Published in final edited form as:
J Neurochem. 2010 April ; 113(1): 14–26. doi:10.1111/j.1471-4159.2010.06580.x.

Hydrogen Sulfide as a Gasotransmitter

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Abstract

Nitric oxide (NO) and carbon monoxide (CO) are well established as messenger molecules throughout the body, gasotransmitters, based on striking alterations in mice lacking the appropriate biosynthetic enzymes. Hydrogen sulfide (H_2S) is even more chemically reactive, but till recently there was little definitive evidence for its physiologic formation. Cystathionine β-synthase (CBS, EC 4.2.1.22), and Cystathionine γ-lyase (CSE; EC 4.4.1.1), also known as cytathionase, can generate H_2S from cyst(e)ine. Very recent studies with mice lacking these enzymes have established that CSE is responsible for H_2S formation in the periphery, while in the brain CBS is the biosynthetic enzyme. Endothelial-derived relaxing factor (EDRF) activity is reduced 80% in the mesenteric artery of mice with deletion of CSE, establishing H_2S as a major physiologic EDRF. H_2S appears to signal predominantly by *S*-sulfhydrating cysteines in its target proteins, analogous to *S*-nitrosylation by NO. Whereas *S*-nitrosylation typically inhibits enzymes, *S*-sulfhydration activates them. *S*-nitrosylation basally affects 1–2% of its target proteins, while 10–25% of H_2S target proteins are *S*-sulfhydrated. In summary, H_2S appears to be a physiologic gasotransmitter of comparable importance to NO and CO.

Keywords

cystathionine γ-lyase; cystathionase; cystathionine β-synthase; EDHF; EDRF; GAPDH; K_{ATP}; *S*-sulfhydration; hydrogen sulfide; hydropersulfide

The notion that gases can serve as messenger molecules stems largely from research indicating that nitric oxide (NO) is a physiologic vasodilator and mediates the tumoricidal/bactericidal actions of macrophages (reviewed in Moncada *et al.* 1991). Subsequently, NO was established as a neurotransmitter/neuromodulator in the brain and peripheral nervous system (Bredt & Snyder 1989Bredt & Snyder 1990; Bredt *et al.* 1990, 1991a,b, 1992; Burnett *et al.*, 1992; Nelson *et al.* 1995). Soon thereafter, evidence accumulated establishing carbon monoxide (CO) as physiologically generated and mediating non-adrenergic non-cholinergic (NANC) neurotransmission in the intestine as well as neural activity in the brain (Verma *et al.* 1993; Zakhary *et al.* 1997; Xue *et al.* 2000; Boehning *et al.* 2004). Both of these gaseous molecules are well accepted as gasotransmitters; a term which, as used here, does not necessarily imply that the gaseous molecule is a neurotransmitter but rather that it transmits information between cells in various parts of the body.

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It was easy to accept that NO and CO are physiologically relevant, once the biosynthesis of both substances was established from reasonably well characterized enzymes. In the case of NO, three isoforms of nitric oxide synthase (NOS; EC 1.14.13.39), derived from three distinct genes, convert arginine to NO and citrulline, with neuronal NOS (nNOS) highly localized to the brain and peripheral nerves as well as a few non-neural tissues, endothelial NOS (eNOS) generating NO that regulates blood vessels, and inducible NOS (iNOS) occurring ubiquitously throughout the body, but with highest densities in inflammatory cells such as macrophages. nNOS and eNOS are constitutive enzymes activated by calcium-calmodulin which explains their rapid augmentation in response to depolarizing events (Bredt & Snyder 1989). By contrast, iNOS is inducible, largely in response to inflammatory stimulation, and is not notably influenced by calcium (Lowenstein *et al.* 1992, 1993; Cho *et al.* 1992). Mice with targeted deletion of the three enzymes lose the capacity to generate NO in the relevant target organs (Huang *et al.* 1993; Huang *et al.* 1995; Wei *et al.* 1995; MacMicking *et al.* 1995; Shesely *et al.* 1996; Son *et al.* 1996; Morishita *et al.* 2005).

CO has long been known to be formed by two isoforms of heme oxygenase (HO) which derive from distinct genes (Maines 1988). HO-1 is a markedly inducible enzyme whose formation is stimulated by diverse stressors, including heme, and is abundant in liver, kidney and spleen; organs responsible for degradation and heme catabolism of aged red blood cells (Poss and Tonegawa 1997). By contrast, HO-2, localized to neurons in the brain and the endothelial layer of blood vessels, is constitutive and activated by calcium-calmodulin, much like nNOS and eNOS (Verma *et al.* 1993; Zakhary *et al.* 1996; Boehning *et al.* 2004). Although HO-2 is constitutive, glucocorticoids (Weber *et al.* 1994; Raju *et al.* 1997) and opiates (Li and Clark 2000; Panahian and Maines 2001) have been shown to increase HO-2 expression. HO-1 was first identified in aging red blood cells where it degrades the heme ring of hemoglobin generating biliverdin, which is rapidly reduced by biliverdin reductase to bilirubin. When the heme ring is cleaved at the α-meso carbon bridge, the one carbon fragment is liberated as CO by oxidation, a process that was well documented but largely overlooked by biologists until appreciation of NO led to demonstration that CO is also a gasotransmitter. Recently, mitochondrial soluble adenyl cyclase was found to be regulated by carbon dioxide/bicarbonate, indicating that carbon dioxide too might be a gasotransmitter (Acin-Perez *et al*. 2009).

Awareness of hydrogen sulfide (H_2S) precedes by centuries the appreciation of NO and CO. It was referred to as *aer hepaticus* (hepatic air) by alchemists (Myers 2007). In 1777 Carl Wilhelm Scheele was the first chemist to prepare and characterize H_2S, describing it as "sulfuretted hydrogen," in *Chemische Abhandlung von der Luft und dem Feuer* (*Chemical Treatise on Air and Fire*). H_2S is odoriferous at concentrations less than 1 ppm, causes headaches at 4 ppm and is lethal at high levels (Reiffenstein *et al.*, 1992). It is about 5 times more potent as a toxin than CO, acting largely by inhibiting cytochrome C oxidase (Lloyd 2006). All of us possess abundant levels of H_2S in our gut derived predominantly from bacteria that can form H_2S by the reduction of sulfate as well as the decomposition of sulfur containing amino acids such as cysteine and methionine, sulfated polysaccharides and sulfur containing lipids. Actions upon the gut of bacterially generated H_2S are of some interest (Lloyd 2006). However, most biomedical researchers would be more disposed toward investigating a substance generated by mammalian enzymes under physiologic circumstances. Several pathways for the physiologic formation of H_2S have been widely discussed and inhibitors of these enzymes influence H_2S levels. However, none of the inhibitors have been extraordinarily potent or selective. Woody Allen apocryphally commented, "Ninety percent of life is showing up." In the absence of definitive evidence for the physiologic formation and function of H_2S, the world of biomedical science would not be persuaded of a physiologic role for H_2S. Very recently, deletion of a putative biosynthetic enzyme for H_2S, cystathionine γ-lyase (CSE; EC 4.4.1.1), also known as cystathionase, was

shown to deplete endogenous H_2S levels and to markedly alter vasorelaxation and blood pressure (Yang *et al.* 2008). Hence, H_2S now warrants inclusion in the family of gasotransmitters.

Metabolism

The two principal enzymes proposed as a physiologic sources of H_2S both metabolize cystathionine. Cystathionine is well established as an intermediate in various cycles involving sulfur-containing amino acids but has not had a prominent role in biomedical research. It is formed by the enzyme cystathionine β-synthase (CBS; EC 4.2.1.22), which condenses homocysteine with serine to generate the thiol ether cystathionine (Fig. 1a). In the condensation, the hydroxyl group of serine is replaced with the thiolate of homocysteine. The gene of human CBS is localized to chromosome 21 at 21q22.3 (Münke *et al.* 1988). In human and rat CBS exists primarily as a homotetramer with a subunit molecular weight of 63 kDa. Each subunit also binds the cofactors pyridoxal 5′-phosphate (PLP), *S*-adenosyl methionine (SAM) and heme (Miles and Kraus 2004;Banerjee and Zou 2005). The heme appears to be a redox sensor, while SAM is an allosteric activator of the enzyme. The C-terminal portion of CBS contains a tandem repeat of two “CBS domains” which appear to act as inhibitors of enzymatic function, as their deletion activates CBS (Shan and Kruger 1998;Kery *et al.* 1998). The CBS domains have been proposed to act as energy sensors (Scott *et al.* 2004).

Recently CBS has been shown to be sumoylated at lysine 211 in the “CBS domain” (Kabil *et al.* 2006). Sumoylation often elicits nuclear localization of proteins and may explain the substantial levels of CBS in the nucleus. Sumoylation inhibits the catalytic activity of CBS (Agrawal and Banerjee 2008). Interestingly, CBS physiologically binds huntingtin, the protein mutated in Huntington’s Disease (Boutell *et al.* 1998). Huntingtin itself is also sumoylated which enhances the neurotoxicity of mutant huntingtin (Steffan *et al.* 2004; Subramaniam *et al.* 2009).

Heme binds to the N-terminal portion of CBS comprising about 70 amino acids. In its ferrous state, this heme binds both CO and NO (Taoka and Banerjee 2001). CO binds with higher affinity, with a K_i of about 5.6μM, while NO (K_i ~ 360μM) is only about two percent as potent so that its binding probably is not physiologically relevant (Taoka *et al.* 1999; Taoka and Banerjee 2001). CO inhibits CBS activity. The interaction of CO with CBS is analogous to its interaction with heme in the transcription factor neuronal PAS domain protein 2 (NPAS2) wherein CO disrupts the DNA binding activity of NPAS2 (Dioum *et al.* 2002). The potent influence of CO upon CBS raises the possibility of cross-talk between CO and H_2S as messenger molecules.

SAM activates CBS several fold by binding to the CBS domain in the carboxyl terminus of the enzyme (Shan and Kruger 1998; Kery *et al.* 1998). Thus, truncated CBS, lacking the C-terminus, displays 5 fold greater catalytic activity than the native enzyme and is no longer stimulated by SAM (Taoka *et al.* 1999). The biologic rationale for activation of CBS by SAM is unclear. One possibility is that the CBS domain is an energy-sensing domain. This notion is based on findings that AMP-activated protein kinase (AMPK) binds CBS at its CBS domain (Scott *et al.* 2004). One wonders whether SAM regulation of CBS reflects some sort of reciprocal link between signaling by H_2S and signaling by SAM’s methylation of multiple targets.

CBS can catalyze H_2S formation from cysteine through a β-replacement reaction with a variety of thiols (Braunstein *et al.* 1971; Porter *et al.* 1974) (Fig. 1b). This is coupled with the formation of the corresponding thiol ether. CBS levels are relatively high in the brain where it is postulated to be the physiologic source of H_2S (Abe and Kimura 1996). Using

both cysteine and homocysteine as co-substrates simultaneously, the V_{max} of H_2S production for human CBS is 22–40 fold higher than for cysteine alone (Singh *et al.* 2009). In this reaction the K_m values for cysteine and homocysteine are 6.8 mM and 3.2 mM respectively. Accordingly, homocysteine might be a preferred co-substrate for H_2S generation. In determining whether CBS physiologically generates H_2S, many investigators have relied upon the inhibitors, hydroxylamine and amino-oxyacetate (Abe and Kimura 1996). These do inhibit the generation of H_2S from cysteine in brain homogenates, but both are general inhibitors of all PLP-dependent enzymes.

CSE can also form H_2S from cyst(e)ine (Cavallini *et al.* 1962a,b; Szczepkowski and Wood 1967) (Fig. 1b), though the classical function of CSE is to hydrolyze cystathionine into cysteine with ammonia and α-ketobutyrate as byproducts (Fig. 1a). The enzyme converts cystine to thiocysteine, pyruvate and ammonia, in a β-disulphide elimination reaction, with the thiocysteine then reacting with cysteine or other thiols to produce H_2S and cystine or the corresponding disulfide (Fig. 1b). In most peripheral tissues CSE levels are much higher than those of CBS, while in the brain, CBS predominates (Yang *et al.* 2008; Mustafa *et al.* 2009a,b; Abe and Kimura 1996).

CSE inhibitors have been employed to examine the enzyme's role in generating H_2S physiologically. The two principal inhibitors utilized are DL-propargylglycine (PAG) (Abeles and Walsh 1973; Washtien and Abeles 1977) and β-cyano-L-alanine (β-CNA) (Pfeffer and Ressler 1967). They influence other enzymes such as cystathionine γ-synthetase (EC 2.5.1.48) (Marcotte and Walsh 1975), methionine γ-lyase (EC 4.4.1.11) (Johnston *et al.* 1979), aminotranferases (Marcotte and Walsh 1975; Burnett *et al.* 1980; Tanase and Morino 1976; Alston *et al.* 1980) and D-amino acid oxidase (EC 1.4.3.3) (Horiike *et al.* 1975; Marcotte and Walsh 1976). Thus, one must be cautious in interpreting results utilizing such agents. However, it is of interest that PAG and β-CNA do suppress H_2S production by the liver and kidney but not by the brain; fitting with other evidence that CBS is the predominant source of H_2S in brain tissue (Abe and Kimura 1996).

Like CBS, CSE is a PLP-dependent enzyme. If CSE were to generate H_2S as a physiologic signaling molecule, one might expect it to be influenced by signaling systems such as calcium. Indeed, CSE is selectively activated by calcium-calmodulin similar to the activation of eNOS, nNOS and HO-2 (Yang *et al.* 2008).

Definitive evidence that CSE is a physiologic source for H_2S comes from experiments employing CSE knockout mice (Yang *et al.* 2008). H_2S levels in aorta and heart of homozygous CSE knockout mice are reduced by about 80% with a 50% reduction in heterozygous knockouts. Serum H_2S levels in homozygous and heterozygous CSE knockouts are reduced 50% and 20% respectively. The residual H_2S in mutant serum may reflect non-enzymatic reduction of elemental sulfur to H_2S or H_2S generated from other tissues by CBS. The studies with CSE knockouts establish that H_2S is a product of normal mammalian physiology.

H_2S is presumed to exist in an ionized form in most tissues as HS^-. Kimura and associates (Ishigami *et al.* 2009; Shibuya *et al.* 2009) have characterized a form of H_2S which they refer to as "bound sulfur." This material presumably arises when the sulfur of H_2S is incorporated into proteins, bound to other sulfur atoms to form persulfides. Presumably this bound sulfur releases H_2S under reducing conditions. These authors showed that the bound H_2S was not depleted in CBS knockout mouse brain (Ishigami *et al.* 2009). It was possible to generate this H_2S pool from cysteine by the coordinate actions of two enzymes, 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) and cysteine aminotransferase (EC 2.6.1.3). The physiologic significance of this pool of sulfur is unclear. Definitive evidence awaits

studies with deletion of the postulated enzymes utilizing techniques such as RNA interference or mutant mice.

Signaling Mechanisms

Signaling by NO was first characterized in terms of its relaxation of blood vessels. NO binds with high affinity to heme in the active site of soluble guanylyl cyclase (sGC), altering the enzyme's conformation and enhancing its catalytic activity. Generated cyclic GMP then leads to smooth muscle relaxation through activation of cyclic GMP-dependent protein kinase which results in protein phosphorylation, a decrease in cytosolic calcium, and dephosphorylation of the myosin light chain. CO also activates soluble sGC but is substantially less potent than NO. Its potency is dramatically increased in the presence of certain agents such as YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), a benzyl indazole derivative (Friebe *et al.* 1996). Conceivably, conformational alterations such as those elicited in the enzyme by YC-1 occur in intact organisms and lead to enhanced and physiologic potency of CO *in vivo*. Such a view would be consonant with direct evidence that cyclic GMP levels in various tissues are markedly depleted in HO-2 knockout mice (Zakhary *et al*. 1997; Watkins *et al.* 2004).

H_2S also binds with high affinity to heme. However, it does not appear to physiologically stimulate sGC (Abe and Kimura 1996). Moreover, the ability of H_2S to relax blood vessels is not impaired in the presence of inhibitors of sGC (Zhao *et al*. 2001).

If H_2S does not act through sGC, how does it signal? A clue comes from NO, which can *S*-nitrosylate cysteines of various proteins (Stamler *et al.* 1992a,b; Stamler *et al.* 1997). Because both NO and the thiol groups of cysteines are chemically reactive, armchair chemistry would predict nitrosylation of cysteines in proteins (Fig. 2). Stamler and associates (Jia et al. 1996; Xu et al. 1998; Mannick et al. 1999) showed such modification for a wide range of proteins. Demonstration of physiologic nitrosylation of numerous proteins under basal conditions by endogenously generated NO was rendered feasible by development of the biotin switch assay (Jaffrey *et al.* 2001; Jaffrey and Snyder 2001). In this procedure free thiols are blocked by the sulfhydryl-reactive compound, methyl methane thiolsulfonate; the nitrosylated thiols are then exposed by treatment with ascorbate, labeled with biotin, coupled to streptavidin, and nitrosylated proteins are then separated by gel electrophoresis. A substantial number of proteins are basally nitrosylated, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.7.6), glycogen phosphorylase (EC 2.4.1.1), creatine kinase (EC 2.7.3.2), sodium/potassium adenosine triphosphatase (EC 3.6.1.3), *N*-methyl-D-aspartate (NMDA)-glutamate receptor, β-tubulin and actin. Nitrosylation of these and other proteins is abolished in nNOS knockout mouse brain (Jaffrey *et al.* 2001).

In the absence of ascorbate some proteins were still labeled; indicating that in addition to *S*-nitrosylation, ascorbate-dependent labeling, there was another thiol modification of cysteine that was labelled independent of ascorbate. Mass spectrometric analysis indicated that the labeling reflects *S*-sulfhydration, attachment of an additional sulfur to the thiol (–SH) groups of cysteines yielding a hydropersulfide (–SSH) moiety (Mustafa *et al.* 2009b) (Fig. 2). This is not to be confused with *S*-thiolation or *S*-thionylation, in which a protein thiol forms a mixed disulfide with a small-molecular weight thiol such as glutathione or cysteine (Thomas *et al.* 1995). *S*-thiolation blocks the protein thiol rendering it non-reactive, whereas *S*-sulfhydration yields a hydropersulfide (–SSH) moiety which has enhanced chemical reactivity.

Numerous proteins, such as β-tubulin, actin, and GAPDH, are basally sulfhydrated. For most proteins, especially GAPDH in the liver, sulfhydration is substantially more prevalent than

nitrosylation. Sulfhydration is abolished in CSE knockout mouse liver, but is unaffected in livers of nNOS, eNOS and iNOS knockouts. Sulfhydration occurs at physiologic levels of L-cysteine with maximal stimulation of GAPDH, β-tubulin and actin at about 0.6–1 mM L-cysteine, comparable to its physiologic concentrations in the liver.

Nitrosylation of most enzymes and receptors inhibits their activity. This fits with the importance of cysteine thiols for activities of many proteins and nitrosylation masking the critical reactive thiol groups. By contrast, sulfhydration merely changes an –SH to an –SSH which would enhance chemical reactivity and might even afford greater access to targets. Indeed, whereas nitrosylation of GAPDH abolishes its catalytic activity (Hara *et al.* 2005), H_2S elicits a 7-fold increase in GAPDH activity (Fig. 3a; Mustafa *et al*. 2009b). DTT reverses GAPDH activation by H_2S (Fig. 3b), and H_2S fails to increase the activity of C150S mutant GAPDH (Fig. 3c), consistent with the H_2S augmentation of GAPDH activity occurring via sulfhydration at C150 (Mustafa *et al*. 2009b). H_2S increases the V_{max} of GAPDH with no effect on K_m (Fig. 3D; Mustafa *et al*. 2009b). Activation of GAPDH by H_2S enzymatically generated from L-cysteine by CSE is observed in HEK 293 cells transfected with CSE (Fig. 3E; Mustafa *et al*. 2009b). Similarly, sulfhydration directly enhances actin polymerization with no effect on its depolymerization (Mustafa *et al*. 2009b).

Sulfhydration is a prominent posttranslational modification with 10–25% of endogenous GAPDH, β-tubulin and actin basally sulfhydrated (Mustafa *et al*. 2009b). By contrast, physiologic nitrosylation levels affects only 1–2% of target proteins (Jaffrey *et al*. 2001). The physiologic relevance of sulfhydration is evident in the reduction of GAPDH activity by about 25–30% in livers of CSE knockout mice despite normal levels of GAPDH protein (Fig. 3f; Mustafa *et al*. 2009b). This finding corresponds reasonably well with the extent of activation elicited by H_2S and the proportion of total GAPDH which is sulfhydrated.

The fact that a very large number, perhaps the majority, of proteins are basally sulfhydrated and that sulfhydration alters protein function, suggests that sulfhydration is an important physiologic signal.

Physiologic roles of H_2S

Blood vessels

The best known physiologic role for NO is as endothelial-derived relaxing factor (EDRF). EDRF activity was defined by the classic studies of Furchgott (Furchgott and Zawadzki 1980). Whereas norepinephrine constricts blood vessels by directly contracting the smooth muscle, Furchgott showed that the vasorelaxant action of acetylcholine is lost when the endothelial layer of blood vessels is removed. A substance with the properties of NO was released by endothelial tissue, and NO's actions fit with the properties of EDRF. With the development of eNOS knockout mice, direct verification of the NO-EDRF hypothesis was possible. eNOS knockouts display elevated blood pressure and diminished EDRF activity in some vascular beds (Huang *et al*. 1995). CO also behaves like an EDRF. Like eNOS, HO-2 is localized to the endothelial layer of blood vessels whose endothelial-dependent relaxation is blocked by HO inhibitors (Zakhary *et al*. 1996).

H_2S has long been known to relax blood vessels (Zhao *et al*. 2001). Direct evidence bearing upon a potential EDRF activity for H_2S awaited investigations employing CSE knockout mice (Yang *et al*. 2008). These mice develop age-dependent hypertension peaking at 12 weeks of age with blood pressures 18 mm Hg greater than control mice (Fig. 4a), similar to the hypertension of eNOS knockouts (Yang *et al*. 2008; Huang *et al*. 1995). Interestingly, the hypertension of CSE knockouts is age dependent. Blood pressure of heterozygotes resembles that of homozygotes at early ages, but by 10 weeks of age the homozygous mice

display levels 10 mm Hg greater than the heterozygotes (Fig. 4a). The age-dependent hypertension parallels the ontogeny of CSE which attains peak levels three weeks after birth (Ishii *et al*. 2004).

H_2S satisfies the principal properties of an EDRF (Yang *et al*. 2008). It is selectively localized to the endothelial layer of blood vessels (Fig. 4b). In CSE knockout mesenteric arteries the contractile effects of phenylephrine (Fig. 4c), exerted upon α-adrenoceptors of vascular muscle, and the direct relaxing effects of NO donors are the same as in wild-type animals. H_2S more potently relaxes mesenteric arteries of CSE knockouts than wild-type, indicating super-sensitivity associated with decreased endogenous H_2S. By contrast, methacholine relaxation of the mesenteric artery is reduced by about 80% in homozygous CSE knockout vessels and about 50% in heterozygotes (Fig. 4d). The methacholine relaxation reflects EDRF activity, as it is abolished by removal of the endothelium.

Thus, most EDRF activity of the mesenteric artery can be attributed to H_2S. Muscarinic cholinergic treatment of blood vessels activates eNOS to produce NO. Similarly, methacholine treatment of endothelial cells triples H_2S levels which are abolished by depletion of CSE utilizing RNA interference.

If the great majority of mouse mesenteric artery EDRF activity is attributable to H_2S, what is the role of NO? NO is well established as an EDRF in numerous vascular beds, but EDRF activity in many vessels is only partially diminished by NOS inhibitors and in eNOS knockouts (Brandes *et al*. 2000; Félétou and Vanhoutte 2007). EDRF activity attributable to NO is most prominent in large vessels such as the aorta, while in resistance vessels that regulate blood pressure more directly, NO's effects are less evident (Brandes *et al*. 2000). Differences among diverse vascular beds and species variations may account for discrepant observations. Determining the relative roles of NO, CO and H_2S in mediating physiologic EDRF activity will require side-by-side comparisons of HO-2, eNOS and CSE knockout mice as well as studies in multiple species.

How does H_2S relax blood vessels? NO is well established to act by stimulating sGC. CO does elevate cyclic GMP levels. However, endogenous CO-induced vasodilation occurs via a cyclic GMP-independent mechanism (Naik and Walker, 2003). It appears likely that CO acts via the large-conductance calcium-activated potassium channels (BK_{Ca}). Thus, inhibitors of BK_{Ca} channels block endogenous CO-elicited vasodilation (Naik and Walker, 2003). Moreover, HO inhibitors reduce BK_{Ca} channel activity in several vascular beds (Kaide *et al*. 2001; Zhang *et al*. 2001; Li *et al*. 2008). Inhibitors of sGC do not influence CO-induced BK_{Ca} channel activation (Kaide *et al*. 2001; Xi *et al*. 2004). Interestingly, the actions of CO on BK_{Ca} may involve binding to heme, analogous to NO binding to heme in sGC. Thus, the α-subunit of BK_{Ca} contains a heme-binding pocket, and binding of heme to the channel inhibits its activity, CO binds to channel-associated heme to elicit channel activation (Jaggar *et al*. 2005).

A major component of EDRF activity involves hyperpolarization, a phenomenon that is not elicited by sGC. Thus, to fully explicate EDRF, investigators have sought an endothelial-derived hyperpolarizing factor (EDHF). Compounds postulated to mediate EDHF activity include prostacyclin generated from arachidonic acid by cyclooxygenase (EC 1.14.99.1), epoxyeicosatrenoic acids generated from arachidonic acid by cytochrome P450 epoxygenase (EC 1.14.14.1), hydrogen peroxide, potassium ions, C-type natriuretic peptide, electrical coupling through myoendothelial junctions mediated by connexins, and NO itself (reviewed in Bellian *et al*. 2008; Luksha *et al*. 2009). For none of these substances has definitive evidence been provided employing genetic mutant animals provided.

In mouse mesenteric artery and aorta, inhibition of eNOS and cyclooxygenase reduces cholinergic EDRF activity only about 20% (Mustafa *et al.* in preparation). The remaining 80% of cholinergic relaxation reflects pronounced hyperpolarization with resting membrane potentials approximating the potassium equilibrium potential. This hyperpolarization is virtually abolished in CSE homozygous knockout mice.

EDHF activity reflects opening of potassium channels (Bellian *et al*. 2008; Luksha *et al*. 2009). The vasorelaxant effects of H_2S are blocked by inhibitors of the ATP-sensitive potassium channel (K_{ATP}) (Zhao *et al*. 2001; Zhao and Wang 2002; Cheng *et al*. 2004). Glibenclamide, a potent and selective inhibitor of K_{ATP}, reduces cholinergic hyperpolarization of the mesenteric artery smooth muscle cells by about 70% (Mustafa *et al.* in preparation). By contrast, glibenclamide doesn't affect relaxation elicited by NO donors.

How does H_2S stimulate K_{ATP}? K_{ATP} possesses 9 cysteines with C43, that lies close to the surface, selectively influenced by oxidative insults. K_{ATP} is sulfhydrated with the sulfhydration abolished by mutations of C43 (Mustafa *et al.* in preparation). Thus, H_2S vasorelaxation reflects hyperpolarization mediated by the opening of K_{ATP} channels via their sulfhydration at C43. K_{ATP} is physiologically activated by binding of the phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) (Shyng and Nichols 1998; Baukrowitz *et al*. 1998). PIP2 binding to K_{ATP} is abolished in cells lacking CSE or containing catalytically inactive enzyme, and H_2S donors markedly stimulate PIP2-K_{ATP} binding (Mustafa *et al.* in preparation). The PIP2-K_{ATP} binding involves the sulfhydrated C43, as binding is markedly reduced in K_{ATP}-C43S mutants.

As physiologic vasodilation is thought to be determined largely by EDHF, the evidence that EDHF activity is predominantly determined by H_2S fits with a major role for H_2S as an EDRF/EDHF.

Inflammation

There is abundant literature on potential roles of H_2S in inflammation. Some studies indicate that endogenous H_2S is anti-inflammatory. Thus, one of the earliest events in inflammation is adherence of leukocytes to vascular endothelium and their subsequent migration into underlying tissue. The CSE inhibitor β-CNA markedly increases leukocyte-endothelial adherence as well as carrageenan-induced leukocyte infiltration and paw edema (Zanardo *et al*. 2006). H_2S donors display anti-inflammatory effects, inhibiting leukocyte-endothelium bonding and reducing carrageenan-induced paw edema. H_2S donors reduce visceral pain in a colorectal distension model (Distrutti *et al*. 2006a,b) and diminish colitis in rats (Fiorucci *et al*. 2007).

By contrast, some studies indicate a pro-inflammatory action of H_2S. H_2S levels and CSE expression are increased in several models of inflammation, and the CSE inhibitor PAG reduces inflammation in some of these models (Mok *et al*. 2004; Li *et al*. 2005; Bhatia *et al*. 2005a,b; Collin *et al*. 2005). In rodent sepsis, H_2S increases levels of substance P in the lung (Zhang et al. 2007). Also, H_2S induces the formation of pro-inflammatory cytokines and chemokines by upregulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Zhi *et al*. 2007).

Despite discrepancies, the evidence that H_2S is anti-inflammatory is sufficient that efforts are under way to attack inflammatory diseases with H_2S releasing drugs. For instance, diclofenac derivatives that release H_2S have been developed for use as anti-inflammatory drugs (reviewed in Wallace 2007). An H_2S-releasing mesalamine derivative, ATB-429, displays analgesic and anti-inflammatory effects and has been effective in models of inflammatory bowel disease (Distrutti *et al*. 2006a,b).

Because of its chemical activity and abundant production from bacteria in the colon, there has been speculation that bacterially generated H_2S mediates the pathophysiology of ulcerative colitis (Pitcher and Cummings 1996). Short-chain fatty acids, especially butyrate, are thought to be important in maintaining normal colonic mucosal function (Cummings 1981). Butyrate oxidation provides about 70% of colonic energy whereas the small intestine preferentially utilizes glucose and glutamine (Watford *et al*. 1979; Roediger 1980, 1982; Ardawi and Newsholme 1985; Cummings *et al*. 1987). H_2S donors interfere with colonic butyrate metabolism (Christl *et al*. 1996). It is conceivable that the therapeutic effects of 5-aminosalicylate in ulcerative colitis reflect influences upon H_2S, as patients treated with the drug display substantially decreased levels of sulfide in their feces (Pitcher *et al*. 1995).

Nervous system

The journey to establishing a neural role for any substance commences with ascertaining its localization. In the 1960's histochemical fluorescent techniques that visualize biogenic amines such as serotonin, dopamine and norepinephrine, permitted mapping their neuronal pathways with major functional insights (Carlsson 1987). Immunohistochemistry for a wide range of neuropeptides and neurotransmitter related enzymes established these substances as neurotransmitter candidates (Jones and Hartman 1978). Selective neuronal localizations of nNOS (Bredt *et al*. 1990) and HO-2 (Verma *et al*. 1993) have helped to characterize neurotransmitter properties for NO and CO respectively. For H_2S, one would hope to localize the biosynthetic enzymes by immunohistochemistry. Relatively little investigation has yet been reported. Szurszewski and colleagues (Linden *et al*. 2008) conducted immunohistochemical studies of both CSE and CBS. For CSE, neuronal localizations were evident in the myenteric plexus of neurons in the small intestine suggesting that like NO and CO, H_2S might be a non-adrenergic non-cholinergic (NANC) neurotransmitter. In the brain, where CSE levels are low, localizations were predominantly in white matter. CBS immunohistochemistry in the brain also revealed prominent white matter localizations with negligible neuronal staining. However, caution is warranted in interpreting these findings. The publication did not display western blots to clarify whether the antibody reacted with substances other than CBS or CSE. A principal control was preabsorption with the immunizing antigen which does not rule out non-specific staining. Further studies employing CBS and CSE knockout mice as controls would be useful.

Influences of H_2S upon neuronal activity in the brain have been explored extensively by Kimura and colleagues (Kimura *et al*. 2005). This group noted that physiologic concentrations of H_2S enhance long-term potentiation (LTP). Sodium hydrogen sulfide (NaHS) applications and weak tetanic stimulation of rat hippocampal slices alone did not elicit LTP, while the simultaneous application of both led to robust LTP (Abe and Kimura 1996). The effect of H_2S on LTP was abolished by NMDA antagonists. Interestingly, NO and CO also induce LTP, but do so even when NMDA receptors are blocked (Zhuo *et al*. 1993). NMDA receptors possess reactive cysteines and are known to be nitrosylated with resulting channel blockade (Lei *et al*. 1992; Choi *et al*. 2000). Conceivably H_2S regulates NMDA transmission by sulfhydrating NMDA receptors.

Besides its actions upon neurons, H_2S also appears to influence astrocytes (Nagai *et al*. 2004). H_2S donors elicit calcium waves in astrocytes and increase intracellular levels of calcium. The increased intracellular calcium occurs rapidly following H_2S exposure and decays slowly, whereas the oscillations of calcium decay rapidly. Effects of H_2S donors are evident both in primary cultures of astrocytes and in glia within hippocampal slices. The increased intracellular calcium in astrocytes following H_2S administration reflects calcium entry, as it is suppressed in calcium-free media and is associated with a direct influx of calcium similar to that elicited by calcium ionophores. The type of calcium channel involved has not yet been established.

H_2S may also serve as a neuroprotectant. Glutamate neurotoxicity in brain cultures involves, at least in part, inhibition of cystine uptake (Tan *et al*. 2001). The cystine/glutamate antiporter couples influx of cystine with efflux of glutamate. This process is blocked by high concentrations of exogenous glutamate which are cytotoxic via a process designated oxytosis (Tan *et al*. 2001). How does H_2S act in this model? Glutamate reduces levels of intracellular glutathione, and H_2S increases them both in untreated and in glutamate-exposed preparations (Kimura and Kimura 2004). In support of this model, buthionine sulfoximine (Griffith 1982), which inhibits γ-glutmaylcysteine synthase (EC 6.3.2.2), a rate limiting enzyme in glutathioine biosynthesis, prevents the H_2S-elicited stimulation of glutathione levels and cell survival. H_2S elicits augmented glutathione by stimulating cystine entry into cells, reversing the inhibition of cystine transport by glutamate (Kimura and Kimura 2004).

Interestingly, the first recognized sign of CBS deficiency in humans is mental retardation (Mudd *et al.* 1999). CBS deficient patients also suffer from seizures, abnormal electroencephalograms, extrapyramidal disturbances and psychiatric disorders (Mudd *et al.* 1985; Abott *et al.* 1987). The role of H_2S in these disturbances is yet to be examined. Another interesting observation is that CBS is enriched in the brains of Down's patients (Ichinohe *et al.* 2005). This is not surprising since the CBS gene is located on chromosome 21. However, the role of CBS and H_2S in the mental retardation found in Down syndrome is also yet to be examined.

The Future

Because H_2S is a chemically reactive substance with toxic actions, its influences upon various tissues have been well characterized for many decades. However, translating pharmacologic effects into evidence for endogenous, physiologic function is a major challenge. Direct evidence that H_2S is physiologically generated by the enzymes CSE and CBS is very recent. Mice with targeted deletion of these two enzymes have been valuable tools in this endeavor, but many basic studies remain to be carried out. Localizing CBS and CSE immunohistochemically in all organs of the body, especially the brain, is a seemingly simple minded task but of immense importance. Phenotypic characterization of the CBS and CSE mutant mice is critical. Using the mice to establish roles for H_2S in nervous system function should be reasonably straightforward. Behavioral analysis, monitoring neurotransmission in various pathways, exploring synaptic plasticity in models such as LTP and long-term depression (LTD), are all approaches that are today the bread and butter of neuroscience. Regardless of what is found in the future, it is likely that H_2S will join NO and CO as an important gasotransmitter. In the vascular system, evidence is strong for a major role of H_2S as a physiologic vasodilator. *S*-sulfhydration as an important mode of posttranslational protein modification is established. As H_2S is generated physiologically in almost all organs of the body, it is likely that functions in diverse tissues, especially the nervous system, will emerge in the not-too-distant future.

Acknowledgments

This work was supported by the National Institutes of Health Medical Scientist Training Program Award (T32 GM007309) to M.M.G., and U.S. Public Health Service Grants (MH018501 and DA000226) and Research Scientist Award (DA00074) to S.H.S.

Abbreviations used

AMPK	AMP-activated protein kinase
BK_{Ca}	large-conductance calcium-activated potassium channels

CBS	cystathionine β-synthase
β-CNA	β-cyano-L-alanine
CO	carbon monoxide
CSE	cystathionine γ-lyase
EDHF	endothelial-derived hyperpolarizing factor
EDRF	endothelial-derived relaxing factor
G3P	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HO	heme oxygenase
H_2S	hydrogen sulfide
K_{ATP}	ATP-sensitive potassium channels
LTD	long-term depression
LTP	long-term potentiation
NaHS	sodium hydrogen sulfide
NANC	non-adrenergic non-cholinergic
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NPAS2	neuronal PAS domain protein 2
PAG	DL-propargylglycine
PIP2	phosphatidylinositol (4,5)-bisphosphate
PLP	pyridoxal 5′-phosphate
SAM	*S*-adenosyl methionine
sGC	soluble guanylyl cyclase
–SH	thiol
–SSH	hydropersulfide
YC-1	3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole.

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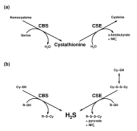

Fig. 1.

(a) The classically described roles of CBS and CSE in sulfur metabolism. CBS condenses homocysteine with serine to generate the thiol ether cystathionine. CSE hydrolyzes cystathionine into cysteine, α-ketobutyrate and ammonia. (b) H_2S producing reactions catalyzed by CBS and CSE. CBS catalyzes the β-replacement reaction of cysteine (Cy–SH) with a variety of thiols (R–SH) to generate H_2S and the corresponding thiol ether (R–S–Cy). CSE catalyzes the β-disulfide elimination reaction of cystine (Cy–S–S–Cy), this is followed by a reaction with a variety of thiols, to generate H_2S and the corresponding disulfide (R–S–S–Cy).

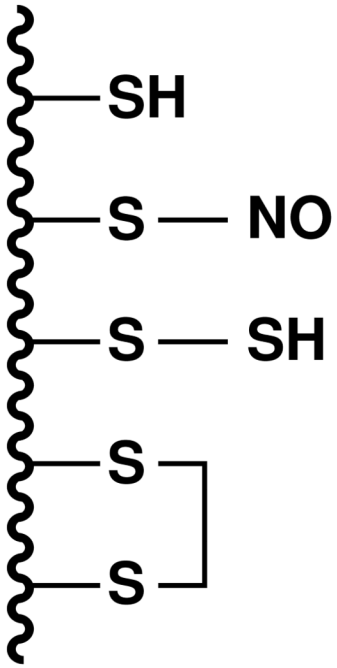

Fig. 2.

A model protein with some of the possible states of the cysteine thiol groups. From the top to the bottom, a free thiol (–SH), an *S*-nitrosylated thiol (–SNO), an *S*-sulfhydrated thiol (hydropersulfide) (–SSH) and a disulfide is shown.

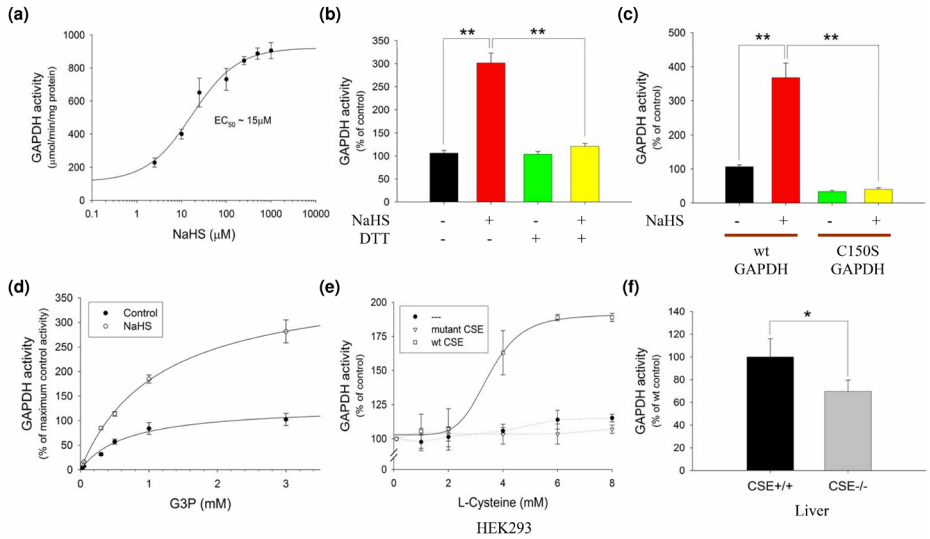

Fig. 3.

(a) Sulfhydration physiologically increases the catalytic activity of GAPDH. GAPDH activity assay *in vitro* at 37 °C with increasing NaHS levels. NaHS dose-dependently activates GAPDH. (b) DTT (1 mM) reverses GAPDH activation by 10 μM NaHS *in vitro*. All results are mean ± SEM. $^{**}P < 0.01$. (c) Wild-type versus C150S mutant GAPDH activity *in vitro* with 15 μM NaHS. Wild-type (wt) but not C150S GAPDH is activated by NaHS. All results are mean ± SEM. $^{**}P < 0.01$. (d) GAPDH activity with increasing substrate, glyceraldehyde 3-phosphate (G3P), levels with or without 10 μM NaHS. NaHS increases overall V_{max} without affecting K_m (~0.8 mM). (e) GAPDH activity in HEK293 cells transfected with nothing, or plasmids endcoding wild-type CSE, or catalytically inactive CSE and incubated with increasing concentrations of L-cysteine in the media for 1 h at 37 °C. GAPDH is activated in a dose-dependent manner in the presence of wild-type CSE. (f) *In vivo* GAPDH activity from wild-type versus $CSE^{-/-}$ liver. $CSE^{-/-}$ mice show decreased GAPDH activity (n = 6 animals). All results are mean ± SEM. $^{*}P < 0.05$. Reproduced with permission from Mustafa *et al*. 2009.

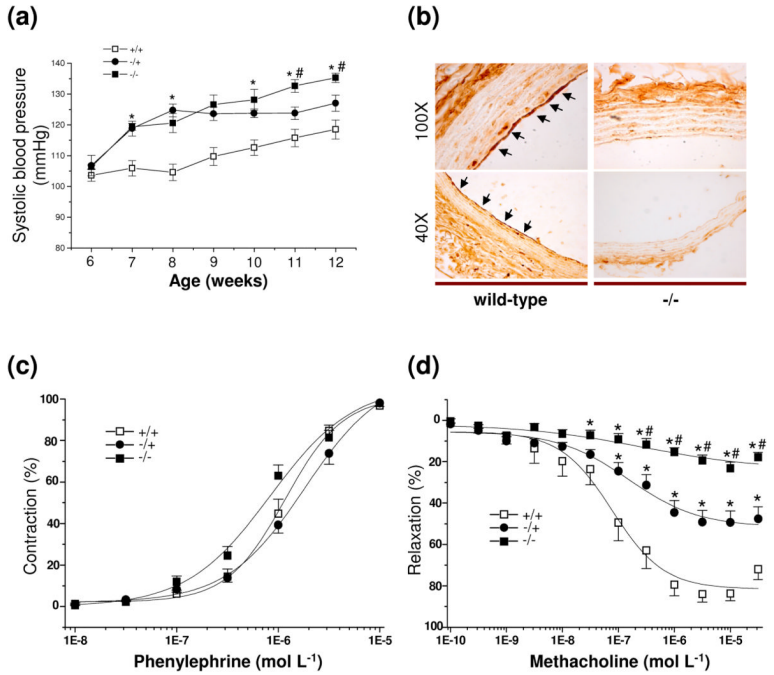

Fig. 4.

(a) Age-dependent hypertensive phenotype of CSE male knockout mice. The hypertensive phenotype peaks at 12 weeks of age with blood pressures 18 mm Hg greater than wild-type control mice (+/+). Blood pressure of heterozygotes (−/+) resembles that of homozygous knockouts (−/−) at early ages, but by 10 weeks of age the homozygous knockout mice display levels 10 mm Hg greater than the heterozygotes (n = 12). (b) Immunohistochemical localization of CSE to the endothelium of arterial blood vessels (black arrows) in wild-type mice. The signal is abolished in CSE knockout mice. (c) The contactile effects of phenylephrine on the mesenteric artery is the same in wild-type, heterozygous and homozygous knockout mice (n = 15). (d) Methacholine relaxation of the mesenteric artery is reduced by about 80% in homozygous CSE knockout vessels and about 50% in heterozygotes (n = 15). All results are means ± SEM. *P < 0.05 versus wild-type; #P < 0.05 versus heterozygote. Reproduced with permission from Yang *et al*. 2008.