J Mol Med (2012) 90:255–263
DOI 10.1007/s00109-012-0873-4

REVIEW

Hydrogen sulfide: a gasotransmitter of clinical relevance

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Received: 15 December 2011 / Revised: 22 January 2012 / Accepted: 26 January 2012 / Published online: 8 February 2012
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Abstract Though the existence of hydrogen sulfide (H_2S) in biological tissues has been known for over 300 years, it is the most recently appreciated of the gasotransmitters as a physiologic messenger molecule. The enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) had long been speculated to generate H_2S, and inhibitors of these enzymes had been employed to characterize influences of H_2S in various organs. Definitive evidence that H_2S is a physiologic regulator came with the development of mice with targeted deletion of CSE and CBS. Best characterized is the role of H_2S, formed by CSE, as an endothelial derived relaxing factor that normally regulates blood pressure by acting through ATP-sensitive potassium channels. H_2S participates in various phases of the inflammatory process, predominantly exerting anti-inflammatory actions. Currently, the most advanced efforts to develop therapeutic agents involve the combination of H_2S donors with non-steroidal anti-inflammatory drugs (NSAIDs). The H_2S releasing moiety provides cytoprotection to gastric mucosa normally adversely affected by NSAIDs while the combination of H_2S and inhibition of prostaglandin synthesis may afford synergistic anti-inflammatory influences.

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Keywords Sulfhydration · Cardio protection · Inflammation · Cystathionine γ-lyase · Cystathionine-β-synthase

Introduction

Hydrogen sulfide (H_2S) is the most recently appreciated of the three gasotransmitters, joining nitric oxide (NO), and carbon monoxide (CO). Though only recently recognized as being physiologically formed in mammalian tissues, H_2S has been known to exist in animal tissues for many years. Like NO and CO, H_2S is toxic, about five times more so than CO [1]. Recently, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) have been established as the major physiologic sources of mammalian H_2S based on studies showing that their deletion or inhibition markedly diminishes mammalian H_2S levels [2, 3]. As with NO and CO, identification of the biosynthetic enzymes now provides a firm basis for elucidating how H_2S is produced, signals to intracellular targets, and affects diverse physiologic processes. In the interest of brevity, the review will be limited to a few areas of H_2S disposition: focusing on physiologic roles in the cardiovascular system and inflammation and a brief discussion of regulatory mechanisms and signaling modalities.

Understanding the disposition of H_2S can be facilitated by comparisons with NO and CO. NO was identified as endothelial-derived relaxing factor and as regulating macrophage function years before the first NO synthase (NOS) was purified and cloned [4, 5]. NO is formed by a family of NO synthase isoforms. Cloning and characterization of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) greatly facilitated research in the field and led to a vast expansion of NO literature [6]. The NOS isoforms are all heme-requiring enzymes, a property they share with CBS.

nNOS and eNOS are constitutively expressed but activated in response to Ca^{2+}/calmodulin [7], which also activates CSE [8].

NO relaxes blood vessels by binding to heme in the active site of guanylyl cyclase to facilitate formation of cyclic GMP which, via protein kinase G, relaxes blood vessels [9]. *S*-nitrosylation is a more prominent and ubiquitous physiological signaling mechanism for NO whereby NO reacts with the SH group of cysteines in target proteins to inhibit or activate them [10]. As described below, H_2S appears to signal predominantly by an analogous mechanism—sulfhydration of target proteins, whereas no major action via cyclic nucleotides has been reported for H_2S [11].

nNOS is highly localized to discrete neuronal systems in the brain and to autonomic nerves in the periphery [12]. eNOS occurs in the endothelial layer of blood vessels and the respiratory system, while iNOS occurs in all cells of the body, but is notably enriched in macrophages [4]. Localizations of CBS and CSE are less well characterized, though the enzymes are highly expressed in liver and kidney and at lower levels in pancreas, adipose tissue, small intestine and brain [13]. In the brain, CBS is largely glial while CSE occurs in neurons and endothelial cells [14, 15].

CO also displays some analogies to NO. It is generated by two isoforms of heme oxygenase (HO) with HO1 being inducible, similar to iNOS while HO2 is constitutive. Like nNOS and eNOS, HO2 is activated by calcium/calmodulin [16]. HO2 is highly localized to neurons in the brain and the periphery and fulfills many characteristics of a neurotransmitter [17]. In the intestine HO2 and nNOS are co-localized in myenteric neurons where both appear to serve as neurotransmitters of non-adrenergic-non-cholinergic neurotransmission [18, 19]. As related below, there is some evidence for myenteric localization of CSE which might fulfill similar functions as the other two gasotransmitters in the gut. A conjunction of all three gasotransmitters occurs in the carotid body, where nNOS is expressed in nerve fibers, HO2, and CSE colocalize in glomus cells and regulate carotid body afferent discharge in response to hypoxia [20].

H_2S metabolism

There has been much controversy over endogenous, mammalian levels of H_2S, and the extent to which the gas derives from exogenous sources or reflects influences of biosynthetic enzymes (Figs. 1 and 2). Estimates for H_2S concentrations have ranged from the high micromolar to the low nanomolar with recent appreciation that physiologic levels are probably relatively low [21]. A major confounding factor in H_2S measurement has been the large endogenous stores of sulfane sulfur, which is artifactually reduced to H_2S during assays [22, 23]. Another difficulty relates to sensitivity and specificity of the various techniques employed to measure H_2S. Recently, several groups have developed fluorescent probes which may be substantially more sensitive and may permit imaging of H_2S in intact cells [24, 25].

CBS and CSE, the enzymes generally acknowledged as the principal sources of physiologic mammalian H_2S, were both first known as participants in metabolism of cystathionine, which is formed by CBS via the condensation of homocysteine with serine to generate cystathionine as a thiol ether. The markedly elevated levels of homocysteine in patients with homocystinemia, a genetic deletion of *Cbs*, lead to substantial cardiovascular disability [26]. CBS forms H_2S from cysteine or homocysteine with a combination of the two substrates providing maximal yields in vitro [27]. Inhibitors of CBS, such as hydroxylamine or amino-oxyacetate impair the generation of H_2S from cysteine in the brain, but they are non-specific, affecting all pyridoxal phosphate enzymes. Moreover, because the K_m of CBS for cysteine and homocysteine is 3–7 mM, high concentrations of these amino acids are employed in studies of H_2S formation, whereas physiologic levels are less than 10% of the K_m values [27].

The heme in CBS binds CO with high affinity, at least 100 times that of NO [28]. Hence, CO appears to be a physiologic inhibitor of CBS, which, as described below, may account for vasodilation of the cerebral circulation. CBS is also activated by *S*-adenosyl methionine, whose function is unclear but might reflect some relationship between signaling by H_2S and biologic methylation [29].

CSE was first characterized as cystathionase, responsible for the pyridoxal phosphate dependent hydrolytic degradation of cystathionine [30]. CSE was proposed as a physiologic generator of H_2S in peripheral tissues such as the liver, because inhibitors, such as propargylglycine and β-cyanoalanine, diminish H_2S formation. While these inhibitors are relatively non-selective, more recent studies of CSE-deleted mice have definitively established that CSE is the predominant source of H_2S in peripheral tissues [8]. Evidence supporting CSE as generating H_2S for signaling purposes comes from the finding that CSE, like nNOS, eNOS, and HO2, is activated by calcium/calmodulin [8].

Less well characterized than CBS and CSE as a source of H_2S in mammalian tissues is the enzyme 3-mercaptyopyruvate sulfotransferase (3-MST). Kimura and associates [23] developed evidence that 3-MST acts in conjunction with cysteine aminotransferase (CAT) to produce H_2S from cysteine in the presence of α-ketoglutarate. The combination of 3-MST and CAT might be responsible for the generation of H_2S in brain preparations from CBS-deleted mice (Table 1). Because 3-MST is maximally active at very high pH levels, it is not clear to what extent it is responsible for mammalian formation of H_2S.

CBS, CSE, and 3-MST appear to be highly conserved, with the sequences of bacterial forms of these enzymes fairly similar

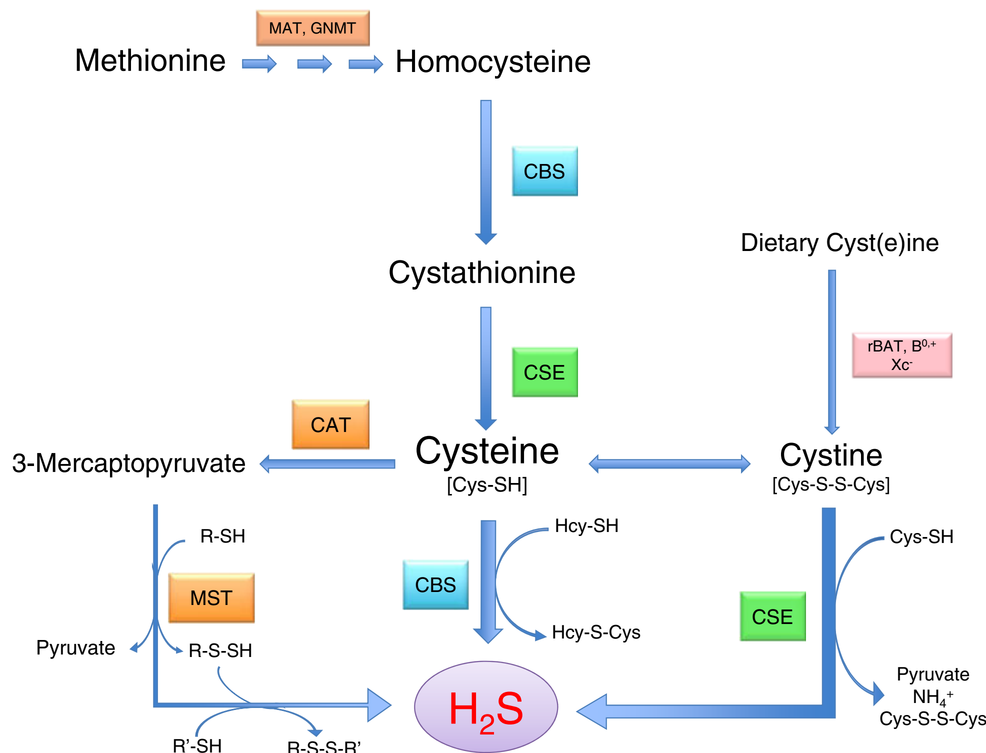

Fig. 1 Pathways of H_2S metabolism. Cysteine metabolism from methionine and dietary cyst(e)ine, which enter cells via specific transporters, leads to H_2S production. H_2S is derived from cysteine, cystine, and 3-mercaptopyruvate (*3MP*). 3-Mercaptopyruvate sulfurtransferase (*3MST*) and 2-cysteine aminotransferase (*CAT*) produce H_2S and pyruvate from 3MP, which is formed from cysteine and α-ketogluterate produced by CAT. Cystathionine-β-Synthase (*CBS*) catalyzes the β-replacement of cysteine with homocysteine (*Hcy*) to generate H_2S and the corresponding thiol ether (*Hcy-S-Cys*). Cystathionine-γ-lyase (*CSE*) catalyzes β-disulfide elimination on cystine, the product of which reacts with available thiols (Cys is shown) to generate H_2S and a disulfide (*Cys-S-S-Cys*)

to mammalian isoforms. Very recently, Nudler and associates [31] have discovered that H_2S is critical for the survival of bacteria and that a wide range of antibiotics, whose initial targets vary markedly, all act via H_2S as a final, common pathway. Thus, bacteria with deletion of the H_2S forming enzymes are markedly more sensitive to antibiotic killing. This discovery may portend a new class of antibiotic-sensitizing drugs that lower the bactericidal concentrations of antibiotics.

H_2S signaling

Unlike NO and CO, H_2S does not appear to stimulate guanylyl cyclase [32], even though it can bind with reasonably high affinity to heme containing domains like that found in guanylyl cyclase. H_2S has been shown to signal via a mechanism analogous to nitrosylation whereby it forms a covalent linkage to the SH of cysteines, a process designated sulfhydration [11]. Sulfhydration was first detected by the biotin switch assay employed to monitor nitrosylation. In this procedure free thiols are blocked by methyl methane thiosulfonate (MMTS). The SH groups of nitrosylated cysteines can then be exposed by treatment with ascorbate and subsequently labeled and identified [33]. Even in the absence of ascorbate, some proteins are labeled by the biotin switch technique, which provided a clue to the existence of sulfhydration. Accordingly, sulfhydration can be detected in a modification of the biotin switch procedure with omission of the ascorbate step [11].

Recently, sulfhydration has been monitored by a new technique which overcomes concerns that some free thiols might not be blocked by MMTS [34]. The newer procedure employs a fluorescent maleimide derivative, which interacts selectively with sulfhydryl groups of cysteines, both sulfhdyrated and non-sulfhydrated. Treatment of samples with dithiothreitol selectively cleaves disulfide bonds, detaching the fluorescent signal from sulfhydrated but not non-sulfhydrated proteins and leading to decreased fluorescence [34]. This technique can be modified to simultaneously detect nitrosylation using a differently colored fluorescent maleimide after treatment with ascorbate to remove NO from nitrosylated cysteines, exposing previously nitrosylated SH groups [34].

Sulfhydration appears to be substantially more prevalent than nitrosylation. Whereas nitrosylation typically affects only about 1–5% of most proteins, 10–25% of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin and actin are basally sulfhydrated [11].

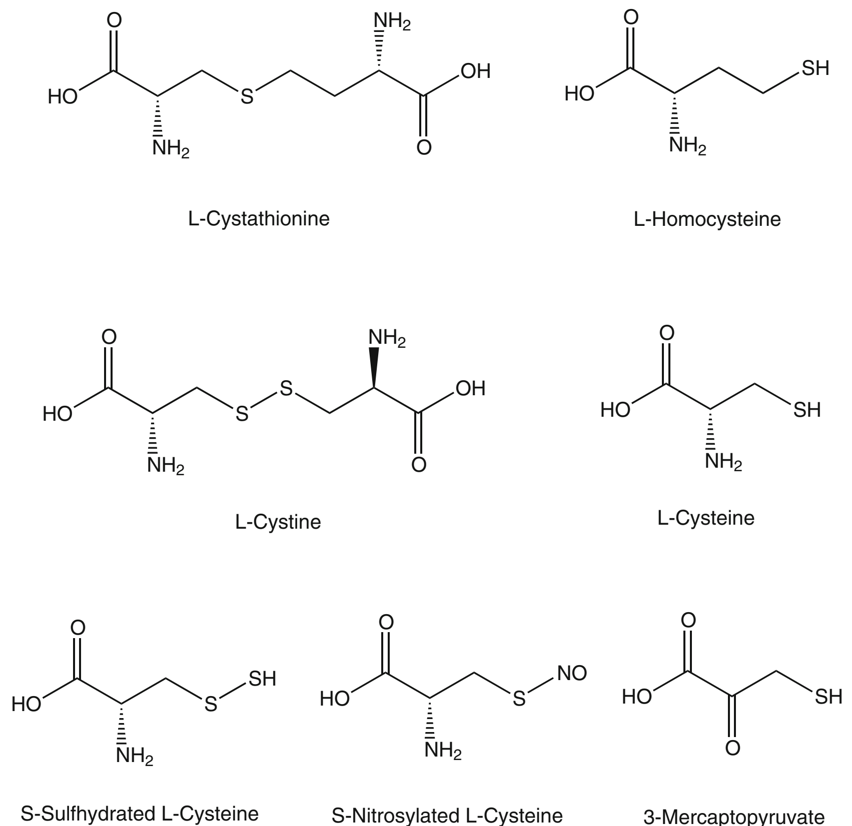

Fig. 2 Structures of molecules involved in H_2S physiology

Sulfhydration can influence protein function differently than nitrosylation. Nitrosylation provides an NO "cap" to reactive SH groups of cysteines typically inactivating proteins, though in some instances it has been shown to have an

Table 1 Models of inflammation

Model	Donor/inhibitor/KO mouse	References
Hypertension: CSE −/− mice	Age-dependent hypertension observed beginning at 7 weeks cholinergic relaxation of mesenteric artery reduced 75–80% in CSE −/− mice	[8]
Ischemia reperfusion injury (rat, mouse, and pig)	NaHS and H_2S donors reduced myocardial infarct size in rat, mouse, and pig models while PAG attenuated this effect and increased infarct size	[45–48, 50]
ischemia reperfusion injury: perfused rabbit heart	H_2S releasing NSAID *S*-diclofenac was found to protect against ischemia-reperfusion injury in isolated rabbit heart	[53]
Burn injury-induced inflammation in mouse	Prophylactic and therapeutic administration of PAG reduced burn-associated systemic inflammation while NaHS was found to increase systemic burn-associated inflammation	[56]
LPS-induced lung and liver inflammation in mouse	NaHS administration resulted in marked increase in lung inflammation and MPO activity in the liver and lung as well as increased TNF levels while PAG exhibited reduced lung and liver MPO activity and ameliorated lung and liver tissue damage	[55]
Mouse airpouch model	NaHS and other donors suppressed leukocyte infiltration which was enhanced under the use of endogenous H_2S inhibitors	[59]
Carrageenan-induced paw edema: mouse	NaHS and other donors suppressed carrageenan-induced paw edema in the mouse to a level similar to KATP channel agonist .	[59, 62]
Carrageenan-induced joint synovitis model: rat	Treatment with H_2S donor Lawesson's reagent attenuated pain response and all inflammatory biochemical changes whereas PAG potentiated synovial iNOS activity and enhanced macrophage infiltration	[61]
TNBSA-induced mouse model of colitis	H_2S donating mesalamine derivative ATB-429 was found to reduce trinitrobenzene sulphonic acid induced colitis severity and granulocite infiltration by 70%	[63]
NSAID-induced gastropathy: rat	NaHS and *S*-diclofenac were found to reduce NSAID-induced gastric injury and to decrease inflammatory mediators TNF, Cox2, and ICAM1 while H_2S inhibitor BCA increased these mediators and enhanced gastric injury	[52, 65]

BCA β-cyanoalanine, *CSE* cystathionine γ-lyase, *iNOS* inducible NOS, *NSAID* non-steroidal anti-inflammatory drug, *PAG* propargylglycine, *TNF* tumor necrosis factor

activating effect [35]. By contrast, in sulfhydration, an SH is converted to SSH which, with its lower pK_a, is more reactive chemically than SH and may have greater exposure to the cellular environment. This notion is substantiated by the finding that sulfhydration of GAPDH increases catalytic activity 700%, and sulfhydration of actin similarly enhances biologic activity. Activation of GAPDH by sulfhydration is physiologically relevant, as total GAPDH activity of liver extracts is reduced about 25–30% in CSE deleted mice despite normal levels of GAPDH protein [11]. It appears that, as with nitrosylation, many, if not most, proteins are sulfhydrated.

Physiologic actions of H_2S

Cardiovascular system

Like NO and CO, H_2S dilates blood vessels. Studies with exogenous H_2S largely report vascular relaxation, though under some conditions, such as high oxygen concentration, vasoconstriction is evident. NO was first elucidated as endothelial derived relaxing factor (EDRF). Investigations employing eNOS knock-out mice and NOS inhibitors reveal only a partial reduction of EDRF activity in certain vascular beds [8, 36, 37]. EDRF activity in HO2 knockout mice has not yet been reported. Studies of H_2S in CSE knockout mice indicate a major contribution to EDRF activity [8]. Immunohistochemical analysis shows that CSE is highly localized to the endothelial layer of blood vessels. Cholinergic relaxation of the mesenteric artery is reduced by about 75–80% in homozygous CSE deleted mice and about 50% in heterozygotes. This cholinergic relaxation reflects EDRF activity being abolished by removal of the endothelium. CSE knockout mice develop age-dependent hypertension with maximal increases in blood pressure of about 20 mmHg, similar to levels of hypertension in eNOS knockouts [8].

The EDRF activity associated with NO is most evident in large vessels such as the aorta, while in the resistance vessels that are the primary determinants of blood pressure, actions of NO are less prominent. In the mesenteric artery, a resistance vessel, H_2S is predominant [8]. Relative roles of H_2S, NO, and CO in various vascular beds may be elucidated by systematic comparison of mice with deletion of HO2, eNOS, or CSE.

NO and H_2S differ markedly in mechanisms whereby they influence blood vessels. NO and CO stimulate cyclic GMP levels while recent studies indicate that H_2S vasodilation largely reflects hyperpolarization elicited by opening ATP-sensitive potassium channels (K_{ATP}) [38–40]. While vasorelaxation by exogenous H_2S has long been known to involve such channels, recent work establishes that physiologic vasorelaxation is mediated by H_2S. Thus, glibenclamide, a potent and selective inhibitor of the K_{ATP} channel, reduces effects of H_2S and diminishes cholinergic hyperpolarization of mesenteric arterial smooth muscle by about 70% while not affecting relaxation elicited by NO donors [40].

H_2S stimulates K_{ATP} channels by sulfhydrating them at cysteine-43. These channels are activated physiologically when bound by phosphatidylinositol(4,5)bisphosphate (PIP2). The binding of PIP2 to K_{ATP} channels is abolished in cells devoid of CSE or containing a catalytically inactive form of the enzyme. Moreover, H_2S donors substantially enhance the binding of PIP2 to K_{ATP} channels, and PIP2 binding occurs at the sulfhydrated cysteine-43 [40].

The observation that H_2S physiologically acts by sulfhydrating and activating the K_{ATP} channel supports the notion that H_2S is a major if not predominant mediator of EDRF activity. Numerous investigators have found much if not most EDRF activity involves cGMP-independent blood vessel hyperpolarization [37] implying that EDRF is primarily dependent upon an endothelial-derived hyperpolarizing factor whose activity is largely attributable to H_2S .

The major role of H_2S in regulating the peripheral circulation suggests that it may be the principal vasoactive gasotransmitter, implying therapeutic relevance. This notion is supported by the limited success of studies devoted to inhibiting or enhancing NO formation respectively to combat endotoxic shock or to treat hypertension [41].

H_2S may also impact the cerebral circulation. Hypoxia is well known to stimulate cerebral blood flow, but underlying molecular mechanisms have been elusive. Very recently, CSE has been identified as a major regulatory factor for cerebral arteriolar vasodilation, acting in conjunction with CO formed by HO2 [42, 43], similar to that seen in the carotid body [20]. HO2 is an established physiologic O_2 sensor, especially in the carotid body where it is exquisitely sensitive to oxygen and is inhibited by hypoxia in a precisely graded fashion [44]. At physiologic concentrations, CO inhibits CBS, the predominant generator of H_2S in the cerebral circulation [29]. Thus, by inhibiting HO2, hypoxia would lead to activation of CBS and generation of H_2S as a vasorelaxant.

Before endogenous H_2S was shown to regulate blood vessels, exogenous H_2S had been shown to exert beneficial cardiovascular actions. Many studies have dealt with myocardial ischemia, which is substantially diminished by administration of H_2S donors during ischemia/reperfusion of the heart [45–47]. Numerous mechanisms had been proposed for these cardioprotective actions [48, 49]. Particularly promising is evidence that H_2S acts by inhibiting apoptosis, as H_2S donors reproducibly diminish poly (ADP-ribose) polymerase cleavage, as well as cleavage of caspase-3 [50]. H_2S also preserves mitochondrial structure and function in response to myocardial ischemia. H_2S may also be cardioprotective by decreasing the “work” of the

heart, analogous to beta-blockers, through diminishing contractility of cardiac myocytes, largely by inhibiting L-type calcium channels [51].

Because of the promising cardiovascular actions of H_2S a variety of drugs have been developed based on this gasotransmitter. Some are simple H_2S donors, such as GYY4137, while others combine an H_2S donating structure with an anti-inflammatory drug such as diclofenac or a classical vasodilator such as sildenafil [52–54].

H_2S and inflammation

The literature on NO, CO, and H_2S has been plagued with conflicting claims for their effects. Nowhere has this been most evident than with H_2S and inflammation. Prominent pro-inflammatory effects have been reported in association with increased formation of sulfide in neutrophils as well as activation of these cells [55]. Administration of H_2S donors has been reported to accentuate inflammatory factors associated with burns, while burn injuries were reduced by treatment by the CSE inhibitor propargylglycine [56] (Table 1). Lung injury elicited by bacterial sepsis can be alleviated by treatment with propargylglycine and worsened with H_2S donors [55]. By contrast, there are numerous reports of anti-inflammatory effects for H_2S donors as described below. A consensus has emerged in recent years that the apparently contradictory findings largely reflect variations in dose–response relationships. At relatively low, physiologic concentrations H_2S appears to be anti-inflammatory, while high concentrations elicit inflammation, a pattern reminiscent of NO, which is anti-inflammatory in low concentrations and pro-inflammatory at high levels. CO, well known to be lethal in high doses, is also often beneficial when administered in low doses [57, 58].

What physiologic mechanisms underlie influences of H_2S on inflammation? One of the best characterized involves the disposition of leukocytes, especially their adherence to vascular endothelium as well as their extravasation. H_2S donors and sulfide salts diminish lymphocyte and neutrophil infiltration in models of inflammation, whereas inhibitors of H_2S biosynthesis increase leukocyte adherence [59]. H_2S donors diminish edema, presumably due to inhibition of plasma exudation, while CBS and CSE inhibitors increase the formation of edema in response to inflammatory stimuli [59]. A molecular mechanism underlying anti-inflammatory roles of H_2S may include its scavenging peroxynitrite, a toxic derivative of NO, as well as other oxidants [60].

H_2S has been shown to exert beneficial influences in disorders of joints, including resolving synovitis in rodents [61] and alleviating the pathology of carrageenen-associated arthritis [62]. H_2S donors also have been extensively explored in intestinal disorders, with beneficial effects in several models of colitis [63].

H_2S may participate in some actions of tumor necrosis factor alpha (TNF-α). While TNF-α is regarded as pro-inflammatory, it does display anti-apoptotic actions mediated via nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). The anti-apoptotic actions of NF-κB appear to be mediated by H_2S generated by CSE [34]. TNF-α treatment (10 ug/kg for 4 h) in peritoneal macrophages triples H_2S generation by stimulating the binding of the transcription factor SP1 to the CSE promoter increasing CSE protein levels. The H_2S generated by CSE enhances the binding of NF-κB to promoters of downstream genes, whose signaling is markedly diminished in CSE knockout mice. H_2S acts by sulfhydrating the p65 subunit of NF-κB, which promotes its binding to the co-activator ribosomal protein S3. The anti-apoptotic influences of NF-κB are substantially reduced in CSE-deleted mice [34].

The anti-inflammatory influences of H_2S have led to efforts to develop therapeutic agents. Classic non-steroidal anti-inflammatory drugs (NSAIDs) often cause gastric irritation by inhibiting the formation of prostaglandins, which are physiologic cytoprotectants of the gastric mucosa. H_2S, on the other hand, reduces mucosal inflammation, protects the gastrointestinal mucosa from injury and also augments tissue repair. In direct comparisons of naproxen and its H_2S-linked derivative, the latter exerted comparable therapeutic efficacy with reduced gastric damage [64, 65]. Several other NSAIDs have been combined with H_2S donors. Mechanistic studies have been conducted with some of these drugs, with particularly extensive investigations utilizing *S*-diclofenac [52, 66, 67]. *S*-diclofenac has been shown to inhibit cell proliferation [68, 69] and to protect against ischemia-reperfusion injury in perfused hearts [53].

New directions

Evidence for H_2S as a physiologic gasotransmitter has lagged behind CO and NO, but H_2S is rapidly catching up. Therapeutic applications may emerge in the not-too-distant future, especially in the area of anti-inflammatory drugs. Definitive understanding of how H_2S participates in inflammatory processes may come from studies of inflammation in mice with deletion of CSE and/or CBS. In the gastrointestinal system and liver, CSE levels greatly exceed those of CBS. Because many major proteins are physiologically sulfhydrated, it is possible that many metabolic functions of the liver are determined in notable part by the actions of H_2S, as is evident by the substantial decrease in GAPDH activity in livers of CSE knockout mice, due to the loss of the activating influence of GAPDH sulfhydration [11].

One area not addressed in this review is the role of H_2S in the brain, which was discussed in a previous review [70]. Studies with mice lacking CBS and CSE suggest that the majority of H_2S in the brain derives from CBS rather than

CSE. The limited immunohistochemical studies thus far performed reveal CBS predominantly in glia [14]. CSE may have neuronal as well as glial localizations so that even if it generates a smaller amount of H_2S, this enzyme might be the source of a neurotransmitter pool [15]. In the intestine, there is evidence that CSE is localized to the myenteric plexus of neurons and may exert physiologic influences on intestinal motility [15, 71]. Conceivably, neuronal CSE in the gut occurs in the same neurons known to possess HO2 and nNOS, which are co-localized in neuronal populations [72].

Acknowledgments This work has been supported by National Institutes of Health Medical Scientist Training Program Award (T32 GM007309) to M.S.V. and US Public Health Service Grants (MH018501) to S.H.S.

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