H_2S Synthesizing Enzymes: Biochemistry and Molecular Aspects

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Contents

Abstract

Hydrogen sulfide (H_2S) is a biologically active gas that is synthesized naturally by three enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST). These enzymes are constitutively present in a wide array of biological cells and tissues and their expression can be induced by a number of disease states. It is becoming

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increasingly clear that H_2S is an important mediator of a wide range of cell functions in health and in disease. This review therefore provides an overview of the biochemical and molecular regulation of H_2S synthesizing enzymes both in physiological conditions and their modulation in disease states with particular focus on their regulation in asthma, atherosclerosis and diabetes. The importance of small molecule inhibitors in the study of molecular pathways, the current use of common H_2S synthesizing enzyme inhibitors and the relevant characteristics of mice in which these enzymes have been genetically deleted will also be summarized. With a greater understanding of the molecular regulation of these enzymes in disease states, as well as the availability of novel small molecules with high specificity targeted towards H_2S producing enzymes, the potential to regulate the biological functions of this intriguing gas H_2S for therapeutic effect can perhaps be brought one step closer.

Keywords
H_2S synthesizing enzymes • CSE • CBS • 3-MST • Hydrogen sulfide

1 Introduction

Research into the biology of hydrogen sulfide (H_2S) over the last decade has exponentially increased our understanding of the way in which this gasotransmitter influences physiological and pathophysiological (i.e. disease) processes in a wide range of biological systems. With the characteristic and undoubtedly obnoxious odour of rotten eggs and an early history which appeared to mark H_2S as nothing more than a poisonous gas (Beauchamp et al. 1984), it is perhaps surprising that H_2S is synthesized endogenously in mammalian cells by both enzymatic and non-enzymatic mechanisms and that, once formed, H_2S produces the wide array of biological effects which are now being reported (Li et al. 2011). H_2S has therefore followed an interesting 'development curve' in the minds of researchers starting as an agent which was of toxicological (sometime devastatingly so) impact, via a gas of interest to pharmacologists to a molecule which now occupies a very much more central position in gas physiology and with growing therapeutic potential.

The last decade has seen multiple advances in our knowledge of the mechanisms by which H_2S is synthesized, how these are regulated, the tissue and cellular distribution of H_2S synthesizing enzymes in tissues and how this distribution changes in disease. These various areas form the central themes of this present review.

H_2S can be produced naturally in the body by at least three enzymes. Cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) are known to be significant producers of endogenous H_2S. Collectively they have been termed 'H_2S synthesizing enzymes'. Both CSE and CBS are pyridoxal-5′-phosphate (PLP) dependent and utilize L-cysteine as substrate whilst 3-MST is non-PLP dependent and uses 3-mercaptopyruvate as substrate (Shibuya et al. 2009a; Steegborn

et al. 1999). Endogenous H_2S can also be produced non-enzymatically. For example, erythrocytes, supplemented with glucose and sulfur and containing electron carriers such as NADH, NADPH and reduced glutathione (GSH), spontaneously react with sulfur to produce H_2S (Searcy and Lee 1998). Iron-sulfur cluster containing proteins carrying Fe_2S_2, Fe_3S_4 or Fe_4S_4 clusters are another source of non-enzymatically generated H_2S. These include proteins such as ferredoxins and 'Rieske' proteins among others (Beinert et al. 1997). In the presence of reducing agents such as glutathione, H_2S can also be released from bound sulfur in, for example, persulfides in neurons and astrocytes (Ishigami et al. 2009). Although non-enzymatic routes for H_2S generation have been identified, it seems likely that the bulk, if not all, of the H_2S which is generated for the purpose of carrying out biological functions in the body is derived enzymatically. This no doubt explains the huge emphasis in recent years in understanding as much as possible about the biochemistry, molecular biology and physiological relevance of the H_2S synthesizing enzymes.

The list of reported biological effects of both endogenous and exogenously administered H_2S is far reaching and expanding on an almost weekly basis. Numerous molecular targets for this gas have also been identified. The biological effects of H_2S are outside the scope of this present review and are dealt with elsewhere in this volume. Rather, we will provide here an overview of the H_2S synthesizing enzymes. This will encompass a discussion of the way in which H_2S synthesizing enzymes are regulated in health and in disease, as well as a consideration of the limitations of the currently available H_2S synthesizing enzyme inhibitors which have been widely used to probe the biological roles of this gas.

2 H_2S Synthesizing Enzymes: The Beginnings

The detection of sulfide in brain tissue in 1989 prompted the suggestion that H_2S, produced endogenously, may have a role to play in physiology (Goodwin et al. 1989). The subsequent demonstration that H_2S produced by the enzyme CBS in the mammalian brain selectively enhanced NMDA receptor-mediated responses and facilitated the induction of hippocampal long-term potentiation led to the first proposed biological role of this gas as a neuromodulator (Abe and Kimura 1996). Shortly thereafter, the second H_2S producing enzyme, CSE, was found to be expressed in guinea pig ileum, rat portal vein and thoracic aorta, and the smooth muscle relaxant effect of H_2S was noted for the first time (Hosoki et al. 1997). This seminal work led to a number of detailed investigations which rapidly established the vascular (Zhao et al. 2001a, b) and gastrointestinal (Teague et al. 2002) muscle relaxant properties of this gas. Several years later, the third H_2S synthesizing enzyme (3-MST) was discovered when brain homogenates of CBS knockout mice were shown to produce H_2S at levels similar to that of wild-type mice in the absence of PLP, suggesting redundancy of CBS and the existence of yet another H_2S producing enzyme. This new enzyme, 3-MST, produced H_2S from 3-mercaptopyruvate, which is derived from cysteine in the presence of α-ketoglutarate. This latter reaction is catalysed by the enzyme cysteine

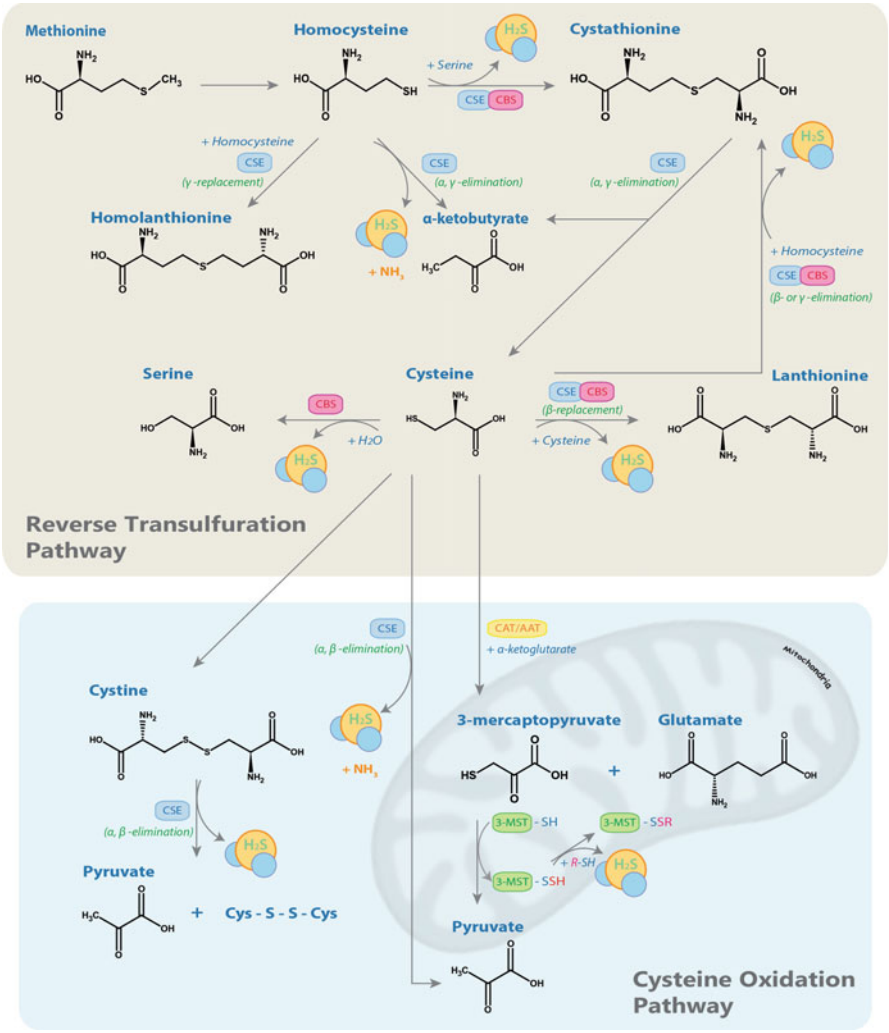

Fig. 1 Pathways for H_2S biosynthesis in the cell. The endogenous production of H_2S occurs via two main pathways—reverse transsulfuration and cysteine oxidation which take place partly inside mitochondria. 3-MST, 3-mercaptopyruvate sulfurtransferase; CBS, cystathionine β-synthetase; CSE, cystathionine γ-lyase; H_2S, hydrogen sulfide; NH_3, ammonia

aminotransferase (CAT), which is identical with aspartate aminotransferase (AAT) (Fig. 1) (Shibuya et al. 2009b).

Over recent years, considerable attention has been focused on the biochemistry and molecular biology of the H_2S synthesizing enzymes. Much is now known about the mechanics of H_2S biosynthesis in terms of substrate specificity, cofactor requirement and tissue and cell distribution, and the molecular topology of these enzymes has also been described. Interested readers are referred to several excellent

reviews on this topic (e.g. Kabil and Banerjee 2014; Kabil et al. 2014; Nagahara et al. 2007; Singh and Banerjee 2011). In contrast, there have been few concerted attempts to review changes in the expression or activity of the H_2S synthesizing enzymes with disease states and this review will thence, in part, concentrate on this aspect.

3 Endogenous Synthesis of H_2S and Regulation of Its Synthesizing Enzymes Under Physiological Conditions

CBS catalyses the formation of cystathionine via two separate pathways. The first pathway involves linking homocysteine with serine (Stipanuk 1986) and the second pathway links homocysteine with cysteine (Chen et al. 2004). H_2S is produced only from the second reaction. Besides generating cystathionine, CBS also converts cysteine to serine and lanthionine, and in the process yields H_2S. These reactions are part of the reverse transsulfuration pathway of H_2S biosynthesis (See Fig. 1).

Although found in many organs including brain, kidney, liver, ileum, uterus, placenta (Patel et al. 2009) and pancreatic islets (Kaneko et al. 2006), mouse tissue-specific quantification of CBS revealed that this enzyme is predominantly expressed in the brain and kidney (Kabil et al. 2011a).

The regulation of CBS activity is dependent on the presence of several cofactors and activators. Besides requiring PLP to function, the NH_2 terminal end of CBS contains the binding site of a heme cofactor group that further regulates the activity of CBS by functioning as a redox-sensitive gas sensor (Banerjee and Zou 2005). Because CBS contains iron, the oxidation state of CBS can be toggled, viz. ferric (Fe^{3+}) to ferrous (Fe^{2+}) CBS by reducing agents such as NADPH in the presence of diflavin methionine synthase reductase (Kabil et al. 2011b). In the ferrous (Fe^{2+}) state, CBS can be bound by other gasotransmitters like CO (Puranik et al. 2006) and NO (Taoka and Banerjee 2001), resulting in downregulation of the activity of CBS. Subsequently, the oxidation of the ligand-bound CBS (i.e. CO-CBS or NO-CBS) by air to the ferric (Fe^{3+}) state restores the catalytic activity of CBS. Such a regulatory mechanism suggests a complex interplay between the different gasotransmitters in controlling endogenous H_2S biosynthesis at least via the CBS route. This would further imply that in conditions such as inflammation or hypoxia in which cellular NO and CO levels may be either increased or decreased, the precise effect on CBS activity and hence H_2S generation by this enzyme will be difficult to predict.

CBS activity is also regulated by allosteric binding of S-adenosylmethionine (SAM) (Finkelstein et al. 1975), which is a co-substrate that is involved in a multitude of other metabolic pathways including transmethylation, transsulfuration and aminopropylation (Cantoni 1952). Using mice in which the *MAT1A* gene (which encodes for SAM) has been disrupted and human hepatocellular carcinoma cells which have the *MAT1A* gene silenced, it was demonstrated that the binding of SAM to CBS stabilizes the protein against proteolysis (Prudova et al. 2006). In vitro, SAM increased CBS enzyme activity and thence H_2S production (Finkelstein et al. 1975).

CBS activity is not only regulated by cofactors and activators but also by its location within the cell. Using yeast cells as a model system, human CBS has been shown to be post-translationally modified by the small ubiquitin-like modifier-1 (SUMO-1) protein at its C-terminal regulatory domain. SUMOylation enables CBS to be shuttled into the nucleus where it associates with the nuclear scaffold (Kabil et al. 2006). SUMOylation of CBS was enhanced by human polycomb group protein 2 (hPc2). It has been proposed that nuclear localization of CBS occurs when local glutathione demand is high, since CBS generates cysteine, which is the rate-limiting amino acid in glutathione synthesis (Agrawal and Banerjee 2008). Coupled with the observation that regulation of CBS was correlated with the rate of proliferation in human and yeast cells through a redox-sensitive mechanism (Maclean et al. 2002), it is possible that the presence of CBS in the nucleus provides a source of an antioxidant (i.e. H_2S) in the cell when, and if, required. This possibility warrants further investigation to determine whether such a mechanism does indeed exist in cells either in vitro or in vivo.

Like CBS, cystathionine γ-lyase (CSE) uses homocysteine as substrate to produce H_2S, α-ketobutyrate, ammonia and homolanthionine. CSE can also generate H_2S from cysteine, forming cystathionine and pyruvate (See Fig. 1). Although both homocysteine and cysteine are substrates of CSE, at physiological concentrations of cysteine and homocysteine, kinetic simulations employing physiologically relevant concentrations of homocysteine and cysteine using the dithiobisnitrobenzene (DTNB) assay showed that α- and β-elimination of cysteine catalysed by CSE accounts for approximately 70 % of the H_2S produced while the α,γ-elimination of homocysteine contributes about 29 %. However, once homocysteine levels are elevated to those apparent in hyperhomocysteinemia, then about 90 % of the H_2S generated by CSE occurs via α,γ-elimination and γ-replacement reactions of homocysteine (See Fig. 1) (Chiku et al. 2009). This indicates that the identity of the main substrate for CSE-dependent generation of H_2S in cells is likely to depend on the relative availability of cysteine and homocysteine.

CSE is located in a wider range of tissues than CBS. CSE activity is several-fold higher in human brain than in mouse brain (Diwakar and Ravindranath 2007). The mouse small intestine, stomach (Ishii et al. 2004), rat portal vein and thoracic aorta (Hosoki et al. 1997), mouse pancreas (Kaneko et al. 2006), rat uterus, human placenta, myometrium, amnion and chorion (Patel et al. 2009) all express CSE. As compared to CBS, CSE is more abundant in mouse liver where it is the principal enzyme responsible for H_2S generation (Kabil et al. 2011a).

Like CBS, the activity of CSE is also regulated by a number of cofactors. CSE was initially reported to be activated physiologically by calcium-calmodulin after stimulation of muscarinic cholinoceptors in vascular endothelial cells in culture, as determined by an increase in CSE activity and H_2S production. Through co-immunoprecipitation experiments, recombinant CSE was found to bind directly to calmodulin (Yang et al. 2008). Although Ca^{2+} has been suggested to activate CSE, the precise concentration of Ca^{2+} is critical as too much Ca^{2+} inhibits purified rat CSE-mediated H_2S production in the presence of PLP. At physiologically relevant Ca^{2+} concentrations (i.e. ~100 nM) H_2S was efficiently produced in the

presence of PLP. However, H_2S production was decreased at 300 nM Ca^{2+} and reduced even further at concentrations up to 3 μM. The involvement of calmodulin in regulating CSE activity however is not yet clear since a recent study showed that neither calmodulin nor a calmodulin antagonist, W-7, affected the production of H_2S by purified rat liver CSE (Mikami et al. 2013).

SUMO, besides decreasing CBS activity, also targets CSE in vitro, in that recombinant human CSE can also be SUMOylated (Agrawal and Banerjee 2008). However, whether the SUMOylation of CSE occurs within the cell and has a physiological effect in consequence is not clear. If this is indeed the case then the re-localization of both CBS and CSE into the nucleus may be a strategy to increase cysteine levels inside the nucleus when the demand for glutathione is increased such as during early phases of cell proliferation (Markovic et al. 2007) or when telomerase activity is high as observed in 3T3 fibroblast cells (Borrás et al. 2004).

As an important intracellular antioxidant protecting the cell against oxidative stress, physiological glutathione levels are well controlled via glutathione synthesis in the transsulfuration pathway. Without glutathione, numerous cell types including lymphocytes (Franco and Cidlowski 2009), embryos (Dalton et al. 2004) and hepatocytes (Ghibelli et al. 1999) undergo apoptosis due to excess reactive oxygen species (ROS) generation. Since activation transcription factor 4 (ATF4) controls expression of genes responsible for amino acid metabolism and redox status, ATF4 might, by increasing glutathione synthesis through the transsulfuration pathway, be a key cellular survival factor. Indeed, the absence of ATF4 in mouse embryonic fibroblasts resulted in a substantial fall in glutathione levels due to downregulation of CSE expression (Dickhout et al. 2012). Thus, if the amount of H_2S synthesizing enzymes in the nucleus are reduced, the levels of glutathione would subsequently be reduced, thereby affecting the fate of the cell.

The most recently discovered H_2S synthesizing enzyme 3-MST, unlike CBS and CSE, is PLP independent. 3-MST catalyses the conversion of 3-mercaptopyruvate to pyruvate by degrading cysteine. In this pathway, cysteine is first converted by cysteine aminotransferase (CAT), also known as aspartate aminotransferase (AAT), to 3-mercaptopyruvate via the incorporation of α-ketoglutarate into the reaction. 3-MST then forms a persulfide by transferring a sulfur from 3-mercaptopyruvate onto itself which in the presence of a reductant like thioredoxin produces pyruvate and H_2S (Nagahara et al. 2007; Yadav et al. 2013). Dihydrolipoic acid has also been identified to associate with 3-MST to release H_2S (Mikami et al. 2011a).

Like CBS and CSE, 3-MST is found in many tissues with high activity in the proximal tubular epithelium of the kidney, pericentral hepatocytes in the liver, cardiac cells and neuroglial cells (Shibuya et al. 2009b). Within the cell, 3-MST is located both in the cytoplasm as well as the mitochondria. However, because the concentration of cysteine is much higher in the mitochondria, as compared to the cytoplasm, it is plausible that most of the H_2S generated by 3-MST occurs in mitochondria (Nagahara et al. 1998).

H_2S production by 3-MST can be significantly inhibited by aspartate which is a potent inhibitor of CAT (Akagi 1982). H_2S produced by 3-MST and CAT in retinal neurons is also regulated by Ca^{2+}. A negative feedback mechanism has been

proposed whereby H_2S produced after activation by Ca^{2+} in turn suppresses Ca^{2+} influx into photoreceptor cells by activating the vacuolar-type $H(_+)$-ATPase (V-ATPase). The activated V-ATPase then releases protons into the synaptic cleft thereby suppressing Ca^{2+} channels found in the photoreceptor cells (Mikami et al. 2011b).

Unlike CBS and CSE, the activity of which is regulated for the most part by binding of other factors, 3-MST activity is probably regulated intrinsically by its redox state. Structural studies have shown that 3-MST contains catalytic site cysteines (Cys^{247}, Cys^{154} and Cys^{263}) each of which is redox active (Nagahara 2013). Cys^{154} is unique to rat 3-MST whilst Cys^{263} is conserved in mammalian 3-MST (Nagahara et al. 2007). The activity of 3-MST is regulated by both intermolecular and intramolecular redox-sensing switches as reviewed elsewhere (Nagahara 2013). Nevertheless, it is important to note that the redox-dependent modulation of 3-MST may not be relevant in human MST since the cysteine residues (i.e. Cys^{154}) which are involved in the cross-linking in rat MST are not conserved in the human enzyme (Nagahara et al. 2007).

4 Molecular Regulation of H_2S Synthesizing Enzymes

Hydrogen sulfide contributes to cell signalling by regulating the function of a number of molecular targets including ion channels (Sun et al. 2008; Zhao et al. 2001b), kinases (Hu et al. 2007) and transcription factors such as signal transducer and activator of transcription (STAT) (Calvert et al. 2009; Li et al. 2009a) as well as nuclear factor-kappaB (NFκB) (Li et al. 2011; Oh et al. 2006). However, the expression of the enzymes that are responsible for producing H_2S (i.e. CSE, CBS and 3-MST) are also frequently regulated at a transcriptional and subsequently at a protein level by multiple molecular factors. In many disease states, the expression of these enzymes is both inducible and dynamic. With a better understanding of how the H_2S synthesizing enzymes are regulated in disease states, a more targeted effort could be developed to identify drugs to affect endogenous production of H_2S in disease states with a view to restoring physiological H_2S levels.

To date, changes in expression (mRNA and/or protein) of H_2S synthesizing enzymes have been identified in a number of cells and tissues in a variety of different disease states and involving the intermediacy of a range of different signalling pathways (summarized in Table 1). Most evidence collected to date suggests a pivotal role for NFκB in the control of cellular CSE and CBS expression, and this interaction will therefore be described in some detail below.

In an early study seeking to identify the molecular targets of H_2S, it was shown that H_2S inhibited the activation of NFκB in lipopolysaccharide (LPS)-stimulated mouse macrophages. The mechanism is believed to be via increased heme-oxygenase-1 (HO-1) expression under the control of an ERK1/2-dependent pathway. Increased HO-1 expression then led to reduced IκB phosphorylation and degradation and impaired translocation of NFκB into the nucleus

Table 1 Examples of modulated H_2S producing enzymes in selected disease states

Disease/stress	Model	Factors/signalling involved	Location	Regulation of H_2S producing enzyme	Reference
Atherosclerosis	RAW264.7 macrophages	Akt signalling	–	↑ CSE (mRNA & protein)	Xu et al. (2014b)
	High-fat diet in mice	–	Liver, lung Liver, kidney	↓ CSE (protein) ↑ CBS (protein) ↓ 3-MST (protein)	Peh et al. (2014)
	High-fat diet in mice	–	Liver	↑ CSE & CBS (mRNA)	Hwang et al. (2013)
Asthma	Ovalbumin-treated mice	–	Lung	↓ CSE (protein)	Zhang et al. (2013)
	Ovalbumin-treated rats	–	Lung	↓ CSE (protein)	Chen et al. (2009)
Bowel syndromes	Visceral hyperalgesia in rats	Voltage-gated sodium channel	Colon	↑ CBS (protein)	Wang et al. (2012b)
	Chronic visceral hyperalgesia in rats	NFκB	Colon	↑ CBS (protein)	(Li et al. 2012)
Cancer	Colon cancer	–	Colon	↓ 3-MST (protein)	Ramasamy et al. (2006)
	Human hepatocellular carcinoma (HCC) cell lines	Sp1, PI3K/Akt (PKB)	–	↑ CSE (protein)	Yin et al. (2012)
	Down's syndrome megakaryocytic leukaemia cell line (CMK)	Sp1, Sp3	–	↑ CBS (mRNA)	Ge et al. (2003)
Diabetes	Streptozotocin-treated rats	–	Liver	↓ CSE (protein)	Manna et al. (2014)
	INS-1E cells, rat pancreatic islets	Glucose	–	↓ CSE (mRNA & protein)	Zhang et al. (2011)
	Streptozotocin-treated rats	Insulin	Liver	↑ CBS (mRNA)	Ratnam et al. (2002)

(continued)

Table 1 (continued)

Disease/stress	Model	Factors/signalling involved	Location	Regulation of H_2S producing enzyme	Reference
Down's syndrome	Patients with Down's syndrome	–	Astrocytes	↑ CBS (protein)	Ichinohe et al. (2005)
Hypoxia	U87-MG human glioblastoma cells, PC12 rat cells, rats exposed to hypoxic conditions	Hypoxia-inducible factors (HIFs)	Cerebral cortex	↑ CBS (mRNA & protein)	Takano et al. (2014)
Inflammation	LPS-treated mammalian cell lines (HEK-293, COS-7, J774.1A, RAW264.7)	NFκB	–	↑ CSE (mRNA & protein)	Wang et al. (2014)
	Temporomandibular joint pain in rats	Inhibition of voltage-gated potassium currents	Trigeminal ganglion neuron	↑ CBS (mRNA & protein)	Miao et al. (2014)
	LPS-induced inflammation in rats	Dexamethasone	Neutrophils	↓ CSE (protein)	Li et al. (2009b)
	LPS-induced inflammation in mice	–	Liver, kidney	↑ CSE (mRNA)	Li et al. (2005)
Oxidative stress	Mammalian cells (A549, SMMC-7721)	H_2O_2	–	↑ CSE (mRNA & protein)	Wang et al. (2012a)
	Rat mesangial cells	Platelet-derived growth factor (PDGF)-BB, STAT, Nrf2	–	↑ CSE (mRNA & protein)	Hassan et al. (2012)
Renal ischaemia	Renal ischaemia/reperfusion in rats	Sp1	Kidney	↓ CBS (mRNA & protein)	Wu et al. (2010)
Ulcerations	Gastric ulcers in rats	–	Stomach	↑ CSE & CBS (protein)	Wallace et al. (2007)

3-MST 3-mercaptopyruvate sulfurtransferase, *Akt*/*PKB* protein kinase B, *CBS* cystathionine β-synthetase, *CSE* cystathionine γ-lyase, H_2S hydrogen sulfide, H_2O_2 hydrogen peroxide, *Nrf2* nuclear factor like 2, *PI3K* phosphoinositide 3-kinase, *Sp1* specificity protein 1, *STAT* signal transducer and activator of transcription

(Oh et al. 2006). Later studies confirmed that H_2S affects NFκB but by a different mechanism. In this case, H_2S was shown to sulfhydrate NFκB on the p65 subunit at the cysteine-38 residue. This molecular event then increased NFκB binding to a co-activator ribosomal protein, S3 (RPS3), which triggers the transcription of antiapoptotic genes (Sen et al. 2012). Interestingly, recent work has shown that NFκB also regulates CSE expression by binding to its promoter region in macrophages challenged with LPS (Wang et al. 2014). In a separate study, rats with chronic visceral hyperalgesia displayed upregulated colonic CBS expression. An inhibitor of the p65 subunit of NFκB, pyrrolidine dithiocarbamate (PDTC), reduced the expression of CBS, suggesting that activation of the p65 subunit of NFκB upregulates CBS expression (Li et al. 2012). Thus, it appears that H_2S may act to maintain the levels of CSE and CBS inside the cell in check during an inflammatory response by regulating the levels of NFκB.

Drugs have also been shown to regulate CSE expression. In the case of atherosclerosis, statins, a class of drugs used to lower cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), were recently reported to upregulate CSE transcription and subsequent H_2S generation. This effect was mediated by activation of Akt signalling (Xu et al. 2014b). In inflammation, dexamethasone, a glucocorticoid steroid, reduced CSE expression in neutrophils isolated from LPS-challenged rats. Dexamethasone, in this model, was proposed to inhibit the formation of pro-inflammatory H_2S, most probably through reduced NFκB-mediated CSE expression (Li et al. 2009b).

In certain disease conditions, the effect of the disease itself could also have a role to play in directly regulating the expression of H_2S synthesizing enzymes. For example in diabetes, high glucose significantly reduced CSE mRNA and CSE activity levels in freshly isolated rat pancreatic islets and in the rat pancreatic cell line, INS-1E. This was mediated by stimulating phosphorylation of the transcription factor SP1 via p38 MAPK activation which subsequently led to decreased CSE promoter activity (Zhang et al. 2011).

5 H_2S Synthesizing Enzymes in Diseases States

5.1 Asthma

Asthma is a chronic inflammatory disease characterized by shortness of breath, wheezing and recurring cough. It is caused by a combination of excess airway mucus production and reversible airflow obstruction. Immune cells such as eosinophils and neutrophils play a major role in this inflammatory state although different patients with severe asthma may exhibit different phenotypes that need to be properly defined if the most effective treatment is to be provided (Gibeon and Chung 2012). Current methods for distinguishing different phenotypes of asthma involve detecting sputum eosinophils (eosinophilic asthma), serum periostin levels which is a biomarker for eosinophilic asthma (Jia et al. 2012) and breath nitric oxide (NO) levels (Dweik et al. 2011). H_2S is one such potential biomarker of asthma

given that it was found to be elevated in the sputum of asthmatics (Chung 2014; Saito et al. 2013).

Endogenous serum H_2S levels were found to be altered in chronic obstructive pulmonary disease (COPD) in man almost a decade ago (Chen et al. 2005). This group reported that endogenous serum H_2S levels were higher in patients with COPD as compared to patients with acute exacerbation of COPD (AECOPD). Serum levels decreased in patients with stable COPD as the condition became clinically more severe. Subsequently, serum H_2S levels in asthmatic children were reportedly decreased compared with a matched healthy control group (Tian et al. 2012).

A more recent clinical study examined levels of H_2S in serum and sputum from 40 patients with varying degrees of severity of asthma and compared the data obtained with that of 15 healthy subjects (Saito et al. 2013). H_2S levels in sputum of asthma patients were significantly higher than those in sputum from healthy subjects. Serum H_2S concentration in asthmatics was also 10 times higher than that in sputum. Sputum measurements are more likely to be indicative of asthma as measurement of serum H_2S will be confounded by the presence of H_2S generated by non-respiratory tissues. Moreover, a positive correlation was identified between sputum H_2S levels and sputum neutrophil number. These observations raise the possibility that sputum H_2S concentration may perhaps be a biomarker of neutrophilic asthma.

Since endogenous H_2S levels are modulated during asthma, it is conceivable that the activity or expression of H_2S synthesizing enzymes may also be altered at one or other time in the course of the disease (See Table 1). To date, there have been few studies to investigate this possibility, although there are clues in the literature that this may be the case. For example, it has recently been shown, using mouse lung slices, that exogenous H_2S impeded airway contraction of smooth muscle cells by inhibiting intracellular Ca^{2+} release evoked by inositol-1,4,5-triphosphate (InsP3). This effect was also apparent when murine lung tissues was treated with L-cysteine (an endogenous H_2S precursor) which effect was reversed by the irreversible CSE inhibitor, DL-propargylglycine (PAG) (Castro-Piedras and Perez-Zoghbi 2013). More direct evidence comes from experiments using an ovalbumin (OVA)-induced mouse model of acute asthma. In this case, CSE expression was downregulated in the lungs of wild-type mice challenged with OVA, whilst mice in which CSE was knocked out ($CSE^{-/-}$) displayed augmented airway inflammation following ovalbumin treatment as indicated by higher levels of Th2 cytokines such as IL-5, IL-13 and eotaxin-1. Exogenous H_2S administration, in the form of NaHS, effectively 'rescued' $CSE^{-/-}$ mice from the exacerbated symptoms of asthma strongly implying that an upregulated CSE/H_2S system has a protective role during the development, or perhaps the maintenance, of asthma in this model (Zhang et al. 2013). Whether such a mechanism also occurs in man is not yet known. Considerable evidence now exists, reviewed elsewhere in this volume, that H_2S exhibits both pro- and anti-inflammatory effects in a range of animal models. Thus, whether an H_2S donor administered may prove beneficial or deleterious in asthma remains an open question.

5.2 Atherosclerosis

H_2S exhibits pronounced vasodilator activity both in vitro and in vivo, and it is hence no surprise that deranged H_2S biosynthesis has been implicated in a number of cardiovascular diseases including atherosclerosis. H_2S dilates blood vessels by various mechanisms, including the opening of potassium-activated ATP channels (K_{ATP} channels) in vascular smooth muscle (Zhao et al. 2001a) as well as potentially via intracellular acidification by activating of the Cl^-/HCO_3^- exchanger (Lee et al. 2007). Atherosclerosis is a chronic and complex inflammatory condition. Its pathogenesis involves an intricate tapestry of prolonged immune cell recruitment and cytokine/chemokine secretion coupled with the presence of reactive oxygen species (ROS), as well as vascular smooth muscle cell proliferation and migration (Ross 1999). Intriguingly, each of these separate events has been reported to be regulated by H_2S (Du et al. 2004; Yan et al. 2006). However based on the evidence available thus far, the link between H_2S and atherosclerosis and the involvement of the H_2S synthesizing enzymes in the process is far from clear.

In a recent study, Peh and colleagues reported reduced expression of CSE and CBE protein in liver, kidneys and lungs of mice fed a high-fat diet. 3-MST expression was also reduced in liver. In addition, CSE/CBS and 3-MST enzyme activity was also diminished as determined by ex vivo H_2S synthesis using either cysteine or 3-mercaptopyruvate as substrates. It should be noted that these animals showed no evidence of frank atherosclerosis in that plasma levels of serum amyloid A (SAA) and C-reactive protein (CRP) were normal and the histological appearance of blood vessels revealed no ongoing disease. Changes in the expression of these enzymes were therefore likely to be a consequence of fat feeding and not a reaction to vascular disease. If this is the case, then changes in H_2S synthesis may be presumed to occur before the onset of atherosclerosis (Peh et al. 2014). Whether measurement of these enzymes in susceptible individuals might be a surrogate biomarker of impeding vascular damage due to atherosclerosis remains to be determined. Intriguingly, in a separate study, mice fed a high-fat diet leading to the development of fatty liver exhibited higher levels of CSE and CBS in the liver (Hwang et al. 2013). The mechanisms which control CSE, CBS and 3-MST expression in animals consuming fat in their diet warrant further study.

Other researchers have studied H_2S synthesizing enzyme expression and activity in apolipoprotein E (ApoE) knockout mice. These animals have increased plasma levels of cholesterol, triglyceride and low-density lipoprotein cholesterol, develop a frank atherosclerotic state and also show evidence of defective H_2S biosynthesis including lower production of H_2S in aorta and reduced plasma H_2S concentration. Interestingly, CSE mRNA expression in the aorta was actually increased. Administration of exogenous H_2S (NaHS) elevated plasma H_2S and reduced aortic CSE mRNA thereby suggesting an inverse correlation between CSE expression and H_2S levels in the aorta which may be a consequence of a positive feedback mechanism in which decreased H_2S production (due to vascular disease) is compensated for by increased CSE gene expression (Wang et al. 2009). Interestingly, elevated plasma H_2S has also been reported in patients with atherosclerosis (Peter et al. 2013), whilst

diminished production of endogenous H_2S which is apparent in $CSE^{-/-}$ mice is associated with accelerated atherosclerosis (Mani et al. 2013). In this latter work, the rate of development of atherosclerosis in these animals could be reduced by replacement therapy with NaHS. The current consensus is therefore that lack of H_2S contributes to accelerated progression of atherosclerosis (Szabó et al. 2011; Xu et al. 2014a; Zhang et al. 2012).

5.3 Diabetes

Altered expression of H_2S synthesizing enzymes as well as endogenous H_2S levels has been observed in diabetic animals. In an early study, plasma H_2S and the expression of CSE and CBS were elevated in the pancreas of streptozotocin (STZ)-induced diabetic rats (Yusuf et al. 2005) which suggests a possible correlation between Type 1 diabetes (Schnedl et al. 1994) and increased pancreatic and liver H_2S synthesis. To evaluate more precisely the role of H_2S in diabetes, a subsequent study made use of $CSE^{-/-}$ mice injected with STZ to provoke a diabetic state. Since the pathogenesis of diabetes is associated with reduced number, but increased activity, of K_{ATP} channels in β-pancreatic cells, these authors sought to compare the mass and K_{ATP} channel activity of β-pancreatic cells in $CSE^{-/-}$ mice with that of wild-type mice (Yang et al. 2011). CSE deficiency protected mice from STZ-induced diabetes as well as the damage and dysfunction of pancreatic islets—as determined by measurement of the changes in insulin secretion and electrophysiological recording of K_{ATP} channel currents after induction of a diabetic state.

Curiously, other studies have reported diametrically opposite results. For example, it was previously noted that circulating H_2S levels were lower (c.f. control, non-diabetic animals) in both STZ-treated rats and non-obese diabetic (NOD) models of Type 1 diabetic mice (Brancaleone et al. 2008; Jain et al. 2010). A similar conclusion was reached from the examination of blood H_2S levels in humans with diabetes (Jain et al. 2010). CSE enzyme activity was also reduced in the liver of Type 1 diabetic rats as well as in peripheral blood mononuclear cells from Type 1 diabetic patients (Manna et al. 2014).

In contrast to Type 1 diabetes which is caused by the death of β-pancreatic cells, Type 2 diabetes arises, usually later in life, with the development of insulin resistance. To study the possible role of H_2S in this process, 6-month-old $CSE^{-/-}$ mice were fed for 8 weeks with a high-fat diet in order to induce hyperglycaemia (Okamoto et al. 2013). The mice developed impaired glucose tolerance and decreased insulin content in their pancreatic cells as compared to their wild-type counterparts. It was noted that the lack of CSE in these animals promoted thioredoxin-binding protein-2 (TBP-2) gene expression which increases insulin insensitivity and causes glucose intolerance (Yoshihara et al. 2010). These results indicate that changes in expression or activity of CSE (and perhaps other H_2S synthesizing enzymes) are implicated in the outcome and severity of both Type 1 and 2 diabetes.

6 Frequently Used Inhibitors and Mouse Knockout Models of H_2S Synthesizing Enzymes

To study the biological effects of endogenously synthesized H_2S, as well as to study the characteristics and behaviour of the H_2S synthesizing enzymes, the use of selective inhibitors of H_2S synthesizing enzymes is crucial. Currently, several inhibitors of CSE and CBS have been used (See Fig. 2) although to date, pharmacological inhibitors of the enzyme 3-MST have yet to be identified. In this context, L-aspartate inhibits the production of H_2S by 3-MST indirectly by inhibiting CAT/AAT (Akagi 1982). Mice with genetic deletion of one or other of the H_2S synthesizing enzymes are also beginning to show their value to the field as well. Of the three known H_2S synthesizing enzymes only $CSE^{-/-}$ mice have been widely used as 90 % of $CBS^{-/-}$ mice died during the first 2 weeks of neonatal life because of growth retardation and severe hepatopathy (Watanabe et al. 1995) and only lately has it been possible to produce 3-MST knockout mice (Nagahara 2013). However, recently a non-neonatal fatal mouse model with the *cbs* gene inactivated but instead expresses low levels of the human CBS transgene and exhibits classical homocystinuria could provide a useful, alternative to a $CBS^{-/-}$ mouse model (Maclean et al. 2010).

Some of the commonly used pharmacological inhibitors of CSE are DL-propargylglycine (PAG) (Marcotte and Walsh 1975) which irreversibly inhibits CSE by physically obstructing the access of the substrate to the active site of the enzyme (Sun et al. 2009) and the reversible inhibitor β-cyanoalanine (BCA) which has also been proposed as a competitive inhibitor of this enzyme (Pfeffer and Ressler 1967). However, these compounds have frequently been claimed to exhibit poor selectivity, require high concentrations and have limited ability to permeate the cell membrane (Szabó 2007). For example, PAG was reported many years ago to cause an irreversible inactivation of both aspartate aminotransferase (Tanase and Morino 1976) and alanine transaminase (Burnett et al. 1980). Aminooxyacetic acid (AOAA) is also very commonly used and is often stated to be a selective inhibitor of CBS (d'Emmanuele di Villa Bianca et al. 2009; Oh et al. 2006; Roy et al. 2012). However, experiments using recombinant human CSE and CBS enzymes have revealed that AOAA inhibits both CSE and CBS. Indeed, AOAA appeared to be

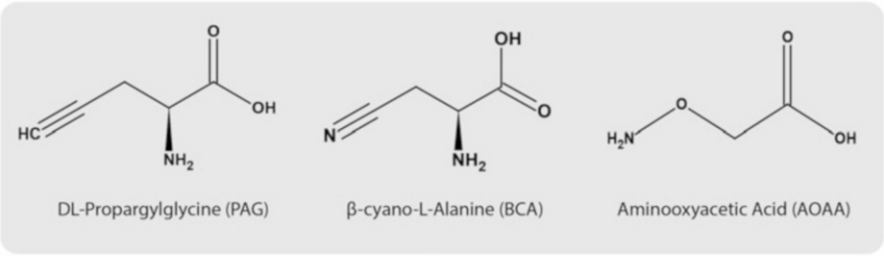

Fig. 2 Chemical structures of compounds that inhibit CSE or CBS

an even more potent inhibitor of CSE than CBS at least under these experimental conditions (Asimakopoulou et al. 2013). Moreover, AOAA, like PAG, is a general inhibitor of pyridoxal phosphate-dependent enzymes and has been reported to inhibit enzymes such as 4-aminobutyrate aminotransferase (GABA-T) (WALLACH 1961) and aspartate aminotransferase (Kauppinen et al. 1987).

7 Conclusion

Much has been learnt about the physiological and pathophysiological implications of H_2S since it was realized that this evanescent gas is formed naturally in cells and tissues in the 1990s. Indeed, research in the last two decades has thrown H_2S very much into the spotlight and it is now widely referred to as the so-called third gasotransmitter after CO and NO. Many mechanisms governing the regulation of H_2S synthesizing enzymes both in homeostatic and disease conditions have been elucidated. However, many questions still remain to be answered. For example, the quantitative contribution that each of the three H_2S synthesizing enzymes makes to the levels of H_2S found naturally in cells in health and disease remains unclear. This is important since, as detailed in this review, the presence and/or activity of such enzymes may be useful biomarkers of disease progression in some cases and it is critical to understand which H_2S synthesizing enzyme is important in which cell at which time point and how this is affected by disease. Moreover, whilst the biological significance of H_2S has been under intense scrutiny for more than two decades, there are still no reliably potent and selective inhibitors of any of the three H_2S synthesizing enzymes. For comparison, potent and selective inhibitors of both cyclooxygenase and nitric oxide synthase are available and have played crucial roles in understanding the complex biological roles of prostanoids and nitric oxide, respectively. Potent and targeted inhibitors of CSE, CBS and 3-MST could be expected to fill a similar niche. Finally, the precise intracellular signalling pathways which determine the degree of expression of H_2S synthesizing enzymes in healthy and in diseased cells have yet to be fully elucidated.

While many advances have been made, more selective and potent H_2S synthesizing enzyme inhibitors as well as cells in which the H_2S synthesizing enzymes (both alone and in combination) have been knocked out would certainly provide powerful tools to aid the understanding of the role of endogenous H_2S in biology. With the recent advent of high-throughput tandem-microwell assays that can screen enormous libraries of potential H_2S producing enzyme inhibitors (Zhou et al. 2013) as well as the introduction of 3-MST$^{-/-}$ mice and novel transgenic mouse models of CBS-deficient homocystinuria, there is certainly much to look forward to in moving the field of H_2S biology forward towards its therapeutic potential.

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