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REVIEW

# Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools

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## ABSTRACT

$H_2S$ (hydrogen sulfide) is a well known and pungent gas recently discovered to be synthesized enzymatically in mammalian and human tissues. In a relatively short period of time, $H_2S$ has attracted substantial interest as an endogenous gaseous mediator and potential target for pharmacological manipulation. Studies in animals and humans have shown $H_2S$ to be involved in diverse physiological and pathophysiological processes, such as learning and memory, neurodegeneration, regulation of inflammation and blood pressure, and metabolism. However, research is limited by the lack of specific analytical and pharmacological tools which has led to considerable controversy in the literature. Commonly used inhibitors of endogenous $H_2S$ synthesis have been well known for decades to interact with other metabolic pathways or even generate NO (nitric oxide). Similarly, commonly used $H_2S$ donors release $H_2S$ far too quickly to be physiologically relevant, but may have therapeutic applications. In the present review, we discuss the enzymatic synthesis of $H_2S$ and its emerging importance as a mediator in physiology and pathology. We also critically discuss the suitability of proposed 'biomarkers' of $H_2S$ synthesis and metabolism, and highlight the complexities of the currently used pharmacological $H_2S$ 'donor' molecules and 'specific' $H_2S$ synthesis inhibitors in their application to studying the role of $H_2S$ in human disease.

## INTRODUCTION

$H_2S$ (hydrogen sulfide/dihydrogen sulfide [1,2]) is a well known and pungent gas with the distinctive smell of rotten eggs. The toxicology of high concentrations of $H_2S$ as an environmental pollutant has been studied extensively [3]. Since it was first discovered to be synthesized in mammalian and human tissues, it has attracted considerable interest in a relatively short period of time as an endogenous gaseous mediator and potential pharmacological and therapeutic tool. Studies in animals and humans have shown $H_2S$ to be involved in diverse

**Key words:** amino-oxyacetate (AOAA), BCA ($\beta$-cyanoalanine), cystathionine-$\gamma$-lyase (CSE), cystathionine-$\beta$-synthase (CBS), GYY4137, hydrogen sulfide ($H_2S$), propargyglycine (PAG).

**Abbreviations:** ADT-OH, 5-(4-hydroxyphenyl)-*3H*-1,2-dithiole-3-thione; AOAA, amino-oxyacetate; BCA, $\beta$-cyanoalanine; BMI, body mass index; CAT, cysteine aminotransferase; CBS, cystathionine-$\beta$-synthase; CHD, coronary heart disease; COPD, chronic obstructive pulmonary disease; AECOPD, acute exacerbation of COPD; COX, cyclo-oxygenase; CRP, C-reactive protein; CSE, cystathionine-$\gamma$-lyase; CSE-s, truncated CSE; DS, Down's syndrome; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; $FEV_1$, forced expiratory volume in 1 s; GABA, $\gamma$-aminobutyric acid; $Hb1_{Ac}$, glycated haemoglobin; IL, interleukin; LPS, lipopolysaccharide; MPST, 3-mercaptopyruvate sulfurtransferase; NMDA, *N*-methyl-D-aspartate; NF-$\kappa$B, nuclear factor $\kappa$B; NSAID, non-steroidal anti-inflammatory drug; PAG, propargyglycine; $PGE_2$, prostaglandin $E_2$; PKC, protein kinase C; PLP, pyridoxal-5′-phosphate; RA, rheumatoid arthritis; SF, synovial fluid; TNF-$\alpha$, tumour necrosis factor-$\alpha$.

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physiological and pathophysiological processes, such as regulation of blood pressure [4,5], inflammation [6], neurodegenerative diseases [7] and metabolic disorders, including obesity and diabetes [8]. These findings have been the subject of several explicitly detailed reviews elsewhere. However, the focus of the present review is to highlight that, as with any emerging field in physiology, pharmacology and medicine, research is limited by the tools available, which have generated significant controversy; specifically, the absence of wholly tissue-selective and enzyme-specific inhibitors to target $H_2S$ biosynthesis, specific donors which release $H_2S$ in a physiological manner and a current lack of suitable 'biomarkers' for $H_2S$ synthesis and turnover *in vivo*.

## $H_2S$ BIOSYNTHESIS: ARE THERE TISSUE-SPECIFIC $H_2S$-SYNTHESIZING ENZYMES?

$H_2S$ is a highly lipophilic molecule able to freely penetrate the membranes of cells of all types by diffusion and without the requirement for specialized membrane transporters [9]. In aqueous solution, $H_2S$ is weakly acidic ($pK_a = 6.76$ at 37 °C) and dissociates to form two dissociation states: $HS^-$ (hydrosulfide anion) ($pK_a = 7.04$) and $S^{2-}$ (sulfide anion) ($pK_a = 11.96$), according to the following sequential reactions:

$$H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S2^- + 2H^+$$

At pH 7.4, approximately 18.5 % of the total sulfide exists as the undissociated acid and 81.5 % as $HS^-$ [10]. It is currently not known whether the biological effects of $H_2S$ are mediated directly by $H_2S$ itself or by derived species that will also exist at physiological pH, predominantly $HS^-$ but also $S^{2-}$. It is unlikely $S^{2-}$ will play a significant role, since it will only be present at a high pH. As a varied mixture of these species will always exist under physiological conditions irrespective of the source of $H_2S$, it is prudent to use the term '$H_2S$' to encompass the sum of these species present under physiological conditions [11,12].

In mammalian and human tissues, the bulk of endogenous $H_2S$ synthesis appears to be from the PLP (pyridoxal-5′-phosphate)-dependent enzymes CSE (cystathionine-$\gamma$-lyase; EC 4.4.1.1) and CBS (cystathionine-$\beta$-synthase; EC 4.2.1.22) via the amino acids cysteine, homocysteine and cystathionine (summarized in Table 1). A PLP-independent pathway has been proposed in neuronal tissue and rodent large vessel vascular endothelial cells utilizing the desulfuration of 3-mercaptopyruvate by MPST (3-mercaptopyruvate sulfurtransferase; EC 2.8.1.2) with CAT (cysteine aminotransferase; EC 2.6.1.75) [13–15]. However, at present, the importance of this third pathway is not as well characterized as CSE and CBS, and its role in determining $H_2S$ synthesis in human tissues is not known.

It been widely suggested that the expression of CSE, CBS, MPST (and CAT) in rodents and humans showed a marked degree of tissue specificity. However, as more researchers investigate the emerging role of $H_2S$ in their particular system, this simple and convenient distinction is no longer as clear as once thought. Up until recently, the current literature consensus was that CBS was the predominant source of $H_2S$ in the brain and nervous tissue, highly concentrated in cerebellar Purkinje and hippocampal neurons, and that in the vasculature (e.g. smooth muscle and endothelium) CSE was the major source of $H_2S$. However, it has been known for some time that neuronal tissue clearly contains CSE and vascular tissue contains CSE and CBS, and the previous assumption on distinct tissue distribution is not clear. Furthermore, CBS has been shown to be preferentially expressed in radial glia/astrocytes of adult and developing mouse brain, but is not present in neurons [16], and at least the rodent macrovasculature has an additional pathway for synthesizing $H_2S$ via MPST/CAT (summarized in Table 1).

In humans, the gene for CSE is located on chromosome 1 (1p31.1). CSE is a member of the $\gamma$-family of PLP-dependent enzymes and is a 405-amino-acid protein consisting of a tetramer formed by two homodimers. The crystal structure of human CSE in the apo form and in complex with PLP has been determined recently [17]. CSE catalyses the $\alpha,\gamma$-carbon elimination of cystathionine to produce cysteine, $\alpha$-oxobutyrate and ammonia (Table 2). Additional cysteine-dependent $\beta$- and homocysteine-dependent $\gamma$-reactions have been suggested [18] to generate $H_2S$ (Table 2). Additionally CSE may catalyse the $\beta$-elimination of cystine (cysteine disulfide) via the formation of thiocysteine, which then decomposes non-enzymatically to $H_2S$ [18]. However, in the presence of physiological concentrations of cysteine ($\sim$100 $\mu$M), homocysteine (10 $\mu$M) or cystathionine (5 $\mu$M), although the catalytic-centre activity number for CSE-mediated cystathionine cleavage was 5-fold greater than for cysteine and 12-fold higher than for homocysteine, the CSE-catalysed $\alpha,\beta$-elimination of cysteine was the predominant source of $H_2S$ accounting for $\sim$70 % of the $H_2S$ produced, whereas the $\alpha,\gamma$-elimination of homocysteine accounted for $\sim$29 % of the measured $H_2S$ [18].

At least two CSE mRNA splice variants have been demonstrated [19,20] producing long (CSE-l) and truncated (CSE-s) CSE proteins. Although CSE-s has been suggested to be inactive [20], the experiments to determine this only measured cysteine accumulation from added cystathionine as an index of enzymatic activity but did not examine $H_2S$ production. As cysteine will also serve as a preferential substrate for CSE (Table 2), the lack of accumulation of cysteine from

**Table 1 Some examples of the tissue distribution of $H_2S$-synthesizing enzymes**

It has been stated in the literature that there is marked tissue specificity for the distribution of CSE and CBS, that CSE is primarily vascular and CBS neuronal. However, the distributions of CSE and CBS are much more widespread, highlighting the importance of $H_2S$ synthesis in a multitude of tissues. The majority of studies have been conducted using animal tissues and it is assumed, but not yet demonstrated, that the distribution of CSE, CBS, CAT and MPST will be similar in humans. Note, $H_2S$ synthesis from CAT/MPST has only been demonstrated in animal tissue. Cysteine lyase has only been shown to be present in non-mammalian species.

| Enzyme | Species | Tissue | Distribution (localization) |
|---|---|---|---|
| CSE (EC 4.4.1.1) | Human | Brain | Astrocytes [163,188], pre-central cortex [163], mRNA expression and catalytic activity in cerebellar and granule and Perkinje cells, pyramidal neurons of CA3 and granule cell layer of dentate gyrus, and reticular neurons in midbrain [189] |
| | | Lens | Epithelium, cortex and nucleus; CBS more abundant in the epithelium of younger individuals (17–21 years) compared with older individuals; CBS is more abundant in the older age group (63–66 years) [190] |
| | | Joint cells | Articular chondrocytes, synoviocytes and trabecular bone-derived mesenchymal cells [187] |
| | | Vasculature | Internal mammary artery [94] |
| | | Lung | Pulmonary artery smooth muscle [108] |
| | | Intrauterine tissue | Chorion, amnion, myometrium and placenta [191] |
| | Mouse | Brain | Purkinje cells (cell bodies and neuronal processes) [192], hippocampus (cell bodies and neuronal processes), optic nerve, cerebral cortex, striatum, thalamus and spinal cord [192–194], neuroblasts (early stages of development) [192], hippocampal dentate gyrus, cerebellar astrocytes and Berman glia [193], cerebellar and granule and Purkinje cells, pyramidal neurons of CA2 and CA3 subfields of the hippocampus, reticular neurons of the midbrain and granule cell layer of dentate gyrus [189] |
| | | Pancreas | Acinar cells [195], exocrine cells and iselets [196] |
| | | Ovary | Ovary follicular cells (cell bodies) [197] |
| | Pig | Lens | Epithelial layer, outer cortex, inner cortex, and nucleus; relative concentration ratio of 3:1:1:1 respectively [190] |
| | | Vasculature | Endothelial cells [198] |
| | Rabbit | Reproductive organs | Vaginal and clitoral cavernosal smooth muscle [199] |
| | Rat | Brain | Hippocampus; CBS highly expressed in the hippocampus and cerebellum compared with the cortex and brain stem [200–203] |
| | | Liver | Induced expression and activity in during acute endotoxaemia [58] |
| | | Kidney | Induced expression and activity in during acute endotoxaemia [58,204] |
| | | Gastrointestinal tract | Liver, stomach, duodenum, jejunum, ileum, colon (Western blotting); diffuse immunostaining in the colon and surrounding blood vessels [202] |
| | | Lung | Airway and vascular smooth muscle [112] |
| | | Intrauterine tissue | Uterus, placenta and fetal membrane [191] |
| | Guinea-pig | Ileum | [205] |
| CBS (EC 4.2.1.22) | Human | Liver | [202,206] |
| | | Colon | Low basal level of expression [191] |
| | | Brain | Neurons in cerebral cortex, cerebellum (Purkinje cells), hippocampus (dentate gyrus and CA3 pyramidal neurons) and mid-brain (reticular neurons) [207] |
| | | Joint cells | Articular chondrocytes, synoviocytes and trabecular bone-derived mesenchymal cells; cytokine and LPS-inducible expression and activity of CSE [187] |
| | | Eye | Lens [208] |
| | | Intrauterine tissue | Chorion, amnion, myometrium and placenta [191] |
| | Rabbit | Genitals | Vaginal and clitoral cavernosal smooth muscle [199] |
| | | Eye | Lens [208] |
| | Guinea-pig | Gastrointestinal tract | Ileum [205] |

**Table 1 Continued**

| Enzyme | Species | Tissue | Distribution (localization) |
|---|---|---|---|
| | Rat | Blood Vessels | Portal vein [205], thoracic aorta [205,209,210], and pulmonary, mesenteric and tail arteries [209] |
| | | Heart | Myocardium [210] |
| | | Brain | Cerebellar, hippocampus [202,211,212] |
| | | Liver | [202,203,209] |
| | | Gastrointestinal tract | Stomach, duodenum, jejunum, ileum and colon; in the colon, CBS immunostaining was primarily localized in muscularis mucosa, sub-mucosa and lamina propria; no immunostaining in crypt, goblet or epithelial cells [202] |
| | | Intrauterine tissue | Uterus, placenta and fetal membrane [191] |
| | Pig | Eye | Lens: mRNA and CSE activity detected; activity and mRNA expression decreased with age [213] |
| | Mouse | Pancreas | Acinar cells [195] and exocrine cells [196] |
| | | Liver, brain and colon | CBS immunoreactivity detected in hippocampal neuronal bodies and neocortical cell bodies [194] |
| CAT (EC 2.6.1.75) | Rat | Brain | Olfactory bulb and cerebellum [214], and cerebral cortex [215] |
| | | Vasculature | Thoracic aorta (cystol and mitochondrial distribution in the endothelium) [216] |
| MPST (EC 2.8.1.2) | Mouse | Brain | Neurons of mitral cell layers, glomerular and external plexiform layers of olfactory bulb, spinal cord (large neurons), cerebral cortex, Purkinje cell somata, proximal dendrites, pons (pontine nuclei) and hippocampus (CA1 and CA3 pyramidal cells) [13] |
| | Rat | Vasculature and liver | Endothelium and smooth muscle of the thoracic aorta [216] |
| | | Liver, kidney and brain | Cytoplasm and mitochondrial expression in proximal tubular epithelial cells in kidney; pericentral hepatocytes in the liver; perinuclear area of myocardial cells, and glial cells in brain [15,217] |
| | Cow | | [218] |
| | Human | Erythrocytes | [219] |
| | | U373 astrocytoma cells | MPST activity only [220] |
| | | Uterus | Myometrium and leiomyomas [221] |
| Cysteine lyase (EC 4.4.1.10) | Amphibians, reptiles and fish | Muscle | Species of batrachian, Caudata, Nothobranchiidae, Neoselachii, *Rivulus* and *Chamaeleo* [27] |
| | Birds | Muscle | *Gallus gallus* [27–29] |

CSE-s suggests it was probably consumed in the generation $H_2S$. However, the precise role of these splice variants in regulating CSE activity in terms of $H_2S$ production requires further attention and may offer therapeutic potential for controlling endogenous $H_2S$ synthesis.

The human CBS gene is located on chromosome 21 (21q22.3) [21]. CBS is a homotetramer consisting of 551-amino-acid subunits which bind two co-factors (haem and PLP) and two substrates (homocysteine and serine) (Table 2). To generate $H_2S$, human CBS can use either cysteine, forming lanthionine (see below), or cysteine plus homocysteine, forming cystathionine. This latter reaction is predicted to predominate under physiological conditions and account for $\sim$96% of the total $H_2S$ generated from CBS, whereas the reactions in the absence of homocysteine represent up to 2.6% of the total $H_2S$ [22]. Although the CBS gene encodes several mRNAs [23], the functional product of these mRNA isoforms in terms of $H_2S$ synthesis have yet to be examined. The haem component of CBS is reported to function as a cellular redox sensor [24], which could increase $H_2S$ generation in response to oxidative and/or nitrosative stress-mediated cellular injury (see below).

In contrast with CSE and CBS, very little information is currently available with regards to human MPST and $H_2S$ synthesis. MPST is a $\sim$33 kDa monomeric or disulfide-linked dimeric protein containing two rhodanese domains. It is located in the cytoplasm and mitochondria, and the human MPST gene is located on chromosome 22 (22q12.3). At least two splice variants of human MPST are present, but as with CSE and CBS, their regulation and role in $H_2S$ synthesis are not understood. Although originally described in 1954 [25], the formation of $H_2S$ from MPST has gained renewed interest and has so far been confined to rodent macrovascular endothelium [14] and brain homogenates [13], where $H_2S$ is generated through

**Table 2 Enzyme substrate specificities and commonly used inhibitors of $H_2S$ synthesis**

An exquisitely detailed analysis of substrate requirement, mechanisms, formation of intermediates and kinetics is provided by Singh and Banerjee [144]. $\rightarrow$ Enzyme-catalysed reaction. It should be noted that the effects of the inhibitors on $H_2S$ synthesis in the model systems have not been investigated and none of the below compounds have been administered to humans.

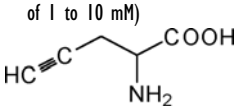
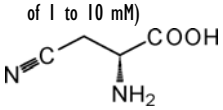
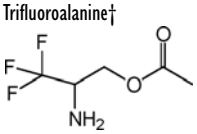
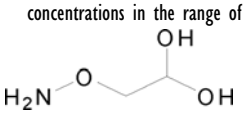
| Enzyme and substrate* for $H_2S$ synthesis and metabolic reactions catalysed | Pharmacological enzyme inhibitor commonly used | Comment/examples of non-specific effects |
|---|---|---|
| CSE<br>(i) Cysteine $\rightarrow$ serine + $NH_4^+$ + $H_2S$<br>(ii) Homocysteine $\rightarrow$ $\alpha$-oxobutyrate + $NH_4^+$ + $H_2S$<br>(iii) Homocysteine + homocysteine $\rightarrow$ lanthionine + $H_2S$<br>(iv) Cystathionine $\rightarrow$ cysteine + $\alpha$-oxobutyrate + $NH_3^+$<br>At physiologically relevant concentrations, the order of preference is cysteine > cystathionine > homocysteine [18,144,222] | D,L-PAG (irreversible inhibitor and is commonly used as a 'selective' CSE inhibitor at concentrations in the range of 1 to 10 mM)<br>  | Target pyridoxal binding (PLP) of CSE and is unlikely to be wholly specific for CSE and will inhibit other PLP-dependent enzymes; for example:<br>(i) ALAT (L-alanine transaminase; a key alanine–glucose cycle enzyme and a clinical marker of liver function) [223]<br>(ii) Chronic dosing induced significant cardiac and hepatosplenomegaly and perturbation of taurine, glycine asparagine and citrulline metabolism. [224]<br>(iii) Metabolized to a toxic renal metabolite by D-amino acid oxidase pathway-dependent processes, leading to proteinuria, glucosuria and polyuria in rats [225,226]<br>(iv) Induced the accumulation of cystathionine and cystathionine ketimine in rat whole brains, in particular in the cerebellum [145]<br>(v) inhibited cyanide metabolism *in vivo* [227]<br>(vi) *in vivo* and *in vitro* pharmacokinetic data lacking |
| | BCA (reversible inhibitor and is commonly used as a 'selective' CSE inhibitor at concentrations in the range of 1 to 10 mM)<br>  | (i) Targets the PLP-binding site of CSE and is unlikely to be specific; BCA is potentially neurotoxic; and it is found in the seeds of sweet peas, legumes and vetchling (for example *Vicia sativa*, *Lathyrus sativus* and related vetch) and potentiates or contributes to the effects of the lathyrism-causing neurotoxins $\beta$-*N*-oxalyl-L-$\alpha$,$\beta$-diaminopropionic acid and found in these plants [228,229]<br>(ii) *In vivo* and *in vitro* pharmacokinetic data are lacking |
| | Trifluoroalanine†<br>  | (i) Probably binds to the PLP-binding site of CSE and thereby conferring non-specificity; for example trifluoroalanine also inhibits biotin metabolism via inactivation of PLP-requiring enzyme 8-amino-7-oxononanoate synthase [230–232]<br>(ii) *In vivo* and *in vitro* pharmacokinetic data are lacking |
| CBS<br>(i) Cysteine + $H_2O$ $\rightarrow$ serine + $H_2S$<br>(ii) Cysteine + homocysteine $\rightarrow$ cystathionine + $H_2S$<br>(iii) Cysteine + cysteine $\rightarrow$ lanthionine + $H_2S$<br>Reaction (ii) is preferential [22,144] | AOAA [also called *O*-(carboxymethyl)hydroxylamine hemihydrochloride (CHH) is an irreversible inhibitor, commonly used as a 'selective' CBS inhibitor at concentrations in the range of 1 to 10 mM]<br>  | AOAA targets the PLP-binding site of CBS and is not specific for CBS; for example:<br>(i) Inhibition of mitochondrial malate–aspartate shuttle, resulting in the inhibition of lactate gluconeogenesis, inhibition of glycolysis and glucose oxidation [233–235]<br>(ii) Inhibition of mitochondrial and cytosolic aspartate transaminase [236,237]<br>(iii) Inhibition of mitochondrial Complex I activity during ischaemia/reperfusion injury [58]<br>(iv) Inhibition of protein synthesis [238] |

**Table 2 Continued**

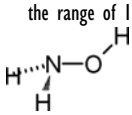
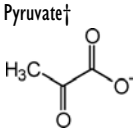
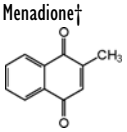
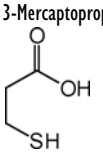
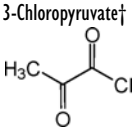
| Enzyme and substrate* for $H_2S$ synthesis and metabolic reactions catalysed | Pharmacological enzyme inhibitor commonly used | Comment/examples of non-specific effects |
|---|---|---|
| CBS cont. | | (v) Inhibition of insulin secretion through $K_{ATP}$-channel-dependent and $K_{ATP}$-independent mechanisms [239]<br>(vi) Induced $\beta$-alaninuria in rats [240]<br>(vii) Reduced oxidative stress in rat hepatocytes [153,235]<br>(viii) Induced NMDA-receptor-dependent excititoxicity in rat striatum, reduced kyneurinine synthesis and potentiated NMDA-receptor-mediated neuronal injury [157,241]<br>(ix) Inhibits mitochondrial oxidative phosphorylation; inhibition of mitochondrial complex I [151].<br>(x) Induced GABA synthesis in dorsal hippocampus and medial septum in rats; inhibition of GABA synthesis following seizure induced by AOAA was also observed [242].<br>(xi) Induced neurotoxicity, seizures and neurodegeneration [152,155,156,243]<br>(xii) Used as a specific inhibitor of 2-oxoglutarate aminotransferase; potentiated clonidine-induced hyotension in rats, but had no effect on systemic blood pressure when used alone [244]<br>(xiii) Potentiated ethanol-induced motor impairment and decreased ethanol metabolism [245]<br>(xiv) *In vivo* and *in vitro* pharmacokinetic data lacking |
| | Hydroxylamine (CBS inhibitor used at concentrations in the range of 1 to 10 mM)<br>  | (i) Hydroxylamine is an endogenous molecule and NO donor [100–105]<br>(ii) Toxic: induces methamoglobin formation, anaemia and reticulocytosis in rats [246]<br>(iii) Induces vasodilation in isolated smooth muscle in mouse, rabbit, rat, cat and dog vascular smooth muscle [165,170,171]<br>(iv) Induces vasodilation in non-vascular smooth muscle [172]<br>(v) Induces hypotension in anaesthetized rats, cats and dogs [165,172,247]<br>(vi) Inhibits semicarbazide-sensitive amine oxidase (SSAO; copper-containing amine:oxygen oxidoreductase) in smooth muscle; an enzyme required for the development of blood vessels and lipolysis<br>(vii) Induces taurine release in the brain stem [166]<br>(viii) Promotes oedema [173]<br>(ix) Promotes eosinophillic inflammation in the lung [174]<br>(x) Promotes the release of the neuromodulator adenosine [248]<br>(xi) Promotes long-term potentiation in rat hippocampal slices [175] |

**Table 2 Continued**

| Enzyme and substrate* for $H_2S$ synthesis and metabolic reactions catalysed | Pharmacological enzyme inhibitor commonly used | Comment/examples of non-specific effects |
|---|---|---|
| MPST/CAT* (i) Mercapatopyruvate → pyruvate + $H_2S$ This reaction was original identified in 1954 [25]; MPST transfers a sulfane sulfur atom from mercaptopyruvate to an active-site cysteine forming an persulfide; $H_2S$ is then liberated from MST-persulfide by an unknown reductant, presumably DTT (dithiothrietol) used in the experimental analysis [15,144] | Pyruvate†  | Uncompetitive inhibitor with 3-mercaptopyruvate at high millimolar concentrations; highly non-specific [249] |
| (i) Cysteine + $SO_3^{2-}$ → cysteate‡ + $H_2S$ (ii) Cysteine + cysteine → lanthionine [29] | Menadione†  | Will also induce intracellular oxidative stress leading to cytotoxicity [220] |
| | 3-Mercaptopropionic acid†  | (i) Non-competitive inhibitor [250] (ii) Also inhibits the key enzyme required for GABA synthesis glutamate decarboxylase [251] |
| | 3-Chloropyruvate†  | (i) 1:1 stoichiometric inhibitor [252] (ii) Also inhibits *N*-acetylneuraminate lyase/sialic acid metabolism and likely to impair synthesis of mucosal, cell and neuronal membranes [253] (iii) Nephrotoxic *in vivo* [254] Additional substrates may include methanethiol and 2-mercaptopyruvate, but the products of these reactions have not been identified [28–30] It is uncertain whether this pathway exists in mammalian or human tissue |

*For reasons of clarity, these substrate requirements are simplified.
†Specific effects with respect to $H_2S$ generation have not been examined.
‡Precursor of taurine.

3-mercaptopyruvate, α-oxoglutarate and cysteine (Table 2). Unravelling the relative contribution of MPST to tissue $H_2S$ production will be complex since there are currently no available MPST inhibitors. The inhibitors that have been used in isolated cell or tissue homogenates would not exhibit any degree of specificity to be useful in cell culture or *in vivo* (Table 2).

A fourth potential pathway for endogenous $H_2S$ synthesis has been proposed [26], via the enzyme cysteine lyase (EC 4.4.1.10) and utilizing cysteine with $CH_3S$

(methanethiol) or $SO_3^{2-}$ (sulfite) as substrates in the presence of PLP (Table 2). However work on this enzyme has exclusively focused on fish, amphibians [27] and birds [28–30]. It is not certain whether this enzyme is present in or is capable of synthesizing $H_2S$ in human (or mammalian) cells and, as a PLP-dependent enzyme, its activity is also likely to be inhibited by commonly used inhibitors of CSE and CBS (see below).

## TISSUE AND BLOOD LEVELS OF $H_2S$

Tissue production and levels of '$H_2S$' in blood has been the subject of much controversy (reviewed in [5]). The predominant method employed to evaluate serum or plasma or tissue levels and synthesis of $H_2S$ in humans and in animal models of human disease has used a spectrophotometric approach based around Methylene Blue. In this procedure, $H_2S/HS^-$ and/or aqueous sulfide in biological samples is 'fixed' or 'trapped' with zinc to prevent loss of $H_2S$ through volatilization and aerial oxidation, resulting in the formation of stable ZnS (zinc sulfide) [31]. $H_2S$ is then released from ZnS under strongly acidic conditions and, in the presence of DMPD (*N*,*N*-dimethyl-*p*-phenylenediamine) and $Fe^{3+}$, results in the formation of the heterocyclic thiazine dye Methylene Blue, which is then either measured by spectrophotometry or HPLC. Under these conditions, plasma or serum $H_2S$ levels in healthy human adults have been reported to be in the range of 20 to 60 $\mu$M [5], although considerably higher levels have been reported using this method, for example >100 $\mu$M [32] and in rodent plasma in excess of 300 $\mu$M [33]. Additional techniques applied to serum or plasma have similarly reported between 20 and 60 $\mu$M '$H_2S$' (or derived species), such as sulfide-selective electrodes [31,34,35], microdistillation and ion chromatography [36], and GC/ion conductance [37]. However, others have provided evidence to suggest that actual levels of 'free' $H_2S$ in plasma were beyond or on the limit of detection for the Methylene Blue assay and electrochemical detectors [38,39]. More recently fluorimetric-based methods have been developed employing monobrombimane to trap 'free' $H_2S$ and the resulting dibimane determined by reverse-phase HPLC to show baseline levels of free '$H_2S$' to be in the region of 0.4–0.9 $\mu$M [40]. It is therefore likely that other methods measure the total sum of $H_2S$-derived species such as $HS^-$ and $S^{2-}$ and possibly other physiological $H_2S$ 'carrier' molecules that exist at physiological pH and which release $H_2S$ under acidic conditions employed in the analytical processes, rather than 'free' $H_2S$ itself and care should be taken to describe the results as such [5].

To date it is not clear what role diet (e.g. cysteine intake) and exercise or past and present medication play in determining tissue and blood levels of $H_2S$ (and its derived species under physiological conditions) and '$H_2S$'-derived metabolites. The influence of these factors and the lack of standardization between laboratories could account for at least some of this biological variation. At present, the 'absolute' level of 'free' or 'bound' $H_2S$ in tissues or in blood is uncertain, but if the field follows a similar progression to that of NO then levels of $H_2S$, the identification and suitability of 'biomarkers' and/or specific metabolites are likely to present controversy for some time. Nevertheless, using the above approaches in combination with inhibitors of $H_2S$ synthesis (see below) have revealed an emerging role for $H_2S/HS^-$ in human disease processes and physiology (see below).

## $H_2S$ METABOLISM: IS THERE A SPECIFIC 'BIOMARKER' FOR $H_2S$ SYNTHESIS OR 'TURNOVER' *IN VIVO*?

Whatever the precise level of systemic and tissue '$H_2S$', measured levels may represent an underestimate of the true extent of $H_2S$ synthesis as pathways for $H_2S$ removal exist, although it is not clear how rapid these processes occur or how $H_2S$ removal is affected by disease. In addition to the controversy over the absolute levels of '$H_2S$' synthesized in tissues and present in blood, the role of $H_2S$ in human physiology and pathology is hampered further by a lack of known specific 'biomarkers' or end products of $H_2S$ metabolism, which are crucial for identifying physiological and pathophysiological processes regulated by $H_2S$ (summarized in Table 3).

Intracellular $H_2S$ is apparently rapidly oxidized to $S_2O_3^{2-}$ (thiosulfate) by mitochondria with the subsequent conversion into $SO_3^{2-}$ and $SO_4^{2-}$ (sulfate) [41,42]. $SO_3^{2-}$ and $SO_4^{2-}$ are also produced upon oxidation of $H_2S$ by activated neutrophils, where $SO_3^{2-}$ induced the respiratory burst leading to further $H_2S$ oxidation and loss [43] by several endogenous oxidant species elevated during disease processes, such as NO [44,45], superoxide [46], $ClO^-$ (hypochlorite) [12], $H_2O_2$ (hydrogen peroxide) [47] and $ONOO^-$ (peroxynitrite) [11]. Furthermore, $SO_3^{2-}$ readily undergoes hepatic metabolism forming $SO_4^{2-}$ [48].

$SO_4^{2-}$ has also been proposed as an index of endogenous $H_2S$, but while stable and reasonably uncomplicated to assay, it is not only formed from $H_2S$ oxidation, but can be derived from the direct oxidation of cysteine by cysteine dioxygenase (EC 1.13.11.20), as well as from the oxidation of $SO_3^{2-}$ by sulfite oxidase (EC 1.8.3.1) [49], limiting its usefulness as a 'biomarker' of $H_2S$ synthesis or metabolism. Similarly, $S_2O_3^{2-}$ measurement in urine and blood has historically been used as an index of environmental $H_2S$ exposure and inhalation [50,51], but its use also requires careful scrutiny. Elevated levels of $S_2O_3^{2-}$ in urine is observed in patients with DS (Down's syndrome) [52] and, although blood levels of $S_2O_3^{2-}$

**Table 3 Proposed 'biomarkers' of $H_2S$ synthesis and metabolism and their limitations**

| 'Biomarker' | Proposed mechanism of formation | Limitation in use as a specific 'biomarker' of $H_2S$ *in vivo* |
|---|---|---|
| Sulfate ($SO_4^{2-}$) | (i) Rapid oxidation of $H_2S$ by mitochondria [40,41] | Also produced by: |
| | (ii) Produced from the oxidation of $H_2S$ by neutrophil respiratory burst [42] | (i) Oxidation of cysteine by cysteine dioxygenase [47] |
| | | (ii) Oxidation of $SO_3^{2-}$ by sulfite oxidase |
| | (iii) Rhodanese-meditated detoxification of $H_2S$ [47] | (iii) Respiratory burst-mediated oxidation of $H_2S$ [42] |
| | (iv) Produced by incubation of plasma and tissue homogenates with $H_2S$ | (iv) Incubation of plasma or homogenates of liver, colon and muscle with $CH_3S$ also leads to $SO_4^{2-}$ formation, suggesting $SO_4^{2-}$ is not specific for $H_2S$ [56] |
| | | (v) Dietary metabisulfite [$S_2O_5^{2-}$; food preservative (E223)] is metabolized to $SO_4^{2-}$ by the liver |
| | | (vi) Urine levels of $SO_4^{2-}$ is not altered by the 'CSE inhibitor' PAG [15]. |
| | | Levels may therefore be modulated through diet or environment, rather than reflect $H_2S$ synthesis or metabolism/degradation |
| Thiosulfate ($S_2O_3^{2-}$) | (i) Produced by incubation of plasma and tissue homogenates with $H_2S$ or $CH_3S$ | (i) Levels in 'healthy' individuals vary greatly and are highly sensitive to small fluctuations in atmospheric $H_2S$ [23] |
| | (ii) Observed in blood and urine after industrial $H_2S$ poisoning [48,49] | (ii) Higher levels of $SO_4^{2-}$ and $SO_3^{2-}$ than $S_2O_3^{-}$ detected in the lung after $H_2S$ gas exposure, suggesting either high $S_2O_3^{2-}$ turnover or it is only a minor product of $H_2S$ exposure [55] |
| | (iii) Elevated levels observed in patients with DS [52] | (iii) Has been detected in blood but not urine after $H_2S$ poisoning, suggesting it is not wholly reliable or robust as an index of $H_2S$ exposure or synthesis [50,51] |
| | | (iv) Incubation of plasma or homogenates of liver, colon and muscle with $CH_3S$ also leads to $S_2O_3^{-}$ formation, suggesting $S_2O_3^{2-}$ is not specific for $H_2S$ [56] |
| | | Levels may therefore be modulated through diet or environment rather than reflect $H_2S$ synthesis or metabolism/degradation |
| Sulfite ($SO_3^{2-}$) | (i) Rapidly oxidation of $H_2S$ by mitochondria [40,41] | (i) Used in food (dried fruits, radish and potato) and drink as an antioxidant and preservative, and naturally present in or added to wine; also used as an excipient in medications (e.g. paracetamol) [255–257] |
| | | (ii) Formed in the lung from the inhalation of the atmospheric pollutant $SO_2$ (sulfur dioxide) |
| | | (iii) Metabolized by the liver [48] |
| | | Levels may therefore be modulated through diet or environment rather than reflect $H_2S$ synthesis or metabolism / degradation. |
| Methanethiol ($CH_3S$) | (i) Methylation of $H_2S$ by thiol-S-methyltransferase [41,42] | (i) Also found in food such as nuts and cheese (including from methionine degradataion) and some vegetables, especially asparagus [258,259] |
| | (ii) Produced by incubation of plasma and tissue homogenates with $H_2S$ [56] | (ii) Can also be formed from the $\beta$-replacement of cysteine with $CH_3S$ by CBS [21] |
| | | Levels may are probably modulated through diet or environment rather than reflect $H_2S$ synthesis or metabolism/degradation |
| Dimethyl sulfide ($CH_3SCH_3$) | (i) Methylation of $H_2S$ by thiol-S-methyltransferase [41,42] | (i) Found in vegetables and dairy products or formed when cooking some foods, for example. beetroot, cabbage, asparagus, corn, butter, celery, seafood etc. [260,261] |
| | | (ii) Used as a food additive as a savoury flavouring [FEMA (Federal Emergency Management Agency) #2746] |

**Table 3 Continued**

| 'Biomarker' | Proposed mechanism of formation | Limitation in use as a specific 'biomarker' of $H_2S$ *in vivo* |
|---|---|---|
| Lanthionine/lanthioinine ketimine | (i) Formed from CSE-catalysed condensation reactions of cysteine and cysteine + serine [17,21]<br>(ii) A metabolic product of lantionine; lanthionine ketimine is found in urine | (i) Lanthionine ketimine is also synthesized from pyruvate kyneurenin aminotransferase and metabolised by ketimine reductase to 1,4-thiomorfoline-3,5-dicarboxylic acid [63]<br>(ii) Lanthionine may also be formed from cysteine without $H_2S$ formation by cysteine lyase [28] |
| Homolanthionine | (i) Formed from CSE-catalysed condensation reactions of homocysteine and homocsyteine + homoserine<br>(ii) Formed by human liver CSE [57].<br>(iii) Present in patients with homocystinuria [65,66] | (i) Metabolized to cystine, and may also be derived from methionine without $H_2S$ formation [66] |
| Sulfhaemoglobin | (i) Formed from the reaction of $H_2S$ with haemoglobin [68] | (i) May also be induced by exposure of haemoglobin to xenobiotics [71–76] and $SO_2$ [71]; however, the roles of $H_2S$ in these phenomenon have not been determined |
| Sulfhydrated proteins | (i) Formed from the reaction of $H_2S$ (and/or $HS^-$) with cysteine residues in proteins [83] | (i) Produced by thiols such as GSH, commensal bacteria and dietary polysulfides [87–89]<br>(ii) Only demonstrated in isolated cells in the presence of high concentrations of NaSH [83] and yet to be confirmed by other groups [144] |

or '$H_2S$' have not yet been assessed, $S_2O_3^{2-}$ has been used to suggest that there is an overproduction of $H_2S$ in DS [52,53]. The location of CBS to chromosome 21 supports this possibility. However, as with $SO_4^{2-}$, it is unlikely that urinary $S_2O_3^{2-}$ is reliable as an index of $H_2S$ as $S_2O_3^{2-}$ levels in blood without detection in urine have also been observed after industrial $H_2S$ poisoning [50,51]. In addition, $S_2O_3^{2-}$ ingestion has been proposed to induce a cardioprotective effect by increasing endogenous $H_2S$ synthesis in a murine model of chronic heart failure [54], suggesting it is inappropriate as a 'biomarker' of $H_2S$. Furthermore, urinary $S_2O_3^{-}$ levels in healthy individuals vary greatly and are highly sensitive to small atmospheric fluctuations in $H_2S$ [23]. In animal studies where $SO_3^{2-}$, $SO_4^{2-}$ and $S_2O_3^{2-}$ have been determined simultaneously, $H_2S$ gas exposure resulted in higher lung levels of $SO_3^{2-}$ and $SO_4^{2-}$ rather than of $S_2O_3^{2-}$ [55]. Similarly, incubation of liver, colonic or muscle tissue or plasma with either $H_2S$ or $CH_3S$ also leads to significant $S_2O_3^{2-}$ and $SO_4^{2-}$ generation [56]. As such, the metabolism of $H_2S$ *in vivo* is highly complex and individually the reliability of $S_2O_3^{2-}$, $SO_4^{2-}$ or $SO_3^{2-}$ as specific indices of endogenous $H_2S$ synthesis and turnover require further attention.

Additional cellular processes for $H_2S$ removal also exist, suggesting the possibility for additional specific 'biomarkers' or molecular 'fingerprints'; however, their use may also not be straightforward. $H_2S$ is rapidly methylated to $CH_3S$ and $CH_3SCH_3$ (dimethyl sulfide) by thiol-S-methyltransferase (EC 2.1.1.9) [41,42], although CBS can also catalyse $H_2S$ production by $\beta$-replacement of cysteine with $CH_3S$ [22]. An additional enzymatic removal process, which is potentially important in colonic tissue and erythrocytes, involves rhodanese (thiosulfate:cyanide sulfurtransferase; EC 2.8.1.1), [49]. Rhodanese catalyses the transfer of $HS^-$ to a thiophilic acceptor (such as cyanide) to form $SCN^-$ (thiocyanate) and $SO_4^{2-}$.

A more promising approach to specific 'biomarkers' of $H_2S$ synthesis and turnover in health and disease may come from the detailed analysis of enzyme-specific metabolic products. The novel amino acids lanthionine and homolanthionine were recently shown to be formed from the CSE-catalysed condensation reactions of cysteine and homocysteine respectively [18,22]. Lanthionine is also formed during $H_2S$ synthesis by CSE and CBS from cysteine alone or cysteine plus serine [17,21]. However, it may also be produced without $H_2S$ formation from cysteine via cysteine lyase [28]. CSE-catalysed homolanthionine from homocysteine and homoserine has been demonstrated in rat and human liver [57], and CSE-mediated $H_2S$ production in rat liver is also induced by bacterial endotoxin [58] and the diabetogenic agent streptozotocin [59], suggesting lanthionine may represent a viable 'biomarker' of $H_2S$ synthesis. Lanthionine is metabolized with $\alpha$-oxo amino acids via glutamine transaminase K/pyruvate kyneurenin aminotransferase to cyclic lanthionine ketimine. This ketimine derivative is present in mammalian brains, including the human cerebral cortex [60], where it may protect neuronal cells from oxidative stress [61] and in human urine [62,63]. However, lanthionine ketimine may be biologically active in its own right [61], since metabolic processes for its removal exist such as ketimine reductases [64] forming 1,4-thiomorfoline-3,5-dicarboxylic acid, although the extent to which this occurs is not clear. It is therefore

likely that the use of lanthionine as an index of CSE/CBS activity and $H_2S$ production will be highly complex.

In sharp contrast, very little is known about the metabolic fate of homolanthionine, but while lanthionine forms the six-membered ring lanthionine ketimine, homolanthionine is unlikely to form the corresponding eight-membered ring homologue. Homolanthionine has been shown to be produced in the human liver by CSE [57] and it is present in urine from patients with homocystinuria [65,66]. Interestingly, homolanthionine production was shown to be increased in a human liver biopsy after 3 months of treatment with pyridoxine HCl, and homolanthionine synthesis in rat liver homogenates was dependent on PLP and cystine [57]. However, rats fed $^{35}$S-labelled homolanthionine on an 8 % casein diet showed [$^{35}$S]cystine in hair, suggesting homolanthionine is metabolically processed, at least in rodents [67]. However, the yield of [$^{35}$S]cystine was low (1.5 %) and the accumulation or metabolism of [$^{35}$S]cystine was not assessed in other tissues. Nevertheless, homolanthionine may offer significant promise for the assessment of overall CSE/CBS activity and $H_2S$ synthesis *in vivo*.

The interaction of $H_2S$ with haemoglobin, forming sulfhaemoglobin, is well known [68] and methaemoglobin has been used as a 'scavenger' of $H_2S$ in *ex vivo* and *in vitro* experiments [69]. Recently, Perna et al. [70] showed significantly lower erythrocyte levels of sulfhaemoglobin in patients with end-stage renal failure and sulfhaemoglobin levels correlated with lower plasma $H_2S$ levels, suggesting sulfhaemoglobin was a marker of endogenous $H_2S$ synthesis. However, there are many additional mechanisms for the formation of sulfhaemoglobin *in vivo*, such as exposure to the atmospheric pollutant $SO_2$ (sulfur dioxide) [71], xenobiotic metabolism (for example, phenacetin [72], acetanilide [72], metoclopramide [73], phenazopyridine [74], dapsone [75], metoclopramide [76,77], *N*-acetylcysteine [76] and neomycin [78], sulfanilamide [79] and sulfanilamide-containing drugs, such as sumatriptan, albeit after ingestion of large quantities [80]), drug overdose (cimetidine, paracetamol, ibuprofen and naproxen) [81] or ingestion of toxic substances (for example paint [82] or shoe dye [83]). As such, any clinical study which proposes to use sulfhaemoglobin as a specific index of $H_2S$ biosynthesis or turnover would require ruling out confounding exogenous mediators of sulfhaemoglobin formation, such as current and past drug therapies or environmental exposure to $SO_2$.

An additional and recently proposed hypothesis is that $H_2S$ and/or species derived from it under physiological conditions further interact with protein thiols via the covalent modification of cysteine (S-sulfhydration) in which an -SH group is transferred to a cysteine-SH residue in a protein, yielding perthiol (-SSH) moieties [84]. Although this hypothesis may explain the signalling mechanisms of intracellular and extracellularly generated $H_2S$, at least in part, it is not clear to what extent perthiol formation accounts for the levels of '$H_2S$' observed in blood and tissues. Detailed discussion on the cellular and molecular signalling pathways induced by $H_2S$ and sulfide salt $H_2S$ donor compounds are expertly reviewed elsewhere [85,86]. However, since perthiol formation would enhance the chemical reactivity of $H_2S$, these species may be too short-lived to represent viable and $H_2S$-specific 'biomarkers' or 'fingerprints' of $H_2S$ production *in vivo*. Owing to this high reactivity, they have previously been proposed as endogenous antioxidant molecules [87]. Furthermore, perthiols may be formed *in vivo* through $H_2S$-independent pathways, such as the general metabolism of thiols (for example glutathione), commensal bacteria [88] or dietary sources including garlic and other *Allium* species [89,90].

## WHAT IS THE ROLE OF ENDOGENOUS $H_2S$ IN HUMAN HEALTH AND DISEASE?

Recent animal models and human clinical studies have shown perturbed synthesis of $H_2S$ in a variety of physiological processes and pathologies, and have highlighted the potential for modulating $H_2S$ synthesis for therapeutic exploitation. These have invariably relied upon: (i) the measurement of '$H_2S$' in body fluids and the production of $H_2S$ by isolated tissues and tissue homogenates; (ii) the use of sulfide salt $H_2S$ 'donors'; and (iii) inhibitors of CSE and CBS activity. The complex physiological problems associated with (ii) and (iii) are discussed in detail further below. With the exception of a few cases to be discussed below, the large majority of studies have only been limited to animal models and the human studies have generally been limited to small patient and volunteer sample sizes. Examples of perturbed synthesis in these few human studies and animal models of disease are summarized in Table 4. For this section, we will focus on studies where both animal models and human clinical studies have been investigated.

### $H_2S$ and the regulation of vascular tone: is $H_2S$ a 'potent' vasodilator?

The majority of evidence for the physiological role of $H_2S$ has been obtained from studies on vascular tissue and has commonly concluded that $H_2S$ is a vasodilatory intermediate and perhaps an (or 'the') EDHF (endothelium-derived hyperpolarizing factor) [86]. However, the precise role of endogenously generated $H_2S$ is not clear and requires critical appraisal. Generally, the application of high micromolar (typically $\geqslant$100 $\mu$M) concentrations of either $H_2S$ gas solutions or $H_2S$ donor compounds, such as $Na_2S$ (sodium sulfide) or NaSH (sodium hydrosulfide), to isolated and pre-contracted blood vessel

**Table 4 Evidence of perturbed synthesis of $H_2S$ in human disease and some recent examples of a possible role of $H_2S$ in animal models of human disease**

A$\beta$, amyloid $\beta$ peptide; apoE, apolipoprotein E; DSS, dextran sodium sulfate; ICAM-1, intercellular adhesion molecule-1; TNBS, trinitrobenzenesulfonic acid; WCC, white cell count.

| Species | Disease/animal model | Evidence for a role of perturbed $H_2S$ synthesis |
|---|---|---|
| Human | Inflammatory joint disease | Increased levels of $H_2S$ in SF aspirates from the joints of patients with RA, reactive arthritis and psoriatic arthritis compared with matched plasma and SF from patients with osteoarthritis, and SF levels of $H_2S$ negatively correlated with neutrophil and total WCC and positively correlated with tender joint score [6,124] |
| | COPD | $H_2S$ levels positively correlated with the percentage of predicted $FEV_1$ and sputum macrophage levels, but negatively correlated with sputum neutrophil count; sputum levels of $H_2S$ were equivalent to levels of $NO_2^-$ [119] |
| | AECOPD | Smoking significantly lowered plasma levels of $H_2S$ in healthy controls and AECOPD patients<br>High levels of NO (measured as total nitrite/nitrate) correlated with higher $H_2S$ levels<br>$H_2S$ levels also correlated with stage of lung obstruction with COPDand $H_2S$ also levels negatively correlated with sputal neutrophil count and correlated positively with lung function (predicted $FEV_1$) [120] |
| | Sepsis | Plasma $H_2S$ levels increased up to 4-fold during sepsis compared with healthy subjects [58] |
| | Hypertension | $H_2S$ levels higher in the control group ($65.7 \pm 5.5$ $\mu$M; mean age, $10.5 \pm 0.73$ years) compared with hypertensive children ($51.9 \pm 6.0$ $\mu$M; mean age, $10.48 \pm 3.2$ years), high plasma levels of $H_2S$ correlated with low homocysteine concentrations, hypertensive children had a lower $H_2S$/homocysteine ratio compared with the control group ($5.8 \pm 2.9$ compared with $11.6 \pm 3.3$), and higher systolic pressure was associated with a lower plasma $H_2S$/homocysteine ratio [256]<br>Plasma $H_2S$ levels negatively correlated with systolic and diastolic blood pressure in men [8] |
| | CHD | Patients with CHD had significantly lower $H_2S$ levels compared to angiographically normal controls; the number of affected vessels correlated with a decrease in $H_2S$ levels, suggesting decreased $H_2S$ levels correlate with disease severity, but could also reflect either $H_2S$ consumption by vascular oxidants; plasma $H_2S$ levels were also significantly negatively correlated with blood glucose levels and were significantly lower in smokers compared with non-smokers [101]. |
| | Obesity/diabetes | Plasma levels of $H_2S$ were significantly lower in patients with Type II diabetes compared with age-matched lean controls and age- and BMI-matched overweight controls, plasma $H_2S$ levels were significantly decreased in overweight compared with lean controls and adiposity determined the levels of $H_2S$ [8]<br>Plasma $H_2S$ levels also negatively correlated with systemic blood pressure, microvascular function *in vivo*, glycaemic control (fasting glucose and $Hb1_{Ac}$ levels), impaired insulin sensitivity (peripheral and central) and measurements of obesity (BMI, waist circumference, waist/hip ratio) [8] |
| | DS | The gene for CBS is located on chromosome 21; increased urinary excretion to $S_2O_3^{2-}$ [53] |
| | Alzhiemer's disease | Plasma levels of $H_2S$ significantly correlated with a decline in cognitive function, assessed by mini-mental state examination, activity of daily living scale, Hachinski ischaemic score and Hamilton's depression score [262] |
| | Renal dialysis/end-stage renal failure | Plasma levels of $H_2S$ and erythrocyte sulfhaemoglobin content were significantly decreased in patients undergoing haemodialysis, plasma levels of cysteine negatively correlated with $H_2S$ levels, and lower plasma $H_2S$ levels were attributed to lower CSE mRNA expression in peripheral blood mononuclear cells [70] |
| | Lung infection (pneumonia) | Serum $H_2S$ levels negatively correlated with serum CRP and predicted the need for antibiotic therapy; patients with purulent sputum, pyretic ($>37.2\,^{\circ}C$) or total WCC $\geqslant 1 \times 10^{10}$ had lower serum $H_2S$ levels than those with non-purulent sputum, apyrexic or with lower WCC [121] |
| | Colon cancer and ulcerative colitis | Decreased colon expression of sulfide 'detoxifying' enzyme rhodanese (thiosulfate sulfurtransferase and mercaptopyruvate sulfurtransferase) in colon biopsies from patients with colon cancer and ulcerative colitis, and decreased tissue expression correlated with disease progression [263] |
| Mouse | Hypertension | Stepwise increased in systemic blood pressure in CSE-knockout animals ($CSE^{-/-} > CSE^{-/+} > CSE^{+/+}$) [34] |

**Table 4 Continued**

| Species | Disease/animal model | Evidence for a role of perturbed $H_2S$ synthesis |
|---|---|---|
| Mouse cont. | Sepsis | Bacterial endotoxin (LPS) induced CSE expression in the lung, liver and kidney, plasma levels of $H_2S$ were markedly increased in LPS-treated mice, and tissue inflammation was significantly reduced after PAG treatment [58] |
| | Chronic renal failure | Renal damage is strongly linked to decreased vascular $H_2S$ synthesis; $CBS^{-/+}$ mice had significantly lower plasma $H_2S$ compared with wild-type animals, glomeruli from $CBS^{-/+}$ mice showed increased oxidative damage compared with wild-type mice, and exogenous $H_2S$ (NaSH) prevented oxidative-stress-induced cell damage [131] |
| | Ischaemic heart failure | Overexpression of CSE preserved cardiac function in the left coronary artery occlusion model, and intra-cardiac $Na_2S$ preserved left ventricular function and attenuated oxidative stress and mitochondrial dysfunction [138] |
| | Burn injury | Burn injury significantly elevated lung and liver $H_2S$ synthesis, CSE and CBS expression, and these effects were significantly inhibited by PAG [264] |
| | Pancreatitis | Caerulin-induced $H_2S$ synthesis in the pancreas and caerulein-induced pancreatitis, lung injury, systemic inflammation and acinar cell death was significantly reduced by the prophylactic and therapeutic treatment of animals with PAG [265,266] |
| | Visceral pain | In colorectal distension-induced pain, $H_2S$ mediated analgaesia by predominantly $\mu$-opoid-receptor-mediated processes [267] |
| | Ulcerative colitis | In the DSS-induced colitis model, DSS induced CSE and CBS mRNA expression and colonic $H_2S$ levels, and PAG increased disease activity scores, mucosal inflammation and levels of myeloperoxidase and oxidative stress [268]; however, this was not observed by others [269] |
| | Diabetes | In non-obese diabetic mice, as diabetes progressed, reduced plasma levels of $H_2S$, aortic $H_2S$ synthesis and reduced responsiveness to $H_2S$ and other endothelium-dependent vasodilators were observed [35] |
| | Atherosclerosis | PAG increased atherosclerotic plaque size and increased plasma and aortic levels of ICAM-1 in $apoE^{-/-}$ mice, and inhibition of atherosclerosis was observed with NaSH treatment [270] |
| Rat | Sepsis | Acute endotoxaemia induced by *Escherichia coli* LPS induced significant expression and activity of CSE and CBS, and prophylactic or therapeutic administration of PAG dose-dependently reduced LPS-induced hepatic, pancreatic and neuromuscular injury, but did not reduce LPS-induced circulatory failure [271] |
| | Hypertension | Spontaneously hypertensive rats had significantly lower plasma $H_2S$ and lower vascular $H_2S$ production than normotensive Wistar–Kyoto rats [272] |
| | Pulmonary hypertension | Hypoxic pulmonary hypertension ($N_2$ hypoxia chamber) decreased plasma $H_2S$ levels and CSE mRNA levels in lung tissue, and pulmonary hypertension and remodelling was inhibited by NaSH [33] |
| | | In the abdominal aorta-inferior cava vein shunting model, plasma levels of $H_2S$ and CSE mRNA expression in pulmonary artery and lung tissue CSE mRNA expression were significantly reduced [97] |
| | Alzheimer's disease | NaSH attenuated LPS-induced cognitive defects [273] and inhibited A$\beta$-induced PC12 cell death [274] |
| | Parkinson's disease | Reduced levels of $H_2S$ in substantia nigra and striatum in 6-hydroxydopamine- and rotenone-treated rats [275] |
| | Heart failure after myocardial infarction | Myocardial infarction was induced by left anterior descending artery ligation; higher mortality was observed in PAG-treated animals, and PAG increased myocardial fibrosis, damage to mitochondrial ultrastructure and increased apoptotic cell death, whereas these processes were inhibited by NaSH [276] |
| | Recurrent febrile seizures | Hydroxylamine, added to inhibit CBS up-regulated $GABA_{B2}$-receptor subunits and c-fos expression, whereas NaSH was inhibitory [277,278] |
| | Cancer chemotherapy | Cisplatin treatment increased CSE expression and activity in outer medulla of the kidney and increased renal inflammatory cell infiltrate, effects reversed by PAG treatment [279] |
| | Diabetes | Streptozotocin-induced diabetes resulted in decreased plasma $H_2S$ levels, but increased expression of CBE and $H_2S$ synthesis in lung, liver and pancreas [59] |

**Table 4 Continued**

| Species | Disease/animal model | Evidence for a role of perturbed $H_2S$ synthesis |
|---|---|---|
| | | In Zucker diabetic fatty rats [280], increased pancreatic levels of $H_2S$ and CSE were observed compared with control animals, and PAG increased serum insulin, and lowered blood glucose and $HbA_{1c}$ levels. |
| | Hepatic ischaemia/reperfusion | Portal vein and hepatic artery occlusion increased serum levels of $H_2S$ and increased hepatic CSE mRNA expression and CSE activity, and PAG potentiated and NaSH inhibited hepatocellular toxicity, oxidative damage and inflammatory cell infiltrate [135] |
| | Oedema | Carrageenan-induced hindpaw oedema was attenuated by NaSH and $Na_2S$, but aggravated by PAG; a $K_{ATP}$-channel-dependent mechanism was proposed [281] |
| | Ulcerative colitis | TNBS model: TNBS increased colonic $H_2S$ synthesis and inhibition of $H_2S$ synthesis with BCA, PAG and AOAA exacerbated colitis; in control animals, inhibition of $H_2S$ synthesis resulted in mucosal inflammation and injury, and decreased prostaglandin synthesis; NaSH resolved colonic inflammation [282] |
| Pig | Renal ischaemia/reperfusion | Aortic occlusion-induced kidney ischaemia/reperfusion injury was attenuated by intravenous $Na_2S$; $Na_2S$ decreased blood levels of $NO_2^-/NO_3^-$, IL-1$\beta$ and IL-6, reduced kidney oxidative DNA damage and nitrosative stress [134] |

preparations, including rodent aorta [45,91], mesenteric [92] and hepatic [93] beds or human internal mammary arteries [94], induce vessel relaxation in an endothelium-dependent and $K_{ATP}$-channel-dependent manner. In agreement with these findings, infusion of NaSH or $Na_2S$ solutions into anaesthetized animals has been observed to induce transient systemic blood pressure reduction [45,58], and conditions associated with hypotension, such as sepsis [56] and haemorrhagic shock [95], have been shown to have increased plasma levels and tissue production of $H_2S$, whereas administration of inhibitors of CSE in these models normalized blood pressure. Conversely, decreased blood levels of '$H_2S$' have been observed in hypertensive rats [96–98], and genetic knockout studies comparing $CSE^{+/+}$, $CSE^{-/+}$ and $CSE^{-/-}$ mice have shown not only a stepwise reduction in plasma $H_2S$ levels with loss of CSE, but also increases in systemic blood pressure [34]. However, it should be noted that this finding has not yet been confirmed [99]. Furthermore, genetic manipulation of CBS also modulates systemic blood pressure and plasma levels of $H_2S$ [100], which again challenges the explicit tissue selectivity of the $H_2S$-synthesizing enzymes CSE and CBS.

Clinical studies are emerging to confirm, albeit indirectly, a vasodilatory role of $H_2S$ in humans and have suggested that modulating systemic $H_2S$ production may represent a viable approach for the treatment of vascular disease. For example, compared with healthy controls, plasma $H_2S$ levels were increased 4-fold in patients with sepsis (up to 200 $\mu$M in one patient [56]), but were substantially reduced in hypertensive children [37]. More recently, plasma '$H_2S$' levels in overnight-fasted men significantly negatively correlated with systemic blood pressure and impaired microvascular function *in vivo* and were significantly lower in overweight volunteers, and lower still in patients with Type 2 diabetes [8]. Furthermore, plasma $H_2S$ levels also negatively correlated with glycaemic control [for example fasting glucose and plasma $HbA_{1c}$ (glycated haemoglobin levels], peripheral and central insulin sensitivity and adiposity [BMI (body mass index), waist and hip circumference and waist/hip ratio]. In one study comparing patients with CHD (coronary heart disease) with angiographically normal subjects, the number of affected coronary vessels (as well as plasma glucose levels) correlated with decreased plasma levels of $H_2S$ [101].

The studies described above have led to the wide and general suggestion that $H_2S$ is a potent vasodilator (some examples [10,102–105]), but are the effects of $H_2S$ on vascular tissue particularly 'potent'? The concentrations of $H_2S$ gas solution or NaSH and $Na_2S$ required to induce tissue relaxation, and added as a bolus, have often been used in excess of 200 $\mu$M. These concentrations may be several orders of magnitude higher than the 'free' levels of $H_2S$ gas in blood and severalfold higher than the reported levels of total $H_2S$ (for example the sum of $H_2S$, $HS^-$, $S_2^-$ and acid-labile sulfur) even in the most optimistic of studies, signifying that $H_2S$ is not a particularly potent vasodilator at all. Furthermore, glibenclamide and other $K_{ATP}$ channel antagonists invariably prevent $H_2S$-induced tissue relaxation, but only when high concentrations of $H_2S$ are used and when used at relatively high concentrations themselves (10–20 $\mu$M), strongly suggesting that at physiological levels $H_2S$ may exert more subtle effects on the vasculature. Additional mechanisms for the regulation of vascular tone have been proposed such as regulation of NO bioavailability [5,106,107], release of NO from nitrosothiols [44,45], inhibition of $Cl^-/HCO_3^-$ channels [108–110], metabolic inhibition [108] and activation of PKC (protein kinase C) or cAMP-dependent pathways [46]. However, these studies have

also used >300 $\mu$M $H_2S$ gas or NaSH to elicit these effects, and these additional pathways have not been demonstrated by others [91,92,111] and generally not investigated using human vessels. As such, the precise mechanism by which endogenously synthesized $H_2S$ regulates vascular tone is not clear.

## $H_2S$ and respiratory smooth muscle

The toxicology of inhaled environmental $H_2S$, leading to ocular and respiratory distress, has been well documented over several decades and reviewed in great detail elsewhere [3]. More recent studies have strongly suggested that $H_2S$ may also function as an endogenous mediator in the lung. CSE and CBS mRNA and protein have been demonstrated in human pulmonary artery smooth muscle [69], and CSE is expressed in the airway and vascular smooth muscle in rat peripheral lung tissues [97,112,113]. As with conduit arteries, the effects of $H_2S$ donors have been investigated on isolated vessels from the lung, but the findings are similarly vague. In pre-contracted guinea-pig bronchial rings, the addition of up to 10 mM NaSH only produced modest tissue relaxation, whereas, in mouse bronchial tissue, significant relaxation was observed, albeit at NaSH concentrations of $\geqslant$500 $\mu$M [114]. Under these conditions, tissue relaxation was resistant to antagonists or inhibitors of $K_{ATP}$, soluble guanylate cyclase, COX (cyclo-oxygenase)-1, COX-2 and tachykinin, and the precise mechanism(s) for lung smooth muscle relaxation in the lung has not been identified and it is unlikely, given the relatively low rate of CSE- and CBS-derived $H_2S$ synthesis [17,18,22,115,116], that bronchial tissue generates $H_2S$ at such high concentrations.

$H_2S$ has also been suggested to play a role in lung remodelling. For example, NaSH inhibited collagen accumulation in the wall of the pulmonary artery in hypoxia and aortocaval shunting rat models [117,118], and inhibition of endogenous $H_2S$ synthesis with AOAA [amino-oxyacetate; also called *O*-(carboxymethyl) hydroxylamine hemihydrochloride] and to a lesser extent PAG (propargyglycine) increased human airway smooth muscle proliferation and IL (interleukin)-8 secretion, whereas $H_2S$ donors substantially inhibited proliferation and IL-8 secretion by inhibiting ERK (extracellular-signal-regulated kinase) 1/2 and p38-dependent signalling pathways [69].

Clinical studies examining plasma levels of $H_2S$ and lung function are starting to emerge, albeit employing small sample sizes. Using a sulfide electrode, Chen et al. [119] showed that plasma levels of $H_2S$ in healthy controls were approximately 35 $\mu$M, but almost doubled in patients with stage I COPD (chronic obstructive pulmonary disease). Plasma $H_2S$ was decreased with increasing lung obstruction (stage I, $\sim$72 $\mu$M; stage II, $\sim$50 $\mu$M; stage III, $\sim$40 $\mu$M; stage IV, $\sim$48 $\mu$M), positively correlated with lung function [predicted $FEV_1$ (forced expiratory volume in 1 s) and negatively correlated with sputum neutrophil count [119,120]. However, plasma $H_2S$ levels were unchanged in patients with AECOPD (acute exacerbation of COPD) [117], were unaffected by theophylline treatment [118] and serum levels were increased compared with control subjects [121]. Intraperitoneal NaSH administration was recently shown to decrease tobacco-smoke-induced emphysema, lung injury and oxidative stress in mice [122], but human studies are similarly less clear cut; smoking either decreased plasma $H_2S$ [101] or had no effect on serum levels of $H_2S$ [119]. Analysis of sputum levels of $H_2S$ would represent a more reliable index of lung $H_2S$ biogenesis or turnover, but, to date, these have not been assessed. As such, the role of endogenous $H_2S$ in the lung is also not clear.

## Is endogenous $H_2S$ a regulator of inflammation?

Much has also been written about the diverse role of endogenous $H_2S$ in inflammatory signalling (reviewed in explicit detail elsewhere; for example [6,123]), but the precise role of endogenous $H_2S$ is not clear. Recently, we showed significantly higher levels of '$H_2S$' in knee joint SF (synovial fluid) aspirates from patients with RA (rheumatoid arthritis) compared with matched plasma or SF obtained from osteoarthritis patients [124]. In that study, SF $H_2S$ negatively correlated with SF total white cell and neutrophil count, and positively correlated with disease activity. Increased SF $H_2S$ was not unique to RA, since subsequent studies also found increased levels in the SF from patients with psoriatic, reactive and septic arthritides [6]. Increased plasma levels of $H_2S$ were also observed in patients with septic shock [58], and plasma $H_2S$ levels negatively correlated with plasma CRP (C-reactive protein) levels in lung infection [121], suggesting increased $H_2S$ synthesis may represent a generalized response to acute as well as chronic tissue injury and inflammation. However, it is not known whether $H_2S$ synthesis was elevated to drive the inflammatory response or was elevated to control or to limit tissue inflammation. Animal model studies employing sulfide salts (for example $Na_2S$ or NaSH) at high concentrations have not been able to clarify this issue; $H_2S$ either induces (or potentiates) inflammation or it is inhibitory. Conversely, inhibition of CSE with PAG (see below) consistently reduced inflammation (reviewed in detail elsewhere [6,123] and summarized in Table 4).

## Is $H_2S$ an endogenous cytoprotective mediator?

Endogenous $H_2S$ has been proposed as a novel cytoprotective mediator [5], and there is growing evidence of direct and indirect antioxidant effects of $H_2S$. In cell culture experiments, $H_2S/HS^-$ generated

from NaSH has been shown to 'scavenge' detrimental pro-inflammatory oxidants, such as $H_2O_2$ [46], $ClO^-$ [12], superoxide [45], $ONOO^-$ [11] and NO [45], inhibit cell death induced by these mediators as well as prevent oxidative modification of intracellular proteins [11,12] and LDL (low-density lipoprotein) [125]. In neuronal cells, NaSH inhibited cell death induced by $\beta$-amyloid, mediated at least in part via antioxidant effects [126] and up-regulating intracellular glutathione synthesis through increasing cysteine uptake and elevating $\gamma$-glutamylcysteine synthetase activity [127]. NaSH is also reported to degrade lipid peroxides [125], inhibit the expression and activity of NADPH oxidase [46,128] and up-regulate thioredoxin-1 expression in vascular endothelial cells [129]. Increased hepatic GSH synthesis and decreased lipid peroxidation are also observed with $Na_2S$ treatment in a murine hepatic ischaemia/reperfusion injury model [130], and hepatocytes isolated from $CSE^{-/-}$ mice showed greater sensitivity to oxidative-stress-mediated injury than wild-type mice [99]. Furthermore, glomeruli isolated from $CBS^{-/+}$ mice showed increased production of endogenous ROS (reactive oxygen species) compared with glomeruli from wild-type animals [131]. In animal models of smoke/burn injury [132], and myocardial [133], renal [134] and hepatic [135] ischaemia/reperfusion, $H_2S$ salt donors reduced the formation of nitrosatively and oxidatively modified cellular proteins, DNA and lipids, suggesting further an 'antioxidant' role for $H_2S$.

However, recent detailed kinetic analysis has shown that the rate constants of the reaction between $H_2S$ ($Na_2S$) and detrimental oxidant species are not sufficiently high enough for oxidant 'scavenging' alone to mediate the cytoprotective effects of $H_2S$ [136], strongly suggesting that other mechanisms must exist. Evidence for this suggestion is in the literature. For example, $H_2S$ from NaSH or $Na_2S$ induced ERK and Akt signalling pathways in cardiac tissue [79,99,100], and preserved mitochondrial ultrastructure and respiratory chain function *in vivo* [73], possibly via a mitochondrial pathway involving the preservation of Bcl-2 signalling, up-regulation of PKC and opening of mitochondrial $K_{ATP}$ channels [100]. $H_2S$ may also mediate cytoprotection via effects on other intracellular organelles such as the ER (endoplasmic reticulum), and has recently been shown to attenuate ER-stress-dependent cardiomyoctye cell death in a rat model of hyperhomocysteinaemia [137]. $H_2S$ has also been proposed as a regulator of angiogenesis by promoting the proliferation of vascular endothelial cells and inhibiting the proliferation or inducing apoptotic cell death in vascular smooth muscle cells by modulation of ERK, p38, $p21^{cip/WAF}$ and caspase-mediated pathways. Furthermore, administration of $Na_2S$ or NaSH *in vivo* prevents myocardial tissue damage and cell loss *in vivo* [138–142] via up-regulation of Nrf2-dependent signalling pathways [138]. Although the precise mechanism(s) mediating these phenomena have not been identified, it is possible that the signalling was mediated through selective protein sulfhydration [143]. Although this is a highly attractive proposal, analogous to S-nitrosation of proteins by NO the extent to which this occurs is uncertain [144] and sulfhydration of intracellular proteins has only been demonstrated in isolated cells after the addition of >100 $\mu$M NaSH. However, that study [143] represents a proof-of-principle demonstration of the potential mechanisms by which $H_2S$ could exerts it physiological (and pharmacological) effects and in time may assist in dissecting the diverse signalling processes mediated by this intriguing biological gas (reviewed in detail in [85]).

## PERILS AND PITFALLS: PHARMACOLOGICAL AND GENETIC TOOLS

### Specific inhibitors of $H_2S$ synthesis?

Much of our current knowledge of the biology of $H_2S$ stems from the use of inhibitors of CSE such as D,L-PAG and BCA ($\beta$-cyanoalanine), and inhibitors of CBS, such as AOAA. These compounds target the PLP-binding site of these enzymes [54] and, while undoubtedly useful, they are not entirely specific and will target other PLP-dependent enzymes, especially over the wide concentration ranges commonly used in the laboratory for simple experiments on isolated tissues and cells (typically used between 1 and 10 mM). As such, the studies described in the immediately preceding section and summarized in Table 4 should be viewed with the following observations discussed below in mind. PAG is used as an irreversible and 'specific' inhibitor of CSE, but it has also been well used over the past few decades as a 'specific' inhibitor of other metabolic processes (summarized in Table 2). For example, PAG also inhibits several transamination reactions in muscle, alters amino acid metabolism and is metabolised to a renal toxin resulting in significant proteinuria, glucosuria and polyuria (Table 2). As such, possible diuretic and other renal effects of this PAG metabolite (or PAG itself) in mediating the effects of PAG (and $H_2S$) *in vivo* should be considered when examining vascular, endocrine and inflammatory pathways. PAG administration to rats also increased whole-brain levels of cystathionine [145], suggesting that PAG may have little effect on endogenous $H_2S$ synthesis in the brain when administered systemically. Furthermore, urinary levels of proposed $H_2S$ 'biomarkers' such as $SO_4^{2-}$ are not reduced in rats after PAG treatment [15].

BCA (Table 2) has also been used to inhibit CSE in a reversible manner [146] and is used at high millimolar concentrations [147]. Feeding BCA to rats results in the accumulation of free BCA and $\gamma$-glutamyl-

$\beta$-cyanoalanylglycine in the brain, liver, plasma and muscle [148], suggesting it is widely bioavailable and metabolically processed. However, this compound is well known for its neurotoxic properties via NMDA (*N*-methyl-D-aspartate)-dependent and -independent mechanisms [149,150] and for its involvement in dietary-induced lathyrism in animals.

Similarly, AOAA is problematic. In other research fields, this compound is used as a general inhibitor of transaminase reactions, mitochondrial oxidative phosphorylation, amino acid transport and protein synthesis (summarized in Table 2). In neuronal systems, AOAA is known to stimulate GABA ($\gamma$-aminobutyric acid) synthesis in rat hippocampus and striatum [151] as well as induce neurotoxicity [152,153], seizures in mice [154] and neuronal cell loss in the striatum [155] and hippocampus [156] through inhibition of mitochondrial respiration [157], processes remarkably similar to that of $H_2S$. However, it is possible that these effects could have been due, at least in part, to some inhibition of endogenous $H_2S$ synthesis. A further complication to the use of high concentrations of AOAA is that it could also perturb $H_2S$ metabolism through inhibition of mercaptopyruvate metabolism and MPST [158–160].

Hydroxylamine has also been used as a 'specific' inhibitor of CBS at millimolar concentrations [161–163], but its specificity and appropriateness is highly questionable (summarized in Table 2). For example, hydroxylamine is an endogenous molecule formed from the oxidation of $NO_2^-$ (nitrites), $NO_3^-$ (nitrates) and $NH_3$ (ammonia) [164,165]. It has also been well established in the literature over several decades that hydroxylamine releases the gaseous mediator NO and forms $NO^-$ (nitroxyl ions) [164,166–170]. As such it is likely that many of the effects observed when using this compound and attributed to inhibition of CBS activity (and decreased levels of $H_2S$) could be due to increased NO and/or HNO (nitroxyl) formation, such as vascular reactivity [165,171,172], promotion of oedema [173] and inflammation [174], glutamate signalling, and learning and memory [175].

Although PAG, AOAA and BCA significantly reduce $H_2S$ synthesis in various animal models and the activity of CSE and CBS in isolated cells and tissues *in vitro*, it has been so far assumed that these compounds are freely cell-permeant. The 'effective' concentrations of each of these inhibitors in any system have not been evaluated and to date these studies are lacking and are entirely overlooked. Therefore, in the absence of any evidence to the contrary, disparities in cellular permeability between cell types and species could at least partly explain the inconsistencies and controversies in the literature.

In the absence of anything better being currently available to researchers in a new and rapidly expanding field, the use of PAG, BCA and AOAA (but clearly not hydroxylamine), with adequate controls, should still provide a valuable insight into the physiological, pathological and pharmacological role of $H_2S$ in the body. However, they must be used with caution and investigators should resist overinterpreting their findings. A detailed analysis of the literature reveals that additional inhibitors have been used to inhibit CSE, CBS and CAT/MPST, but these have not been examined with respect to $H_2S$ synthesis. However, these compounds also either bind to the PLP-binding sites of CSE and CBS or have non-specific effects on other PLP-dependent enzymes (summarized in Table 2).

Genetic models lacking CSE [34,99] or CBS [176] have been generated and have similarly proved controversial. Wang and co-workers [34], using CSE-knockout animals, have largely supported the studies conducted using these 'non-specific' pharmacological tools in the vasculature (e.g. PAG) in that $CSE^{-/-}$ and $CSE^{-/-}$ mice had significantly lower plasma $H_2S$ levels, decreased vascular synthesis of $H_2S$ and had markedly higher systemic blood pressure than wild-type animals. However, rather than clarify the controversial role of $H_2S$ in the vasculature, these studies may have added to them. For example, Ishii et al. [99] showed that CSE-knockout animals were not hypertensive, and more recent studies [131,176] showed that $CBS^{-/+}$ mice also had significantly decreased plasma $H_2S$ levels compared with wild-type animals, again questioning the earlier assumption that CSE is specifically a vascular enzyme and responsible for the vascular synthesis of $H_2S$. These genetic CSE/CBS-knockout studies, the highly non-specific effects of inhibitors of CSE and CBS *in vivo* and their use at high concentrations, the high concentrations of exogenous $H_2S$ required to dilate vascular smooth muscle and the high concentrations of $K_{ATP}$ channel antagonists required to inhibit the vascular effects of high concentrations of added $H_2S$ seriously question the mechanisms by which endogenous $H_2S$ regulates vascular tone.

## $H_2S$ 'donors' and some notes of caution

The vast majority of studies which have examined the potential role of $H_2S$ in health and disease have invariably utilized commercially available sulfide salts such as $Na_2S$ and NaSH. Although these compounds have been useful as they can be conveniently used to prepare standardized solutions of $H_2S$ and circumvents the requirement for $H_2S$ gas cylinders, they are not particularly relevant tools to examine the physiology of $H_2S$ *in vitro* or *in vivo*. For example, we have shown that the addition of $Na_2S$ or NaSH (or saturated solutions of $H_2S$ gas prepared from $H_2S$ gas cylinders) to aqueous solutions results in the instantaneous release of a bolus of $H_2S$ which dissipates in seconds [116,177]. It is highly unlikely that tissues or cells are ever exposed to $H_2S$ generated in such a rapid manner which generates very high local concentrations of $H_2S$ (as well as $HS^-$ and $Na^+$), since endogenously produced $H_2S$ through CSE and CBS is relatively

slow and sustained and may well be synthesized in a steady 'flux' [17,18,22,115,116]. Typically concentrations of $Na_2S$ and NaSH have been employed at concentrations between 50 and 1000 $\mu$M (and often much higher than this [178]) and when administered to animals, blood and tissue levels of added sulfide have only been detailed in very few studies [177]. Authors have generally argued that due to the $pK_a$ of NaSH the final concentration of $H_2S$ is approximately one-third of that of the final concentration of sulfide salt added and $HS^-$ accounts for the remaining two-thirds. However, it is currently not known whether the biological and pharmacological effects of $H_2S$ are determined by $H_2S$ itself or from $HS^-$, which will always be present at physiological pH no matter what the source of $H_2S$ or the donor used. Given that the progression of the field is now towards 'free' levels of $H_2S$ being several orders of magnitude lower than previously considered (nanomolar rather than micromolar) [39], use of $Na_2S$ and NaSH at these concentrations and added or administered as a bolus is physiologically questionable and assumptions that high concentrations of $Na_2S$/NaSH can effectively model the effects of endogenously produced $H_2S$ should not be made. More recent determinations of the levels of 'free' $H_2S$ in plasma are in the region of 0.4–0.9 $\mu$M [40]. As such, compounds which release concentrations of $H_2S$ at this level and over a much longer period of time will most likely be of more physiological relevance. However, as a novel therapeutic approach to reperfusion injury and stroke, clinical-grade $Na_2S$ is showing considerable clinical promise as a pharmacological agent (Table 4), although the precise mechanisms of action have not been defined.

## RECENT ADVANCES: SLOW-RELEASE $H_2S$ DONOR MOLECULES

Novel $H_2S$ donors have been developed and synthesized to circumvent the problem of using sulfide salts such as $Na_2S$ and NaSH as a means to expose animals, tissue and cells to $H_2S$ generated in a physiological manner (reviewed in [6,179,180]). The majority of these studies have modified existing pharmacological compounds such as NSAIDs (non-steroidal anti-inflammatory drugs) with ADT-OH [5-(4-hydroxyphenyl)-*3H*-1,2-dithiole-3-thione], examples of which are illustrated in Table 5. The combination of well-established NSAIDs with ADT-OH derivatives have clearly highlighted the pharmacological potential for $H_2S$, and these novel compounds have shown substantial promise in alleviating and limiting gastrointestinal side effects and toxicity of NSAIDS and in the treatment of inflammatory bowel disease, oedema, endotoxic shock and acute inflammation (Table 5). However, the precise mechanism by which ADT-OH 'releases' $H_2S$ has not been demonstrated and, since dithiolethiones are themselves biologically active, it is possible that some of the observed biological effects associated with ADT-OH derivatives were due to ADT-OH itself rather than released $H_2S$ (reviewed in [6,181]). For example, dithiolethiones have been shown to activate Nrf-2-dependent phase II enzymes, such as $\gamma$-glutamylcysteine synthetase, and elevate intracellular glutathione, NADPH:quinone oxidireductase, gluathione reductase and catalase [182–185]. However, the role of $H_2S$ released from these molecules in these studies was not investigated and it is possible these effects were due, at least in part, to released $H_2S$.

More recently, $H_2S$ donors which do not consist of structurally modified established drug molecules such as GYY4137 have been synthesized and characterized [116,177,186]. GYY4137 is a very slow-releasing $H_2S$ donor compound which releases two molecules of $H_2S$ per molecule of GYY4137 and has been shown to exert prominent endothelium-dependent vasodilatory activity *in vivo* via $K_{ATP}$-channel-dependent mechanisms, as well as exert prominent anti-inflammatory activity *in vitro* and *in vivo* mediated in part via inhibition of NF-$\kappa$B (nuclear factor $\kappa$B)/AP-1 (activator protein-1)-dependent pro-inflammatory signalling (Table 5). In human articular chondrocytes and trabecular bone-derived mesenchymal progenitor cells, GYY4137 protected these cells against oxidative-stress-mediated cytotoxicity, induced Akt phosphorylation and preserved mitochondrial function [187]. GYY4137 offers the additional advantage to the researcher over ADT-OH compounds in that its decomposition products appear inactive [101], allowing the physiological effects of slow release of $H_2S$ to be studied directly in the absence of any possible additive, but therapeutically highly useful, pharmacological effects of NSAID, known drug or ADT-OH. However, it should be noted here that the precise metabolic and pharmacokinetic profiles for any of the ADT-OH-containing $H_2S$ donors or GYY4137 have yet to be fully elucidated so it is possible that at least some of the reported effects *in vivo* could be due to metabolism to biologically active intermediates rather than $H_2S$. As such, detailed control experiments are required to confirm any observations are due to $H_2S$ and not the parent compound or decomposed ('spent') donor.

Despite the recent advancement in the generation of $H_2S$ donor molecules, one clear area that has stagnated is the development of inhibitors that are specific for each of the endogenous $H_2S$-synthesizing enzymes. Although CSE- and CBS-knockout animals and cell lines have been generated in some laboratories, these tools are not widely available, may not be applicable to certain experimental conditions and have generated considerable controversy themselves. Until specific inhibitors are identified, the physiological and pathophysiological role of endogenously generated $H_2S$ will remain unclear.

**Table 5 Applications of pharmacological slow-release $H_2S$ 'donor' molecules**

L-NAME, $N^G$-nitro-L-arginine methyl ester; STAT-3, signal transducer and activator of transcription-3; IFN-$\gamma$; interferon-$\gamma$; TNBS, trinitrobenzenesulfonic acid; AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; HUVEC, human umbilical vein endothelial cell; PKA, protein kinase A; PKG, protein kinase G; HSP27, heat-shock protein 27; ICAM-1, intercellular adhesion molecule 1.

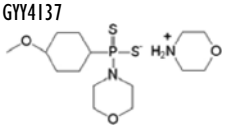
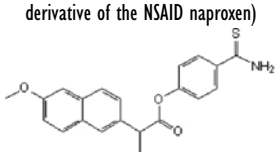
| Compound | Experimental model | Comments | Therapeutic potential |
|---|---|---|---|
| GYY4137  | Isolated aortic rings and perfused kidney; normotensive and hypertensive rats (spontaneously hypertensive and L-NAME-induced hypertension) | Water-soluble compound; GYY4137 induced endothelium and $K_{ATP}$ channel vasodilatation and reduced systemic blood pressure in hypertensive rats (spontaneously hypertensive rats), and inhibited the vasoconstrictor response to angiotensin II and noradrenaline in perfused kidney [177] | Regulation of blood pressure |
| | Bacterial LPS-induced endoxic shock in rats | GYY4137 inhibited endotoxin-induced activation of NF-$\kappa$B and STAT-3, decreased the synthesis of the pro-inflammatory mediators CRP, L-selectin, NO, $PGE_2$, TNF-$\alpha$, IL-1 and IL-6, and induced the synthesis of the anti-inflammatory mediator IL-10; GYY4137 inhibited neutrophil accumulation and activity and decreased tissue damage [186] | Regulation of inflammation and systemic blood pressure in shock |
| | Mouse macrophage cell lines | GYY4137 inhibited LPS-induced synthesis of the pro-inflammatory mediators NO, $PGE_2$, TNF-$\alpha$, IL-1, IL-6 and induced the synthesis of the anti-inflammatory mediator IL-10; modulation of AP-1 and NF-$\kappa$B activation and activity. In sharp contrast, fast release of $H_2S$ via NaSH potentiated LPS-induced synthesis of pro-inflammatory mediators [116] | Regulation of inflammation |
| | Human primary pulmonary smooth muscle cells | GYY4137 inhibited cell proliferation and reduced serum-induced IL-8 synthesis [69] | Regulation of lung inflammation and smooth muscle hyperplasia; for example, asthma, COPD and pulmonary hypertension |
| S-Naproxen (ATB-346) (ADT-OH derivative of the NSAID naproxen)  | Acute gastric damage; ulceration induced by acetic acid; mouse zymosan airpouch oedma model; and adjuvant-induced arthritis in rats | ATB-346 accelerated the healing of gastric ulcers [283]; ATB-346 was significantly more effective than naproxen at suppressing COX-2 activity and leucocyte infiltration | Gastric-sparing properties of S-NSAIDs offer the opportunity to treat inflammatory conditions without the gastrointestinal side effects of traditional NSAIDs; for example, colitis and gastrointestinal inflammation, general oedema and inflammation; arthritis and related chronic inflammatory joint disorders |

**Table 5 Continued**

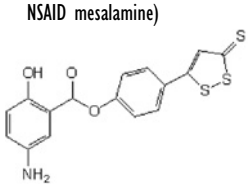
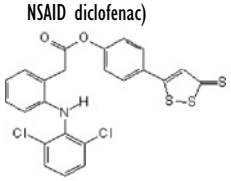
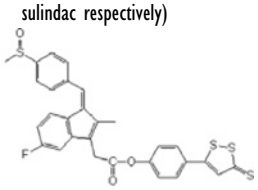
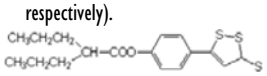
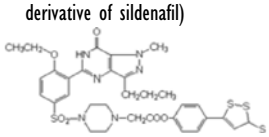
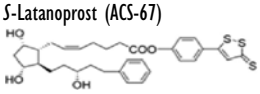
| Compound | Experimental model | Comments | Therapeutic potential |
|---|---|---|---|
| *S*-Mesalamine (ATB-429) (ADT-OH derivative of the NSAID mesalamine)  | Murine colitis induced by TNBS | ATB-429 was significantly more effective at decreasing disease activity, colonic damage, colonic levels of inflammatory cytokines (IFN-$\gamma$, IL-1, TNF-$\alpha$, IL-12 and IL-2), COX-2 and myeloperoxidase activity than the parent mesalamine compound; induction of c-Fos and $K_{ATP}$ channel signalling pathways proposed [284,285] | Gastrointestinal inflammation, ulceration and pain management; general acute and chronic inflammation |
| *S*-Diclofenac (ATB-337/ACS-5) (ADT-OH derivative of the NSAID diclofenac)  | Bacterial endotoxic shock induced by LPS | *S*-Diclofenac decreased LPS-induced synthesis of TNF-$\alpha$ and IL-1$\beta$ and increased IL-10 synthesis, and *S*-Diclofenac decreased NO and $PGE_2$ synthesis in the vasculature, liver and kidney and inhibited the activation of pro-inflammatory transcription factors AP-1 and NF-$\kappa$B [286] | Gastrointestinal inflammation, ulceration and pain management; general acute and chronic inflammation |
| | Carrageenan-induced hindpaw oedema in rats | *S*-Diclofenac decreased carrageenan-induced oedema, myeloperoxidase activity and synthesis of $PGE_2$, NO and $H_2S$ [287] | Swelling and oedema |
| | | *S*-Diclofenac inhibited smooth muscle cell growth and induced p21, p53AIP1 and Bax-dependent apoptosis [288] | Inhibition of smooth muscle cell proliferation may be useful for controlling smooth muscle cell proliferation in diseases such as vascular obstructive and restenosis |
| | Carrageenan-induced hindpaw oedema in rats | *S*-Diclofenac exerted substantially less gastrointestinal toxicity compared with diclofenac alone and, in sharp contrast with diclofenac, *S*-diclofenac did not increase gastric granulocyte infiltration, leucocyte adherance and expression of TNF-$\alpha$, lymphocyte function associated antigen-1 and ICAM-1. *S*-Diclofenac inhibited of COX-1 and COX-2 to the same extent as diclofenac and preserved gastric mucosa [289]. | Inflammation and oedema |
| *S*-Diclofenac (ACS-5) and *S*-sulindac (ACS-18; structure shown) (ADT-OH derivatives of diclofenac and sulindac respectively)  | Carcinogen activation and detoxification enzymes human hepatoma HepG2 and colon LS180 adinocarcinoma cell lines | *S*-NSAIDs down-regulated the AhR signalling pathway by inhibiting AhR from binding to the xenobiotic-responsive element, and inhibited the expression and activity of carcinogen activating enzymes CYP1A1, CYP1B1 and CYP1A2; in contrast *S*-NSAIDs up-regulated carcinogen detoxification enzymes such as glutathione transferase A2, glutamate cysteine ligase and glutathione reductase [161]; these effects were most likely to be due to the dithiolethione moiety rather than $H_2S$ | Cancer chemoprevention and treatment |

**Table 5 Continued**

| Compound | Experimental model | Comments | Therapeutic potential |
|---|---|---|---|
| *S*-Valproate (structure shown), *S*-diclofenac and *S*-sulindac (ADT-OH derivatives of valproic acid, diclofenac and sulindac respectively).  | Human NCI-H1299 and A549 lung carcinoma cell lines, and NCI-H1299 and A549 xenograft in nude mice | *S*-Valproate and *S*-NSAIDs reduced COX-2 activity and inhibited cell proliferation in isolated cells and in nude mice xenografts, and. *S*-valproate and *S*-diclofenac increased the expression of the cell adhesion molecule E-cadherin and down-regulated the expression of an E-cadhedrin transcriptional suppressor ZEB1; NaSH at comparable concentrations did not inhibit cell proliferation, suggesting a lack of direct involvement of $H_2S$ and that the effects were due to the dithiolethione moiety [290] | Cancer chemoprevention and treatment |
| *S*-Diclofenac (ACS-5), *S*-sulindac (ACS-18) and *S*-valproate | HUVECs, human adenocarcinoma HT-29 cell lines C57B16 and Cr:(NCr)-athymic nu fBR mice, and embryonic zebrafish | *S*-NSAIDs and *S*-valproate inhibited vascular outgrowth from muscle and HT-29 adenocarinoma tumour explants, inhibited HUVEC proliferation and induced HSP27 phosphorylation; *S*-NSAIDs and *S*-valproate induced intersomitic vessel defects in zebrafish embryos, and inhibition of angiogenesis was independent of COX inactivation [291] | Inhibition of angiogenesis, cancer chemoprevention and treatment and wound healing |
| *S*-Sildenafil (ACS-6) (ADT-OH derivative of sildenafil)  | Human pulmonary aortic endothelial cells | *S*-Sildenafil inhibited TNF-$\alpha$-nduced superoxide formation and NADPH oxidase activity via PKA, PKG and cAMP-dependent pathways [128] | Acute respiratory distress syndrome |
| *S*-Latanoprost (ACS-67)  | Rat retina and retinal ganglion cells (RGC-5) | In retinal ischaemia, ACS67 reduced retinal and optic nerve damage, stimulated intracellular GSH synthesis and attenuated $H_2O_2$-induced toxicity [292] | Retinal ischaemia/neuroprotection |

## CONCLUDING REMARKS

$H_2S$ is emerging as a highly significant endogenous gaseous mediator which may play a substantial regulatory role in a variety of physiological systems, suggesting the therapeutic potential for manipulation of $H_2S$ in disparate human pathologies (summarized in Figure 1). However, understanding the complex physiology and pharmacology of endogenous $H_2S$ and pharmacological $H_2S$ has, as with any new and emerging field of research, been limited by the availability of specific research tools which has generated the inevitable controversy. In particular, the field is hampered by a lack of wholly enzyme- and tissue-specific inhibitors, $H_2S$ donors which release $H_2S$ at physiologically relevant rates and robust 'biomarkers' of $H_2S$ formation and metabolism. These non-specific tools have generated considerable controversy and often contradictory findings. $CSE^{-/-}$ knockout animals have highlighted a crucial role of $H_2S$ in the regulation of blood pressure [34], but these studies are similarly controversial and recent studies using $CBS^{-/-}$-knockout animals have further suggested that this enzyme is also a significant contributor to vascular $H_2S$ generation [131,176]. Neither of these genetic tools have yet been applied to animal models of human disease.

Molecular biology approaches using RNAi (RNA interference) offers the opportunity to selectively inactivate CSE, CBS and MPST, but this may not be suitable in every system such as primary cell cultures, certain cell lines known to be difficult to transfect, animal models and human clinical studies. The development of selective inhibitors which avoid the non-specific

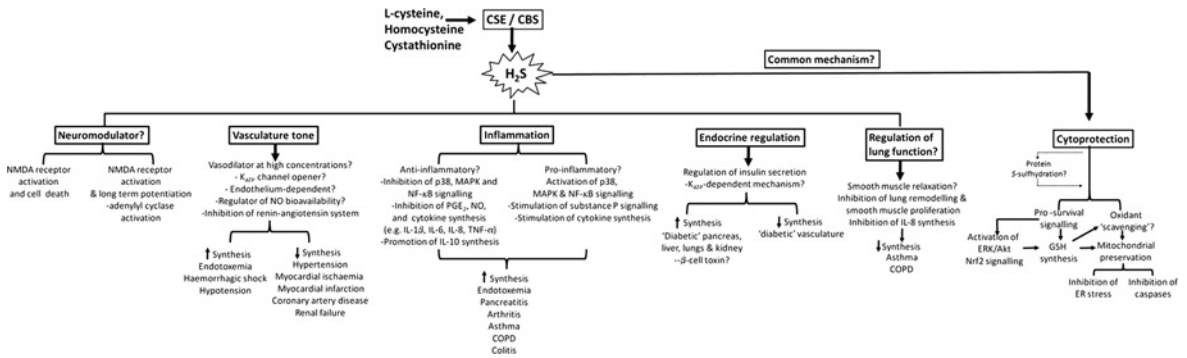



**Figure 1 The emerging physiological importance of $H_2S$ synthesis**

MAPK, mitogen-activated protein kinase.

effects highlighted in Table 2 are crucial for the field to advance. Slow-releasing $H_2S$-releasing molecules offer the opportunity to study $H_2S$ under physiologically relevant conditions, but as with any new experimental tool its use has so far been limited. Similarly, ADT-OH derivatives of NSAIDs have generated a new and exciting class of pharmacological compounds which have shown considerable therapeutic promise for the treatment of inflammatory and vascular conditions. The availability of these new $H_2S$ donors which release $H_2S$ in a slow and sustained manner will greatly increase our understanding of this remarkable endogenous gaseous mediator.

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