSCs). Recently it was shown that NaHS promoted proliferation and neuronal differentiation of NSCs [103]. Further analysis revealed that NaHS-induced proliferation was associated with phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and neuronal differentiation was linked to altered expression of differentiation-related genes [104]. Moreover, administration of NaHS could increase the number of proliferating cells in the dentate gyrus of the hippocampus in the mice after hypoxia [104]. These findings suggest that H₂S may afford a novel therapeutic strategy to intervene in the progression of brain diseases.

Pathological perspectives

Impaired angiogenesis and its induced refractory wound lesions are common complications of diabetes. H₂S has been reported to have proangiogenic effects. We hypothesize that H₂S

improves diabetic wound healing by restoring endothelial progenitor cell (EPC) function in type 2 diabetes [105]. EPC functions of db/db mice were significantly improved after in vitro NaHS treatment [105]. The expressions of Ang-1 in wound skin tissue and in EPCs were upregulated in the NaHS and HTB groups compared with db/db controls but were downregulated by in vivo PAG and in vitro siCSE treatment compared with normal controls [105]. Chronic exposure to high glucose induces the expression of CSE in pancreatic beta-cells, thereby suppressing apoptosis. After 8 weeks of HFD, blood glucose levels were markedly increased in middle-aged CSE-KO mice, insulin responses were significantly reduced, and DNA fragmentation of the islet cells was increased along with an increase in thioredoxin binding protein-2 (TBP-2). Administration of NaHS, reduced TBP-2 gene levels in isolated islets from CSE-KO mice [106]. Diabetic EPC tube formation capacity was significantly inhibited by Ang-1 small interfering RNA before NaHS treatment compared with db/db EPCs treated with NaHS only [105]. Taken together, these results show that H₂S improves wound healing by restoration of EPC functions and activation of Ang-1 in type 2 diabetic mice.

Inflammation and H₂S signaling

Physiological perspective

H₂S has been shown to serve as an important endogenous anti-inflammatory molecule and blocks leukocyte adherence to the vascular endothelium [107]. This was supported by studies where it was shown that H₂S treatment attenuates adherence of leukocytes to the vascular endothelium and impairs extravasation of leukocytes; however Inhibition of endogenous H₂S rescued the phenotype [107, 108]. Recent studies related to sub-dermal inflammation, H₂S treatment provides a protective function by suppressing leukocyte infiltration and edema formation [109]. Moreover, several groups have shown that H₂S suppress the expression of cell adhesion molecules on both the endothelium (e.g., intercellular adhesion molecule (ICAM)-1 and P-selectin) and on the leukocyte (lymphocyte function-associated antigen (LFA)-1) [110, 111]. This finding was further supported by in vivo data where CBS heterozygous mice show increased vascular permeability and increased numbers of adherent leukocytes in mesenteric venules [112]. Other than leukocyte adhesion, H₂S inhibits the activity of the granulocyte enzyme myeloperoxidase (MPO) which catalyzes the conversion of hydrogen peroxide to hypochlorous acid and scavenges peroxynitrite, superoxide anion, hypochlorous acid and hydrogen peroxide [113].

Induction of neutrophil death and conversion of M1 macrophages to M2 by phagocytosis of dying neutrophil are critical for suppressing inflammation. H₂S stimulates both processes and causes a reduction in inflammatory response [114]. Exposure to H₂S also rendered macrophages hyporesponsive to inflammatory stimuli such as bacterial endotoxin or TNF- α [83]. Recently, it was shown that annexin- A1 serves as a pro-resolution mediator and H₂S treatment facilitates this process by translocating annexin-A1 from cytosol to plasma membrane [83]. In annexin-A1 deficient mice, where the expression of CBS and CSE was increased, granulocyte adhesion was inhibited along with a reduction in expression of COX-2 and inducible NO synthase in macrophages [115]. In GI tract, H₂S maintains mucosal integrity by regulating the expression of COX-2 and prostaglandin synthesis [116].

On the other hand, during chronic inflammation, H₂S suppresses upregulation of pro-inflammatory cytokines such as IL-1 β , tumor necrosis factor TNF- α , interferon IFN γ , IL-12, IL-23 and COX-2 and upregulates the expression of anti-inflammatory cytokines such as IL-10 [117, 118]. Although it is anticipated that activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) can explain the diversified role of H₂S in inflammation [119], the exact mechanism is still unknown.

Pathological perspective

Despite its role as an anti-inflammatory molecule, H₂S repairs tissue injury and restores tissue function [120]. As an example, H₂S treatment accelerated healing of ulcers in the stomach of rats, while suppression of H₂S synthesis resulted in a marked delay in healing [121]. This study was further supported by the fact that H₂S-releasing NSAID (ATB-346) provides faster healing of gastric ulcers compared to only NSAID treatment [120, 122]. This occurred despite the compound markedly suppressing gastric prostaglandin synthesis, which is believed to be responsible for COX-2-driven angiogenesis and repair. Thus, H₂S appears to have additional pro-healing mechanisms to those mediated via induction of COX-2 expression [2].

Bowel disease—The pathogenesis of inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis is mostly about an augmentation in the inflammatory response including an increase in the level of pro-inflammatory cytokines such as TNF α . Despite the fact that targeting TNF α provides an efficient therapeutic strategy, the adverse effect of this strategy raised significant concerns. Recently, mesalamine (5-aminosalicylic acid) has been considering for the treatment of IBD, but it has limited anti-inflammatory effects. However, a derivative of mesalamine which can release H₂S exhibits significantly enhanced anti-inflammatory, pro-resolution and pro-healing effects in rodent models of colitis by inhibiting tissue expression of a number of pro-inflammatory cytokines and chemokines (IL-1, IL-8, IL-12, TNF α , RANTES) [123, 124]. Similarly, nonsteroidal anti-inflammatory drugs have been using as anti-inflammatory drugs to reduce acute and chronic pain associated with osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis, however, long-term use of these compounds cause ulceration and bleeding in the GI tract. In contrast, H₂S-releasing NSAIDs provide anti-inflammatory effects by inhibiting COX-1 and COX-2 with similar potency as the parent NSAIDs but without ulceration and bleeding in the GI tract [125, 126].

Multiple sclerosis (MS)—MS is generally considered as an autoimmune disease affecting central nervous system and is characterized by dysfunctional immune system in damaging the brain, impairment of differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes to produce myelin, attenuation of the formation of fibronectin aggregates by astrocytes to inhibit scar formation, and downregulation of the function of healthy endothelial cells (ECs). H₂S upregulated proteins involved in immune system response and downregulated PBMNCs- and EC-related adhesion molecules (LFA-1 and VCAM-1) [127]. Furthermore, it had a cell expansion-inducing effect, altering EC morphology. The effects of H₂S on OPCs and astrocytes were studied compared to mTOR inhibitor rapamycin [127]. In NaHS treated astrocytes the induced fibronectin production

was partially inhibited while rapamycin almost fully inhibited fibronectin production [127]. NaHS slowed but did not inhibit the differentiation of OCPs or the production of myelin compared to rapamycin [127]. Thus these data suggest that H₂S may provide potential therapeutic application against MS.

Hydrogen sulfide on cell cycle

Physiological perspective

The cell cycle is the series of events that take place in a cell leading to its division and duplication of its DNA (DNA replication) to produce two daughter cells. In eukaryotic cells, the cell cycle includes four conventional phases: Gap phase 1 (G₁); DNA synthesis phase (S); Gap phase 2 (G₂), during which the cell prepares itself for division; and mitosis phase (M), during which the chromosomes separate and the cell divides. However, during the development of cancers, the cell cycle process is disrupted. The G proteins, G(s) (stimulatory) and G(i) (inhibitory), are involved in calcium regulation; overexpression has pathological consequences. Interestingly, G(s) G protein was downregulated in cardiac tissue of heterozygous CBS KO mice to 46% that of control hearts. However, the intracellular G(i) G protein content remained the same in heterozygous CBS KO mice [128]. H₂S treatment accelerates cell cycle progression in cell carcinoma through increasing phosphorylation of Akt and ERK [129]. Moreover, H₂S causes a decrease in the G₀-G₁ population and an increase in S phase by regulating protein expression of p21, one of the cyclin-dependent kinase (CDK) inhibitory proteins [130]. Moreover, depletion of CSE protein induces G₁/G₀ phase arrest and decreases the cell population in S phase in hepatoma cells but the process can be reversed by administration of H₂S [131, 132]. These data collectively suggest that H₂S is a pro-proliferative factor in human cancer cells partly by accelerating the progression of the cell cycle.

Cell cycle arrest can be classified into four groups, namely G₁-to-S, G₂-to-M, and M-to-G₁ and H₂S have shown to influence these processes in cancer cells. Treatment with SPARC blocks the proliferation and migration of cancer cells by inducing cell cycle arrest at G₁/S phase [133]. Moreover, administration of GYY4137 inhibits G₁/S cell cycle transition by causing downregulation of cyclin D1 [134]. Similarly, NaHS treatment induces G₁ arrest via up-regulation of CDK inhibitor p21Cip1 in colon cancer cells [135]. Interestingly, treatment with GYY4137 elicits G₂/M arrest in MCF-7 cells after 5–8 days of treatment [136, 137]. However, GYY4137 does not induce cell death indicating that the influence of H₂S on cell cycle is cell type specific but very selective.

H₂S is also involved in modulating cell proliferation of smooth muscle cells where G₁ phase are not affected by the treatment of H₂S and as a result, asynchronized cells become more sensitive than synchronized cells [138]. Other groups have shown that treatment with H₂S results in cell death via stabilization of p53 and an induction of p21 and Bax without affecting antiapoptotic protein level of Bcl2 [138]. However, in synchronized cells, H₂S treatment stimulates cell growth instead of cell death [139]. Interestingly, it was shown that H₂S treatment leads to cell death via DNA damage and alteration in the cell cycle [130]. Consistent with this result, H₂S caused free radical associated DNA damage in Chinese hamster ovary (CHO) cells which was evidenced by using single-cell gel electrophoresis

assay (comet assay) that quantitatively measures genomic DNA damage as single and double-strand breaks [140]. H₂S also functions as an inhibitor of CDK which is the key factor for cell cycle progression of the smooth muscle and lung fibroblast [141, 142].

Pathological perspective

Injury—Moreover, H₂S treatment influences cellular injury and apoptotic cell death induced by rotenone [143]. Treatment with H₂S alters the expression of extracellular signal-regulated kinase (ERK), p38 MAPK and p21 with down-regulation of cyclin D that leads to cell cycle arrest [144]. Other groups have shown that H₂S treatment induced cell death by inducing cytochrome C release and induction of Bax in cancer cells [145]. H₂S treatment causes an inhibition of cell proliferation at G1 phase and these cells were eliminated by the process of apoptosis as indicated by an increase in the number of cells arrested at sub-G1/S phase [49]. Activation of p53 and p21 will be followed by the up-regulation of key cell cycle proteins cyclin-E, CDC-6 (cell division cycle- 6) and decrease in cyclin D1 and p27 involved in cell growth after H₂S treatment suggesting that H₂S controls cell growth regulation. In keratinocytes, H₂S regulates cell proliferation and cell adhesion by influencing MAPK and ERK pathways and modulating the expression of β 4, α 2, and α 6 integrins [139]. Evidence suggests that aberrant p38 MAPK signaling undermines the repair process after injury in aged mice. An interesting study shows that CBS^{-/+} mice, exhibited compromised regenerative function and cell proliferation upon injury. However, there was no significant difference in Pax7 expression levels in the satellite cells from CBS^{-/+} mouse skeletal muscles. In addition, there was enhanced p38 MAPK activation as well as p16 and p21 expression in the CBS^{-/+} mouse satellite cells. Tissue engraftment potential and regeneration after injury were restored to some extent upon treatment with the p38-MAPK inhibitor, SB203580, in the CBS^{-/+} mice [146].

Cell death and H₂S

Physiological perspective

Apoptosis or programmed cell death is a highly regulated cellular suicide program that is critical for the several biological processes such as normal development and maintenance of tissue homeostasis in multicellular organisms. In tumor cells, apoptosis allows these cells to survive against oncogene activation, uncontrolled proliferation, and chemotherapy. It has been shown that H₂S signaling pathway is critical in maintaining the proliferation of hepatoma cells. Inhibition of this pathway prevents the growth of these cells may be due to mitochondrial apoptosis. In support of these studies, it was shown that treatment with NaHS augments cell viability in PLC/PRF/5 hepatoma cells [147]. Similarly, H₂S could inhibit the apoptotic effects in colon cancer cells induced by β -phenylethyl isothiocyanate (PEITC) [148]. Administration of 6-hydroxydopamine also leads to apoptosis in a human neuroblastoma cell line, SH-SY5Y, however, overexpression of CBS or treatment with NaHS reduced cell death [149]. Thus these data indicates that H₂S may serve as an anti-apoptosis agent in the development of diseases that involve excessive cell growth and division, such as cancer and neuroblastoma cells, but the mechanism is not clear. One recent study has shown that in the liver of CBS^{-/-} mice mitochondrial oxidative stress is reduced along with a reduction of a serine/threonine kinase, DYRK1A as an antiapoptotic factor to

counteract these pro-apoptotic signals [70]. Moreover, NaHS treatment also mitigated mitochondrial oxidative stress (NOX4, ROS, and NO) and restored ATP that indicates its protective effects against mitochondrial toxicity [150].

Pathological perspective

Cancer—The balance between the generation of cells and cell death is critical in multicellular organisms and disruption of this balance leads to cancer development. Thus the reactivation of cell death programs by exposure of H₂S could serve as an effective strategy for cancer prevention through increasing the apoptotic percentage of cancer cells such as CA9–22, but signs for apoptosis in normal keratinocytes are not observed [141, 151]. Similarly, overexpression of CSE induces cell death in human melanoma cells by suppressing the activity of nuclear factor- κ B (NF- κ B) and decreasing the expression of anti-apoptotic proteins. This result is in contrary with our results where we have shown that the antiapoptotic actions of NF- κ B are mediated by H₂S synthesized by CSE [28]. H₂S generated by CSE stimulates DNA binding and gene activation of NF- κ B, processes that are abolished in CSE deleted mice. H₂S acts by sulphydration the p65 subunit of NF- κ B, which promotes its binding to the coactivator ribosomal protein S3 (RPS3). Anti-apoptotic influences of NF- κ B, which are markedly diminished in CSE mutant mice [152] (Figure 3, box 3). Thus, sulphydration of NF- κ B appears to be a physiologic determinant of its anti-apoptotic transcriptional activity. These results suggest that the effectivity of H₂S on cell death depends on the context how cell death is induced and the nature of the cells which is functionally different from one to another. The administration of an H₂S donor, GYY4137 exhibit anticancer activities by stimulating cell death although it has no effect on cell survival of normal lung fibroblasts [153].

Diabetic cardiomyopathy (DCM)—An induction of ER-stress leads to an activation of protein kinase RNA-like ER kinase (PERK) by increasing its phosphorylation at Tyr916 residues. Activation of PERK affects protein synthesis by increasing the phosphorylation level of eukaryotic translational initiation factor 2 α (eIF2 α) [154–156]. The phosphorylation of PERK depends on the activity of phosphodiesterase, PTP1B. H₂S sulphydrates PTP1B (Figure 3, box 6) and impairs the catalytic activity of this enzyme [157]. As a result, the phosphorylation of PERK is increased and causes an induction of ER-stress inside cells. In contrary, it was shown that H₂S inhibits endoplasmic ER-Stress in a rat model of diabetic cardiomyopathy (DCM). H₂S exerts cardioprotective effects against AMI-induced apoptosis through the GSK-3 β / β -catenin signaling pathway [158]. NaHS significantly ameliorated hemorrhagic shock caused hemodynamic deterioration, decreased myocardial enzymes elevation, protected myocardial ultrastructure, and inhibited the expression of apoptosis-relevant proteins [159]. It suggested that H₂S might exert its cardioprotective roles via both the extrinsic Fas/FasL/caspase-8/caspase-3 pathway and the intrinsic mitochondria-involved pathways [159].

Ischemia/reperfusion (IR)—The protective effect of H₂S against myocardial ischemia/reperfusion (IR) injury via anti-apoptotic signaling is well established, but the underlying mechanism remains unclear. Recently, miRNAs have been identified as important mediators of myocardial injury by regulating apoptosis-related genes [160]. It was found that HR

injury increased apoptosis of cardiac myocytes, upregulated the expression of miR-1, and down-regulated the expression of Bcl-2 [160]. H₂S preconditioning attenuated cardiomyocyte apoptosis and LDH release, as well as enhanced cell viability following HR injury. MiR-1 was up-regulated by HR and down-regulated by H₂S preconditioning. H₂S also attenuated IR-induced cardiomyocyte apoptosis in vivo. MiR-1 regulated H₂S protection of cardiomyocytes against IR-induced apoptosis by stimulating Bcl-2. These results implicate miR-1 as an important regulator of H₂S on the IR myocardium [160].

Stroke—The N-methyl-D-aspartate receptor (also known as the NMDA receptor or NMDAR), is a glutamate receptor and ion channel protein found in nerve cells. It is activated when glutamate and glycine (or D-serine) bind to it, and when activated it allows positively charged ions to flow through the cell membrane [161]. The NMDA receptor is very important for controlling synaptic plasticity and memory function [162]. The cysteine disulfide bond at the hinge of the ligand binding domain of N-methyl daspartate (NMDA) receptors is important for its activity. However, it was discovered that the reduction of this bond by a reducing agent DTT [20] or treatment with NaHS enhances the activity of NMDA receptors [22, 163]. Thus the intracellular concentrations of H₂S and the redox balance is important to maintain the disulfide bond of NMDAR. Other reports have shown that NaHS improved cerebral energy metabolism after cerebral global ischemia and prolonged survival time of animals with acute cerebral anoxia. In cultured neurons, sodium sulfide increased cell viability and decreased cell apoptosis induced by oxygen-glucose deprivation [164]. Thus, NaHS presents protective effect on acute cerebral ischemia and might be a promising therapeutic drug [164]. Mechanistic studies demonstrated that NaHS-treated rats displayed a significant reduction of malondialdehyde content, and strikingly increased the activity of superoxide dismutases and glutathione peroxidase in the brain tissues compared with I/R group [165, 166]. DATS protects B35 neural cells against OGD-induced cell injury by inhibiting ROS production via upregulating the PI3K/Akt-mediated Nrf2 pathway, which further activates HO-1 [167]. Based on our results, DATS may be a potential candidate for intervention in hypoxic-ischemic brain injuries such as stroke. In addition, Na₂S also enhanced H₂S level and CSE expression associated with upregulation of SIRT6 expression and activity in OGD/R-stimulated brain endothelial cells, whereas CSE inhibitor DL-propargylglycine further deteriorated the decrease of SIRT6 expression and activity as well as the reduction of H₂S level and CSE expression caused by OGD/R [168]. Furthermore, SIRT6 knockdown abolished Na₂S-mediated CSE expression and cytoprotection action in OGD/R-stimulated cells. Na₂S protected brain endothelial cells against simulated ischemic injury through SIRT6-dependent mechanisms [168]. A recent study shows that a topical treatment with DL-propargylglycine (PAG, an inhibitor for cystathionine γ -lyase (CSE)) and aspartate (ASP, inhibitor for cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (CAT/3-MST)), but not O-(Carboxymethyl) hydroxylamine hemihydrochloride (CHH, an inhibitor for cystathionine β -synthase (CBS)), abolished postischemic cerebral vasodilation/hyperemia. Moreover, CSE knockout (CSE^{-/-}) reduced postischemic cerebral vasodilation/hyperemia along with an increase in H₂S production in ischemic side of the cerebral cortex [169].

Injury—H₂S attenuates brain edema in early brain injury after subarachnoid hemorrhage in rats and this could be due to an involvement of MMP-9 induced blood-brain barrier disruption and AQP4 expression [170]. NaHS treatment significantly improved brain edema and neurobehavioral function, and attenuated neuronal cell death in the prefrontal cortex, associated with a decrease in Bax/Bcl-2 ratio and suppression of caspase-3 activation after SAH [171].

Alzheimer's Disease (AD)—Beta-amyloid (A β), a neurotoxic peptide, accumulates in the brain of Alzheimer's disease (AD) subjects to initiate neuroinflammation eventually leading to memory impairment. NaHS exerted a beneficial effect on inhibition of I κ B- α degradation and subsequent activation of transcription factor nuclear factor κ B (NF- κ B), as well as inhibition of extracellular signal-regulated kinase (ERK1/2) activity and p38 MAPK activity but not c-Jun N-terminal kinase (JNK) activity induced by A β [172]. These results demonstrate that NaHS might be a potential agent for treatment of neuroinflammation-related AD. Exogenous stress-induced neuronal cell death in the hippocampus is closely associated with the pathogenesis of depression which is related to the disturbance of endogenous H₂S generation and ER stress in the hippocampus and suggested that endogenous H₂S and ER stress are novel treatment targets of depression [173, 174]. In addition, cytoprotective effect of H₂S-releasing NMDAR antagonists correlated with their ability to increase intracellular sulfane sulfur, but not H₂S, levels [175]. These studies suggest that H₂S-donor compounds that increase intracellular sulfane sulfur are potentially useful neuroprotective agents against neurodegenerative diseases.

Parkinson's Disease (PD)—Another study shows that NaHS administration improves the survival rate and significantly ameliorates the weight loss of MPTP-treated mice [176]. NaHS treatment attenuated MPTP-induced neuronal damage, restored the diminution of DA neurons, and suppressed the overactivation of astrocytes in the mouse striatum [176]. Additionally, NaHS upregulated striatal serotonin levels and modulated the balance of excitatory glutamate and the inhibitory γ -aminobutyric acid system in response to MPTP challenge. The current study indicates that H₂S may function as an effective neuromodulator to regulate striatal neurotransmission and provides insight into the potential of H₂S for PD therapy. Recently it was shown that an E3 ligase, parkin protein is sulfhydrated at the physiological conditions and enhances its catalytic activity [177] (Figure 3, box 2). However, the level of sulfhydration of parkin is decreased in the brains of patients with Parkinson's disease and causes an accumulation of parkin targets such as α -synuclein, a constituent of the characteristic Lewy bodies, suggesting that this loss may be pathologic [177]. H₂S exerts neuroprotection against hypoxia-induced neurotoxicity through its anti-inflammatory effect in microglia. This effect appears to be attributable to inhibition of iNOS, NF- κ B, ERK and p38 MAPK signaling pathways. These results suggest a potential therapeutic application of H₂S releasing drugs in hypoxic brain damage treatment.

Induction of a proinflammatory cytokine, interleukin-1 β (IL-1 β) plays a role in memory impairment associated with various neurological disorders such as AD and PD and brain injury. Previously we have shown that IL-1 β -induced memory impairment in the brain is mediated by H₂S synthesized by CBS. H₂S modifies GAPDH essentially via sulfhydration

in dendrites, which promotes its binding to the E3 ligase protein, Siah. Then Siah binds to a critical synaptic scaffolding molecule, PSD95, and leads it to degradation via ubiquitination. In CBS^{-/-} mice, IL-1 β -induced loss of PSD95 was rescued significantly. Thus, our findings reveal a mechanism where GAPDH sulfhydration appears to be a physiologic determinant of cytokine-induced memory impairment in the brain [27] (Figure 3, box 4).

A crosstalk between Nitric oxide and hydrogen sulfide

Nitric Oxide (NO) is a well-known gaseous transmitter and is synthesized by the enzymatic activity of nNOS, eNOS and iNOS. Thionitrous acid (HSNO), a potential key intermediate in biological signaling pathways, has been proposed to link NO and H₂S biochemistries, but its existence and stability in vivo remain controversial. It was shown that HSNO is spontaneously formed in high concentration when NO and H₂S gases are mixed at room temperature in the presence of metallic surfaces [178]. In vivo, HSNO can be formed if H₂S reacts with S-nitrosothiols [179]. At the cellular level, HSNO can be metabolized to afford NO(+), NO, and NO(-) species, all of which have distinct physiological consequences of their own. HSNO can freely diffuse through membranes, facilitating transnitrosation of proteins such as hemoglobin. Thus these data introduce a new signaling molecule, HSNO, and suggest that it may play a key role in cellular redox regulation [180]. An interesting study shows that physiological concentrations of H₂S abrogated peroxynitrite-induced cell damage as demonstrated by the: (i) inhibition of apoptosis and necrosis caused by peroxynitrite; (ii) prevention of protein nitration; and (iii) inhibition of PARP-1 [poly(ADP-ribose) polymerase 1] activation in cellular models. This implies that a major part of the cytoprotective effects of H₂S may be mediated by modulation of peroxynitrite chemistry, in particular under inflammatory conditions [181]. Glutamate-induced neurotoxicity involves in overproduction of nitric oxide (NO) and oxidative stress. Treatment with NOS inhibitor, ADMA suppressed glutamate-induced neurotoxicity is by promoting endogenous H₂S generation, resulting from suppression in NOS excessive activation and NO overproduction. These findings provide a novel mechanism underlying the protection of ADMA against glutamate-induced neurotoxicity [182]. H₂S is known to inhibit high glucose-induced protein synthesis in kidney podocytes. It was shown that treatment with tadalafil, a phosphodiesterase 5 inhibitor ameliorates high glucose stimulation of matrix proteins by generating H₂S in podocytes through NO-H₂S-AMPK axis [183]. Moreover, the anti-thrombotic efficacy of H₂S involves the NOS-pathway and may be of preventive and therapeutic value for clinical disorders with increased risk of thrombotic events [184].

The interplay between sulfhydration and nitrosylation has well been elicited in studies related with TNF α -induced transcriptional activation of the p65 subunit of NF κ B [108]. In this study it was shown that nitrosylation of p65 is followed by sulfhydration of p65; however nitrosylation of p65 inactivates transcriptional activity of p65 although sulfhydration of p65 stimulates its activity. Upon sulfhydration of p65, it binds with RPS3 and facilitates its transcriptional activity. The other great example of how sulfhydration and nitrosylation affect functional aspects of protein is GAPDH. Nitrosylation of GAPDH leads to cell death by activating p53 through autoacetylation of acetyltransferase p300/CBP [185]; however, sulfhydration of GAPDH prevents its interaction with p300/CBP but induces degradation of PSD95 and facilitates memory impairment [27].

Concluding remarks

Several years ago when nitric oxide was discovered as a gasotransmitter, several investigators had appreciated its importance in both physiology and pathological conditions. However, H₂S is comparatively newly identified exciting gasotransmitter and have already shown its influence in several signaling pathways which are critical for biological processes as discussed here. Unlike nitric oxide, H₂S present as a bound form such as sulfane sulfur and free form inside cells. Most importantly, the discovery of sulfhydration of proteins provides an edge to the field to explain the functions of H₂S in biological processes. Sulfhydration has been proposed as a mode of action of H₂S that causes conformational changes in the target proteins to modify their activity. Interestingly under oxidative conditions cysteine residues are oxidized (SOH, -SO₂H and SO₃H) to cysteine sulfenic acid and in the presence of NO they are oxidized to cysteine S-nitrosothiol (-SNO). The red maleimide assay can detect the sulfhydration of protein, however in a combination of green maleimide the assay can also differentiate the nitrosylated species from sulfhydrated species in vitro and in vivo [28]. The oxidized species will be unable to interact with red maleimide, thus this assay can exclude the oxidized species from the same sample.

The current literature suggest that H₂S provides a promising target for therapeutic intervention. One possible option would be either activate or inhibit CSE/CBS to influence the related signaling pathways. However, it is important to keep in mind that modulation of the physiological level of H₂S may cause adverse effects than anticipated. Moreover, the specificity and selectivity of these compounds are in question too. Instead, targeting the sulfhydrated proteins may provide more specificity towards therapeutic intervention based on H₂S signaling although this could be more challenging.

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Research Highlights

- H₂S, a newly discovered gaso-neurotransmitter, signals inside cells mostly via sulfhydration of proteins.
- Modulation of autophagy by H₂S signaling pathway is implicated in ischemia, myocardial fibrosis, metabolic disorders and TBI.
- H₂S affects cellular metabolism which affects tumor growth and angiogenesis.
- The maintenance, proliferation, and differentiation of stem cells are regulated by H₂S.
- The influence of H₂S signaling in cell cycle and cell death pathways is associated with cancer and neurodegenerative diseases.

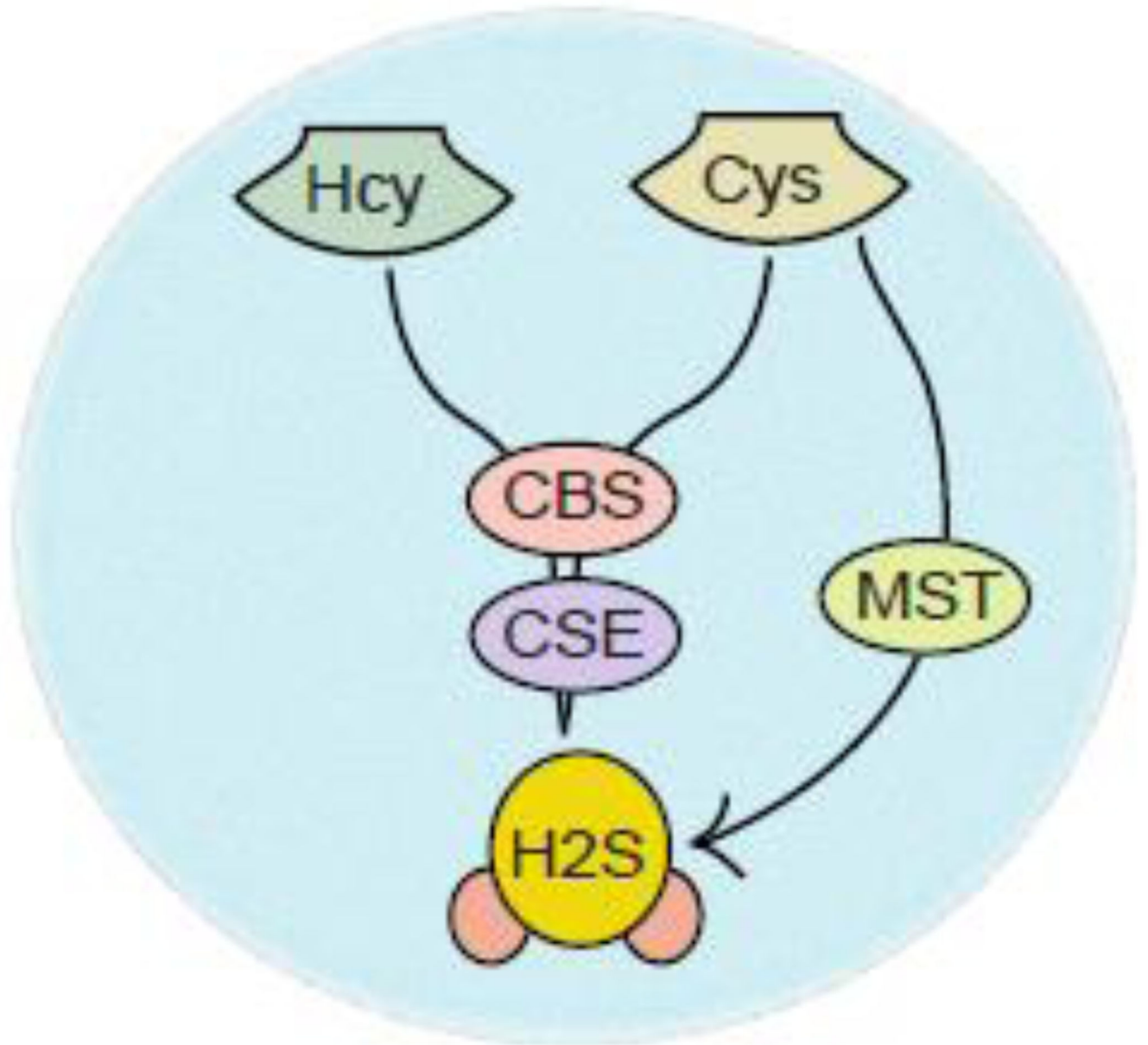


Figure 1.

Intracellular synthesis of H₂S by CBS, CSE and MST. L-cysteine serves as a substrate for all three enzymes to produce H₂S in cells; however, homocysteine can be used as a substrate to synthesize H₂S by the enzymatic activity of CSE and CBS.

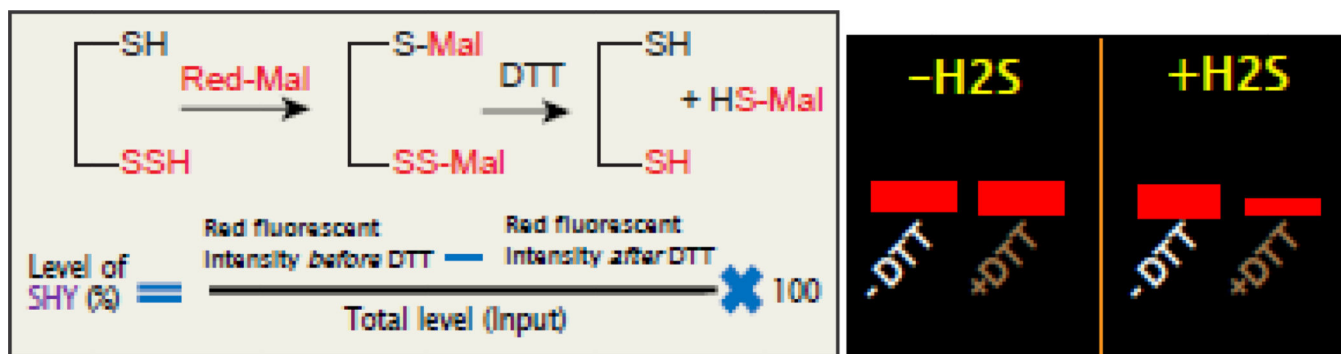


Figure 2.

The principle of red maleimide assay to detect sulfhydrylation of proteins. In this method, red maleimide interacts with both -SH and -SSH groups and forms -S-Mal and -S-S-Mal respectively. Upon treatment with DTT, the -S-Mal will remain unaltered, however, -S-S-Mal will be cleaved off to form -SH and -S-Mal as the products. Thus the sulfhydrated protein will lose the signal of red fluorescence of red maleimide. A cartoon image (right side) of a gel showing that the red intensity of a target protein remains unaltered in the presence or absence of DTT in samples untreated with H₂S. On the other hand, the red fluorescent intensity of protein will be lost after DTT treatment in samples treated with H₂S. The residual red fluorescent intensity before and after DTT treatment of protein will provide the percentage of sulfhydrylation of protein.

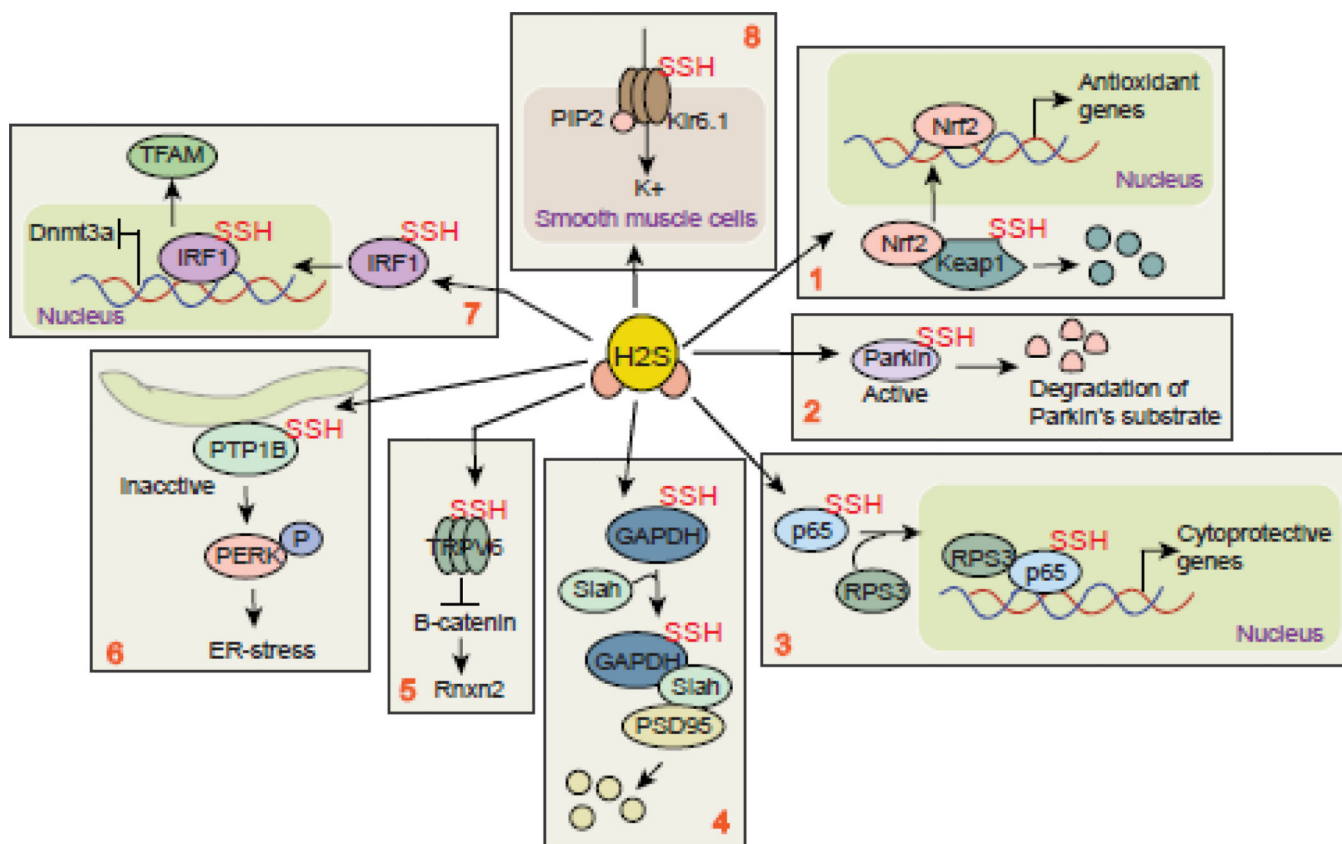


Figure 3. Sulphydration of proteins in general. Box 1: Sulphydration of Keap1 leads Nrf2 to translocate to the nucleus and synthesize antioxidant genes. Box 2: sulphydration of a E3 ligase, Parkin protein leads to degradation of its substrates and provides neuroprotection. Box 3: sulphydration of p65 (NFkB) stimulates its transcriptional activation after its interaction with RPS3 protein. Box 4: sulphydration of GAPDH leads to degradation of PSD95 after its interaction with a E3 ligase Siah protein. This event leads to memory impairment following an induction of a proinflammatory cytokine, IL-1 β . Box 5: sulphydration of a channel protein TRPV6 regulates maintenance of stem cell function via modulating β -catenin inside cells. Box 6: Sulphydration of PTP1B induces PERK phosphorylation and triggers ER stress which has been implicated in several neurodegenerative diseases. Box 7: Sulphydration of IRF1 serves as a transcriptional suppressor and facilitates the transcriptional activation of TFAM which is important for mitochondrial DNA compaction.

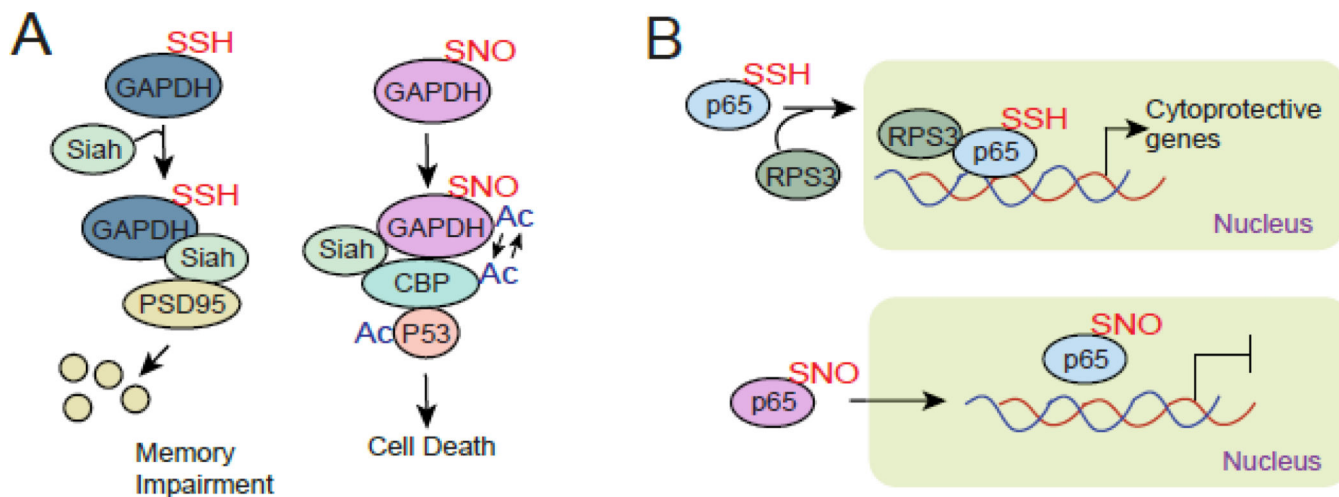


Figure 4. Two contrasting examples how nitrosylation and sulfhydrylation of proteins affect the biological processes. (A) sulfhydrylation of GAPDH leads to degradation of PSD95 via Siah. In contrast nitrosylation of GAPDH leads to cell death via activating p300/CBP and p53 signaling axis. (B) sulfhydrylation of p65 interacts with RPS3 and facilitates transcriptional activation of p65. On the other hand, nitrosylation of p65 prevents its transcriptional activation.