Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability?

Matthew Whiteman a, *, Philip K. Moore b

a Institute of Biomedical and Clinical Science, Peninsula Medical School, St. Luke’s Campus, Exeter, UK
b Pharmaceutical Science Division, King’s College London, London, UK

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Abstract
Hydrogen sulfide (H₂S) is a well known and pungent toxic gas that has recently been shown to be synthesised in man from the amino acids cystathionine, homocysteine and cysteine by at least two distinct enzymes: cystathionine-γ-lyase and cystathionine-β-synthase. In the past few years, H₂S has emerged as a novel and increasingly important mediator in the cardiovascular system but delineating the precise physiology and pathophysiology of H₂S is proving to be complex and difficult to unravel with disparate findings reported with cell types, tissue types and animal species reported. Therefore, in this review we summarize the mechanisms by which H₂S has been proposed to regulate blood pressure and cardiac function, discuss the mechanistic discrepancies reported in the literature as well as the therapeutic potential of H₂S. We also examine the methods of H₂S detection in biological fluids, processes for H₂S removal and discuss the reported blood levels of H₂S in man and animal models of cardiovascular pathology. We also highlight the complex interaction of H₂S with nitric oxide in regulating cardiovascular function in health and disease.

Keywords: nitrosothiol • cardioprotection • KATP • vasodilator • gaseous mediators • reactive nitrogen species • sulfur

Introduction

Hydrogen sulfide (dihydrogen sulfide, H₂S) is a well known and pungent toxic gas and its toxicology as an environmental pollutant has been extensively studied [1]. Since it was first discovered to be synthesized in human tissues a decade ago, it has attracted substantial interest. Studies in animals have shown H₂S to be involved in several physiological and pathophysiological processes as diverse as learning and memory, chronic inflammation and the regulation of blood pressure. It will not have escaped the reader’s attention that these processes are also tightly regulated by the much better understood gaseous mediator, nitric oxide. The similarity in physiological and pathophysiological effects between nitric oxide and H₂S has led to speculation about the existence of cellular ‘cross-talk’ between the two gases and the enzymes responsible for their synthesis.

It should be noted here that although the words sulfur and sulphur are widespread in the scientific literature, predominantly from United States and UK/UK-Commonwealth based researchers, respectively, an etymological basis for the spelling of sulphur is lacking. Sulfur is Latin and not Greek (the Greek for sulfur being thion) although both terms continue to be used interchangeably. However, it should be noted that the International Union of Pure and Applied Chemistry (IUPAC) applied the spelling of ‘sulfur’ in 1990 [2]. This was subsequently adopted by the nomenclature committee of the Royal Society of Chemistry (UK) in 1992 [3]. As such and to avoid confusion, we refer to H₂S as hydrogen sulfide.
Reactive nitrogen species (RNS) in the heart and vasculature

Nitric oxide and peroxynitrite (ONOO⁻) are RNS formed in vivo (extensively reviewed in [4]). Nitric oxide is a free radical gas synthesized enzymatically from the amino acid L-arginine in a number of tissues using the three isoforms of nitric oxide synthase (NOS). Classically, NOS1 and NOS-3 are constitutively expressed and require Ca²⁺/calmodulin to produce low nM quantities of nitric oxide over short periods of time. NOS2 is an inducible Ca²⁺-independent isoform and synthesizes high μM concentrations of nitric oxide over a period of hours. Nitric oxide is important in the endothelium-dependent regulation of blood flow and pressure, inhibiting the activation of blood platelets, neurotransmission and memory (long-term potentiation; LTP). Nitric oxide is also involved with host-defence and the inflammatory cascade (exhaustively reviewed in [4]). Since the half-life of nitric oxide at physiological pH is <5s, its formation in vivo can be quantitated by the measurement of its stable oxidation products, nitrite (NO₂⁻) and nitrate (NO₃⁻) and from the S-nitrosation of free or protein thiols (S-nitrosothiols) [4].

The roles of nitric oxide and ONOO⁻ in the cardiovascular system have been exhaustively studied and reviewed in great detail elsewhere [4, 7]. Briefly, nitric oxide, through the activation of the Ca²⁺/calmodulin-dependent enzymes NOS1 and NOS2 maintains cardiac function and vascular patency through direct endothelial-dependent interaction with soluble guanylate cyclase resulting in cGMP accumulation, intracellular protein phosphorylation and vasorelaxation. In conditions such as sepsis, shock or inflammation, a third isoform, NOS2 is induced resulting in high concentrations of nitric oxide being synthesized and potentially lethal hypotension. Administration of NOS2 inhibitors effectively attenuate this hypotension in animal models. In otherwise healthy individuals or animals, the administration of NOS3 or more recently NOS1 [8] inhibitors alone can increase blood pressure clearly demonstrating a role for nitric oxide in regulating blood pressure homeostasis under physiological conditions. Similarly, nitric oxide donating drugs exert potent hypotensive actions [9, 10].

In the vasculature, ONOO⁻ formation through substantially elevated nitric oxide synthesis can lead to endothelial lipid peroxidation, oxidation, nitration and inactivation of Ca²⁺-dependent vascular smooth muscle cell ATPases. ONOO⁻-mediated oxidation of low-density lipoprotein also leads to the activation of macrophage scavenger receptor to precipitate atherosclerosis as well as directly induce smooth muscle and endothelial cell death. Since this will ultimately lead to impaired vascular response, the physiological removal of ONOO⁻ by antioxidants, decomposition catalysts [4] or thiols (perhaps including hydrogen sulfide [11]) is considered beneficial in maintaining a healthy vasculature.

H₂S biosynthesis

H₂S is rapidly emerging as an important gaseous mediator in the vasculature. In sharp contrast to nitric oxide, and as one would expect from an emerging field of research, its vascular effects, its mechanism of action as well as the processes controlling the regulation of its synthesis are poorly understood. As with nitric oxide, H₂S is a highly lipophilic molecule and freely penetrates cells of all types. Whether or not H₂S and nitric oxide exert their effects in vivo independently or in tandem is currently not known but a growing body of literature is highly suggestive of H₂S involvement in the regulation of nitric oxide mediated signalling events and/or vice versa.

The bulk of endogenous H₂S synthesis in mammalian tissues appears to be from the pyridoxal-5’-phosphate-dependent enzymes cystathionine-γ-lyase (CSE; E.C. 4.4.1.1) and cystathionine-β-synthase (CBS; E.C. 4.2.1.22) and by analogy with NOS use amino acids as substrates; in this case cystathionine, cysteine and homocysteine (summarized in Fig. 1). H₂S may also be formed in vivo from the enzymatic desulfuration of β-mercaptothiopropionate derived from cysteine transamination [12] although it is currently uncertain how this pathway contributes to the levels of H₂S reported in mammalian tissues (see below). In should be noted at this point that in aqueous solution H₂S is weakly acidic (pKa at 37°C, 6.76) and dissociates to form two dissociation states; the hydrosulfide anion (HS⁻), pKa 7.04 and sulfide anion (S²⁻), pKa 11.96 according to the following sequential reactions:

\[ H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2H^+ \]

As such, at physiological pH of 7.4 approximately 18.5% of the total sulfide exists as the undissociated acid and 81.5% as the HS⁻ anion [13]. Therefore, since it is currently not known whether the biological effects of H₂S are mediated directly by H₂S itself or derived species that will also exist at pH 7.4 (such as S²⁻ or HS⁻), we prudently use the term H₂S in this review to reflect the sum of these species present at physiological pH.

The human gene for CSE is located on chromosome 1 (1p31.1) and two possible splice variants of CSE mRNA have been characterized although their precise function in H₂S synthesis are unknown. CSE expression is induced in liver, kidney and lung by pro-inflammatory mediators such as lipopolysaccharide [14] and also in animal models of type I diabetes mellitus [15] and pancreatitis [16] although the precise molecular pathways for the induced expression of CSE and activity remain elusive. Similarly, the human CBS gene is located on chromosome 21 (21q22.3) [17] and encodes several mRNA [18] but the function of these mRNA isoforms in terms of H₂S synthesis are not known. However, it has been suggested that the heme component of CBS functions as a cellular redox sensor [19] perhaps increasing H₂S generation in response to intracellular oxidant load. Interestingly, CBS is allosterically regulated by S-adenosylmethionine [20] and stimulation of CBS activity increases glutathione (GSH) synthesis by promoting cellular cysteine uptake and accompanying increased γ-glutamylcysteine synthetase activity [21, 22] suggesting a role for CBS (and perhaps H₂S) in addressing intracellular redox imbalances due to increased oxidant load. In agreement, H₂S or species derived from it at physiological pH, react with several cytotoxic oxidant species such as hypochlorite [23], peroxynitrite [10], hydrogen peroxide, O₂⁻ [24, 25] and nitric oxide [26] and prevent cell death induced by these oxidants suggesting a cytoprotective function for H₂S.
As with the various isoforms of NOS, CSE and CBS are widely expressed in human tissues and cells although a degree of tissue specificity is apparent. CBS is highly expressed in neuronal tissue, in particular in cerebellar Purkinje and hippocampal neurons. In contrast, CSE activity is most notable in blood vessels and peripheral tissues [27]. The liver of several species, including human beings, contain high levels of CSE and CBS expression and activity. Although there has been considerable interest in CSE and CBS over several decades due to their role in the trans-sulfuration pathway and homocysteine metabolism, very little is known about the precise molecular pathways for H2S synthesis from these enzymes. In neuronal tissue, CBS activity appears Ca2+/calmodulin-dependent suggesting that short-term control of H2S synthesis may be achieved by Ca2+ influx following neuronal depolarization [28] in a similar process to the synthesis of nitric oxide from the calcium/calmodulin-dependent NOS. The high endogenous concentrations of H2S measured in human, rat and bovine brain (between 50 and 160 μM) has led to the suggestion that like nitric oxide, H2S may function as an endogenous neuromodulator [29, 30].

**H2S measurement, catabolism and removal**

At present there are no known specific and reliable stable end products of H2S biosynthesis in the same way that NO2-/NO3- function as a robust index of NOS activity in vivo. The majority of studies which have evaluated serum or plasma levels of H2S have used a spectrophotometric approach based around methylene blue. This assay involves the ‘fixing’ or ‘trapping’ of aqueous sulfide in biological samples with zinc acetate to prevent unnecessary loss of H2S through volatilization or through aerial oxidation resulting in the formation of stable zinc sulfide [31]. Upon subsequent acidification, H2S is released and in the presence of N,N-dimethyl-p-phenylenediamine (DMPD) and of small quantities of ferric ions, the heterocyclic thiazine dye methylene blue produced can be readily measured using standard laboratory spectrophotometers at 670 nm (Fig. 2) [31] or analysed by high-performance liquid chromatography (see Table 1 for comparison of methods and levels of H2S reported in mammalian serum and plasma). It is possible that this spectrophotometric assay measures the sum of
Table 1 Comparison of plasma and serum levels of H₂S determined by several commonly used techniques: relevance to the cardiovascular system

| Species                        | Model                                      | Method of H₂S detection             | Fluid | H₂S level reported | Comment                                                                                                                                       | References |
|-------------------------------|--------------------------------------------|--------------------------------------|-------|--------------------|------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Male Sprague-Dawley rats      | Myocardial injury induced by homocysteine treatment | Commercial sulfide-sensitive electrode | Plasma | Control rats, ~30 µM* Homocysteine treatment, ~50 µM* | Homocysteine is a substrate for CSE. H₂S prevented myocardial injury. CSE expression and activity were lowered by high concentrations of H₂O₂ (0.1–10mM) | [34]       |
| Rats – species not specified  | Isoproterenol-induced myocardial injury    | Zinc acetate / N,N-dimethyl-p-phenylenediamine (DMPD) | Plasma | Control rats, ~65 µM* Isoproterenol treated rats, ~18 µM* | CSE involved in mediating cardiac contraction. Treatment of rats with isoproterenol and NaHS significantly lowered CPK and LDH release from myocardial tissue, lowered the levels of lipid peroxidation products and preserved left ventricular function. NaHS increased survival, induced capillary dilatation and reduced leucocyte infiltration into myocardial tissue. Part of the mechanism for these observations may include H₂S-mediated ‘scavenging’ of H₂O₂ and O₂⁻. | [25]       |
| Male Sprague-Dawley rats      | Endotoxemia induced by bacterial lipopolysaccharide from E. Coli | Zinc acetate / DMPD                  | Plasma | Control rats, ~26 µM* LPS treated rats, ~45 µM* | LPS increase plasma PGE₂ levels; an effect decreased by the H₂S-releasing drug S-diclofenac. LPS increased plasma NO₂⁻ levels; decreased by S-diclofenac suggesting H₂S–nitric oxide cross-talk | [113]      |
| Male Sprague-Dawley rats, WKY rats, Spontaneously hypertensive rats (SHR) | Development of a novel slow releasing H₂S donor (GY4137); Hypertension: induced by L-NAME induced hypertension, normotensive rats and SHR | Zinc acetate / DMPD                  | Plasma | Baseline levels of ~35 µM* rose to ~80 µM* 90 min after i.v. or i.p. administration of H₂S donor. | First demonstration of the biphasic effects of H₂S on cardiac function. Rapid release of H₂S by NaHS reduced cardiac contractility (left ventricular diastolic pressure) by 42% and heart rate by 53%. In contrast, the slow and sustained release of H₂S via GYY4137 had no effect on cardiac contractility or heart rate. NaHS increased mean arterial blood pressure in L-NAME treated rats whereas GYY4137 prevented L-NAME-induced hypertension. Furthermore GYY4137 markedly reduced systolic pressure in SHR but not WKY rats. | [79]       |
| Male Sprague-Dawley rats      | Type I diabetes induced by streptozotocin (STZ) | Zinc acetate / DMPD                  | Plasma | Control rats, ~40 µM STZ treated rats, ~38 µM | Insulin treatment significantly elevated plasma H₂S levels. STZ elevated liver, kidney and pancreas CSE and CBS expression and activity. STZ is a nitric oxide donating molecule and the decreased plasma levels of H₂S after STZ treatment could reflect consumption of H₂S by nitric oxide and / or derived intermediates | [15]       |

Continued
Table 1 Continued

| Species | Model | Method of H2S detection | Fluid | H2S level reported | Comment | References |
|---------|-------|-------------------------|-------|--------------------|---------|------------|
| Male Sprague-Dawley rats | High blood-flow induced pulmonary hypertension induced by abdominal aorta-inferior cava vein shunt | Zinc acetate / DMPD | Plasma | Control rats, 50.9 ± 3.9 µM Pulmonary hypertensive rats, 36.4 ± 2.6 µM | Shunt decreased CSE mRNA expression in lung CSE mRNA localized in smooth muscle cells on small pulmonary muscular arteries with minimal expression in endothelial cells | [110] |
| Male Sprague-Dawley rats | High blood flow induced pulmonary hypertension induced by abdominal aorta-inferior cava vein shunt | Zinc acetate / DMPD | Plasma | Control rats, 50.83 ± 4.01 µM Shut, 36.42 ± 3.12 µM | Shunting decreased CSE mRNA expression in medial and small pulmonary arteries and rate of H2S synthesis in lung tissue | [111] |
| Male WKY and spontaneous hypertensive (SHR) rats | Hypertension | Zinc acetate / DMPD | Plasma | WKY rats 48 ± 13 µM SHR rats 20 ± 9 µM | PAG treatment decreased H2S levels in WKY (to 21 ± 7 µM) and SHR (to 12 ± 10 µM) and also increased the expression of CSE in aorta in WKY and SHR suggesting H2S involvement in regulation of blood pressure | [61] |
| Male Sprague-Dawley rats | Haemorrhagic shock (blood withdrawl, 9-10ml over an hour in 2 min periods) | Zinc acetate / DMPD | Plasma | Prior to blood withdrawl, 28.9 ± 1.4 µM 60 min after blood withdrawl, 37.5 ± 1.3 µM | PAG or β-cyanoalanine injected either prophylactically or therapeutically, inhibited the increase in plasma H2S and drop in mean arterial blood pressure. This response was not inhibited by glibenclamide (KATP channel antagonist). Blood withdrawl increased liver CSE mRNA expression | [62] |
| Wistar rats | Hypoxic pulmonary hypertension (HPH) | Zinc acetate / DMPD | Plasma | Control rats, 301.6 ± 32.41 µM HPH, 92.2 ± 22.1 µM | NaHS increased lung CSE expression; levels of control rats approximately 10 fold higher than the reported findings of others with the same species of rat (see below). Nevertheless, HPH induced a significant decrease in plasma H2S. | [43] |
| Male Wistar rats | Myocardial infarction; ligation of left anterior descending artery from its origin between the pulmonary artery conus and the left atrium | Zinc acetate / DMPD | Plasma | Control rats, 38.2 ± 2.07 µM 48 hrs after MI induction, 59.2 ± 7.16 µM | PAG treatment reduced plasma H2S levels to 39.2 ± 5.02 µM whereas treatment with the H2S donor, NaHS, significantly increased plasma H2S levels to 92.2 ± 12.40 µM. MI and PAG treatment decreased CSE mRNA expression. PAG treatment reduced myocardial oedema and inflammatory cell infiltrate. CSE immunoreactivity detected in infarct area as well as in the endothelium of small vessels of area at-risk. In contrast, CSE was not detected in cardiomyocytes under these experimental conditions. | [70] |
Table 1 Continued

| Species                  | Model                                                                 | Method of H2S detection | Fluid  | H2S level reported                      | Comment                                                                                                                                  | References |
|--------------------------|-----------------------------------------------------------------------|-------------------------|--------|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|------------|
| Male Wistar rats         | Hypoxic pulmonary hypertension                                         | Zinc acetate / DMPD     | Plasma | Control group, 299.6 ± 12.4 µM         | H2S increased lung CSE expression; levels of control rats approximately 10 fold higher than the reported findings of others with the same species of rat. Administration of NaHS to hypoxia treated rats increased plasma H2S levels to 309.2 ± 13.6 µM Potential antioxidant action of H2S: Hypoxia decreased plasma antioxidant enzyme superoxide dismutase (SOD), increased levels of oxidized glutathione (GSSG) and lipid peroxidation markers (malondialdehyde); these effects were reversed by NaHS administration. | [114]      |
| Male Sprague-Dawley rats | Cardiac ischemia reperfusion; occlusion of left anterior descending coronary artery | Zinc acetate / DMPD     | Plasma | Control group, 58.28 ± 7.86 µM Ischemia-reperfusion group 30.32 ± 5.26 µM | NaHS decreased infarct size and improved haemodynamics (increased left ventricular diastolic pressure) Suggested mechanism for cardioprotection via down-regulation of c-fos expression in myocardium. | [115]      |
| Male Sprague-Dawley rats | Method comparison study                                                | Commercial sulfide-sensitive electrode | Plasma | Healthy rats, 45.6 ± 10.59 µM          | This study also shows the rapid removal, metabolism or sequestration of H2S added to vertebrate plasma; half time of decay of 10 µM Na 2S added to plasma at 37°C, 13.0 ± 0.2 sec. | [109]      |
| Lobund-Wistar (LW) rats Harlan-Sprague-Dawley (HSD) rats C57 Black/6 (C57) mice | Method comparison study                                                | Polargraphic H2S sensor and Ion-selective electrode Zinc acetate / DMPD | Plasma | H2S was not detected in LW or HSD rats or C57 mice. H2S was not detected in LW rats but 4.3 ± 0.5 µM detected in HSD rat plasma. | | [38] |
| Male Swiss albino mice   | Septic shock (induced by LPS)                                          | Zinc acetate / DMPD     | Plasma | Untreated animals, ~32µM* LPS 4 hrs, ~40µM LPS 20 hrs, ~65µM | LPS induced liver and kidney CSE expression and activity was inhibited by PAG. H2S levels correlated with myeloperoxidase expression and activity. | [14]       |
| Male Balb/C mice         | Acute pancreatitis induced by caerulein                               | Zinc acetate / DMPD     | Plasma | Control mice, 22.5 ± 1.9 µM Caerulein treatment, 31.1 ± 3.3 µM | Pancreatitis-induced CSE expression elevated plasma H2S levels. PAG treatment lowered plasma H2S levels, levels pancreatic amylase and myeloperoxidase activity, inhibited acinar cell death and lung injury. | [116]      |

Continued
| Species                        | Model                                      | Method of H₂S detection | Fluid   | H₂S level reported                | Comment                                                                 | References |
|-------------------------------|--------------------------------------------|--------------------------|---------|-----------------------------------|--------------------------------------------------------------------------|------------|
| Male Swiss albino mice        | Cecal ligation and puncture-induced sepsis (CLP) | Zinc acetate / DMPD     | Plasma  | Normal, ~10 µM* Sham, ~13 µM* CLP, 20 µM* | Sepsis-induced CSE expression and elevated plasma H₂S levels. Plasma H₂S levels were significantly lowered after prophylactic or therapeutic treatment with PAG. | [118]      |
| Female NOR/Ltj and female NOD/Ljt mice | Non-obese diabetic mouse model and controls | Zinc acetate / DMPD     | Plasma  | ~60 µM* NOD mice stage I, ~60 µM* stage II, ~33 µM* stage III, ~27 µM* | Plasma levels of H₂S decreased markedly as disease progressed. L-cysteine stimulated synthesis of H₂S and vasorelaxant effect in aortic tissue were significantly decreased with increasing disease progression. | [119]      |
| Male C57BL/6J mice            | Genetic knock-out studies; Wild-type (CSE+/+), CSE+/– and CSE−/− | Commercial sulfide ion-selective electrode | Serum   | CSE+/+ mice ~40 µM* CSE+/– mice ~31 µM* CSE−/− mice ~18 µM* | CSE−/− mice had higher systolic blood pressure than CSE+/+ mice; CSE+/− mice had higher systolic blood pressure than CSE+/+ mice. CSE−/− more sensitive to H₂S-induced vasodilatation than CSE+/+. | [55]       |
| Human                         | Chronic obstructive pulmonary disease (COPD); 27 patients with acute exacerbation of COPD (AECOPD), 37 patients with stable COPD | Commercial sulfide-sensitive electrode | Serum   | Smoking significantly lowered plasma levels of H₂S in healthy controls and AECOPD patients. High levels of nitric oxide (measured as total nitrite/nitrate) correlated to higher H₂S levels. H₂S levels also correlated with stage of lung obstruction with COPD (Stage I, ~72 µM; Stage II, ~50 µM; Stage III, ~40 µM; Stage IV, ~48 µM) H₂S levels negatively correlated with sputum neutrophil count and positively with lung function (predicted FEV₁). | Smoking significantly lowered plasma levels of H₂S in healthy controls and AECOPD patients. High levels of nitric oxide (measured as total nitrite/nitrate) correlated to higher H₂S levels. H₂S levels also correlated with stage of lung obstruction with COPD (Stage I, ~72 µM; Stage II, ~50 µM; Stage III, ~40 µM; Stage IV, ~48 µM) H₂S levels negatively correlated with sputum neutrophil count and positively with lung function (predicted FEV₁). | [33]       |
| Human                         | Chronic obstructive pulmonary disease (COPD); 18 patients before and after theophylline treatment | Commercial sulfide-sensitive electrode | Serum   | COPD 30-100 µM* Serum levels unaffected by theophylline treatment. | H₂S levels positively correlated with percentage of predicted FEV₁, sputum macrophage levels but negatively correlated with sputum neutrophil count. Sputum levels of H₂S were equivalent to levels of NO₂⁻. | [120]      |
| Human                         | 40 patients with coronary heart disease (CHD), 17 angiographically normal patients | Commercial sulfide-sensitive electrode | Plasma   | Normal controls, 51.7 ± 11.9 µM CHD, 26.1 ± 14.2 µM Single vessel CHD, 33.0 ± 15.0 µM Double vessel CHD, 16.9 ± 7.9 µM Multi-vessel CHD, 18.4 ± 7.8 µM Unstable angina, 23.6 ± 14.4 µM Acute myocardial infarction, 19.9 ± 7.5 µM Stable angina, 38.4 ± 14.5 µM CHD with coronary artery occlusion, Patients with CHD had significantly lower H₂S levels compared to angiographically normal controls. The number of affected vessels correlated with a decrease in H₂S levels suggesting decreased H₂S levels correlate with disease severity but could also reflect either H₂S consumption by vascular oxidants. Plasma levels were also significantly negatively correlated to blood glucose levels and significantly lower in smokers compared to non-smokers. | Patients with CHD had significantly lower H₂S levels compared to angiographically normal controls. The number of affected vessels correlated with a decrease in H₂S levels suggesting decreased H₂S levels correlate with disease severity but could also reflect either H₂S consumption by vascular oxidants. Plasma levels were also significantly negatively correlated to blood glucose levels and significantly lower in smokers compared to non-smokers. | [60]       |
| Species       | Model                          | Method of H₂S detection                                      | Fluid          | H₂S level reported                        | Comment                                                                                           | References |
|--------------|--------------------------------|---------------------------------------------------------------|----------------|-------------------------------------------|---------------------------------------------------------------------------------------------------|------------|
| Human        | Essential hypertension in children | Ionic conductance meter/sulfide specific detector            | Plasma         | Control group 65.7 ± 5.5 µM (mean age 10.5 ± 0.73)  Hypertensive children (mean age 10.48 ± 3.2yrs), 51.9 ± 6.0 µM | High plasma levels of H₂S correlated to low homocysteine concentrations. Hypertensive children had a lower H₂S: homocysteine ratio compared to the control group (5.8 ± 2.9 cf. 11.6 ± 3.3). Higher systolic pressure was associated with a lower plasma H₂S:homocysteine ratio. | [95]       |
| Human        | Healthy controls                | Gas chromatography-mass spectrometry with pentafluorobenzyl bromide dramatization | Whole frozen blood | 35–80 µM                                   | Method evaluation paper.                                                                                             | [35]       |
| Human        | Healthy volunteers              | Microdistillation and ion chromatography                      | Plasma         | Levels differed with varying dietary meat intake Meat free, 42 ± 15 µM 240 g meat, 47 ± 12 µM 420 g meat, 25 ± 0.8 µM | This study also directly compared the methylene blue assay with this chromatographic technique and found the levels obtained in both systems to be comparable                           | [36]       |
| Human        | Healthy volunteers and septic shock patients | Zinc acetate / DMPD                                      | Plasma         | Age and sex matched healthy controls, 43.8 ± 5.1 µM Septic shock, 150.5 ± 43.7 µM |                                                                                                                  | [14]       |

*Estimated by the authors from published figures.

H₂S-derived species such as HS⁻ and S²⁻ that exist at physiological pH rather than H₂S itself.

However, the majority of the reported studies been useful in generating a substantial amount of data for researchers to ‘benchmark’ their results and clearly highlight the emerging importance of H₂S and closely related species in the cardiovascular system. Levels of H₂S in the plasma measured using this spectrophotometric assay are generally comparable to other approaches (see Table 1). For example, Chen et al. [33] utilized a sulfide-selective electrode and detected serum H₂S levels in man of 37.9 ± 0.9 µM (healthy controls) cf. 33.0 ± 0.7 µM (smokers) and 51.1 ± 3.0 µM (chronic obstructive pulmonary disease [COPD] patients). Using a similar electrochemical method Chang et al. [34] reported plasma H₂S levels in rat of 30–50 µM. Furthermore, the analysis of human plasma by more laborious analytical techniques such as gas chromatography-mass spectrometry with pentafluorobenzyl bromide dramatization or microdistillation with ion chromatography revealed H₂S levels of 35–80 µM [35] and 25–50 µM [36], respectively. We recently reported comparable serum levels of H₂S in healthy human volunteers using the methylene blue spectrophotometric assay and found mean serum levels (±S.D.) of 43.8 ± 5 µM which were increased markedly (up to 200 µM in one case) during septic shock [14]. As such, the methylene blue based spectrophotometric assay appears to be convenient, relatively robust and at least comparable to other techniques for the measurement of plasma H₂S (‘free’ H₂S, HS⁻ or S₂⁻).

However, it is prudent to note that while the majority of laboratories report plasma H₂S in the region of 25–80 µM it is important to note here that a few laboratories have reported markedly different findings using the same analytical techniques. For example, Chenyu et al. [37] have reported levels of in excess of 300 µM in plasma from healthy rats whereas others [38, 39] have suggested that the actual level of H₂S in plasma was beyond the limit of detection for the methylene blue assay and electrochemical detectors (Table 1). Indeed, using rat tissue homogenates and head space gas chromatography Furne et al. [39] failed to detect tissue
release of H₂S from the tissues into the head space after supplementing tissues with L-cysteine. These important studies do not necessarily contradict the majority of the reported findings in the literature (see Table 1) as they would suggest that H₂S is sequestered or carried in plasma and tissues rather than released as ‘free’ H₂S. Indeed, it is intriguing to note here that when solutions of H₂S or the commonly used sulfide salt donors (Na₂S and NaH₂S) are prepared in the laboratory at concentrations reported in plasma, the solutions are characteristically pungent. However, blood, plasma, serum or tissue homogenates, while containing H₂S (Table 1) do not smell, further suggesting that the widely used assays could measure a form of bound or sequestered H₂S rather than ‘free’ H₂S.

Whatever the absolute level of H₂S in plasma, it is likely that any reported plasma levels could represent an underestimate of the true extent of H₂S synthesis as pathways for H₂S removal exist (summarized in Fig. 1) although it is uncertain how rapid this process occurs in the vasculature. The half-life of H₂S in mammalian plasma has been estimated at <30 min. although the mechanism for this has not been identified [38]. A direct reaction with methemoglobin forming sulfhemoglobin has been suggested and it is possible that sulfhemoglobin could act as a ‘sink’ for circulating H₂S as it releases H₂S upon reduction. Intracellular pathways for H₂S removal also exist. In cells, H₂S is rapidly oxidized to thiosulfate (S₂O₃²⁻) by mitochondria and subsequently converted to sulfite (SO₃²⁻) and the major and stable product, sulfate (SO₄²⁻) [40, 41]. Unfortunately, SO₃²⁻ cannot be used as a ‘bio-marker’ of endogenous H₂S production since it can be derived from the direct oxidation of cysteine by cysteine dioxygenase (E.C. 1.13.11.20) and oxidation of SO₂²⁻ by sulfate oxidase (E.C. 1.8.3.1) [42]. Urinary and blood S₂O₃²⁻ has been used as a marker of environmental H₂S inhalation [43, 44] and elevated urinary S₂O₃²⁻ has been measured in Down syndrome patients [45] with the suggestion that these reflected an overproduction of H₂S in Down syndrome patients although blood S₂O₃²⁻ levels were not determined in this study. However, S₂O₃²⁻ may not be completely reliable as an index of H₂S since S₂O₃²⁻ levels in blood without detection in urine have also been observed after industrial H₂S poisoning [46, 47] and urinary S₂O₃⁻ in otherwise healthy individuals vary greatly and are sensitive to small fluctuations in atmospheric H₂S [47]. In animal studies where SO₃²⁻, SO₂²⁻ and S₂O₃²⁻ have been measured simultaneously, H₂S exposure resulted in a greater elevation of SO₃²⁻ and SO₂²⁻ compared to S₂O₃²⁻ in lung tissue [48]. Similarly, incubation of plasma, liver, colonic or muscle tissue with H₂S or one of its metabolites methanethiol (see below) also leads to S₂O₃²⁻ and SO₂²⁻ generation [49]. These findings strongly suggest that the metabolism of H₂S in vivo is complex and the reliability of S₂O₃²⁻ as an index of endogenous H₂S synthesis requires further attention.

Further cellular H₂S removal processes also exist. For example, in the cytosol, H₂S also undergoes methylation in by thiol-S-methyltransferase (E.C. 2.1.1.9) to yield methanethiol (CH₃S) and dimethylsulfide (CH₃SCH₃) [49, 50]. An additional enzymatic removal process which is potentially important in colonic tissue involves rhodanese (thiosulfate : cyanide sulfurtransferase: E.C. 2.8.1.1) [51], an enzyme which catalyses the transfer of the sulfane sulfur atom from HS⁻ to a thiophilic acceptor (such as cyanide) via an enzymic persulfide intermediate to form thio-cyanate (SCN⁻) and SO₄²⁻. H₂S can also be oxidized by activated neutrophils to SO₂²⁻ [52] suggesting H₂S may represent an important source of SO₂²⁻ in vivo. Furthermore, H₂S could also be consumed by endogenous oxidant species in the vasculature such as peroxynitrite [10], hypochlorite [23], superoxide [24, 25], hydrogen peroxide [24, 25] and nitric oxide [26], mediators also known to be produced in elevated amounts in various cardiovascular pathologies.

H₂S in the heart and vasculature

H₂S is emerging as a novel and an important physiological mediator in the cardiovascular system. Much of our current knowledge of the cardiovascular biology of H₂S stems from the use of inhibitors of CSE such as D,L-propargylglycine (PAG), β-cyanoalanine and inhibitors of CBS such as aminoxyacetic acid. These compounds apparently target the pyridoxal phosphate binding site of these enzymes [53] and may not be entirely specific. It should also be noted that although intraperitoneal injection of PAG results in its rapid accumulation into the blood (peak within 2 hrs), an as yet unidentified metabolite of PAG produced through the D-amino acid oxidase pathway injures the renal proximal tubule cells resulting in significant polyuria, proteinuria and glucosuria 5–6 hrs after injection [54]. As such, a possible diuretic and/or other renal effects of this PAG metabolite (or PAG itself) in mediating the effects of PAG (and H₂S) on systemic blood pressure should be considered.

Nevertheless, in the present absence of wholly specific inhibitors in this emerging field of research, studies using PAG when combined with analytical techniques such as PCR, immunohistochemistry and Western blotting or more recently using animal knock-out models [55] have clearly shown CSE to be the more important of the two known H₂S synthesizing enzymes in the cardiovascular system [55, 56], where its expression is induced by pro-inflammatory mediators such as lipopolysaccharide and carragenan but down-regulated during hypoxia, hypertension and myocardial infarction (see Table 1). In sharp contrast, very little is known about the role of H₂S, CSE or CBS in human beings. To date only, one study has shown CSE to be present in human vascular tissue (internal mammary artery) [57] although it is known to be expressed in human liver [58] and leucocytes [59]. Nevertheless, the few emerging studies examining H₂S levels in human blood have clearly shown perturbed H₂S synthesis in coronary heart disease [60] and myocardial infarction, where lower H₂S levels have been suggested to reflect disease severity (see Table 1).

Although experiments in man and animals have clearly shown that removal of endogenous nitric oxide through inhibition of NOS (i.e. with L-NAME) rapidly increases blood pressure, contrasting effects of CSE inhibition (i.e. with PAG) have been reported. For example, injection of PAG exerted little immediate effect on blood
pressure in normotensive anaesthetized rats [61, 62] but increased blood pressure after endotoxic [14] or haemorrhagic shock [52] when administered either prophylactically or therapeutically. This suggested CSE-derived H2S as a pathological mediator in blood pressure homeostasis. In sharp contrast, longer-term injection of PAG in rats for 2–3 weeks significantly increased systolic blood pressure [63] suggesting the role of CSE was regulatory, rather than pathological. These disparities could either reflect the manner in which PAG was administered or merely the extent to which PAG is cell permeable in different animal models and different animal strains. Although PAG reduces H2S levels in the plasma of various animal models (see Table 1) and the activity of CSE in isolated cells in vitro, it has been so far assumed that PAG is freely cell permeable. However, to the best of our knowledge, studies explicitly showing cellular uptake of PAG are lacking. Nevertheless, by far the most compelling evidence for the role of H2S in the vasculature is from recent studies using CSE single and double knock-out mice [55] where CSE−/− and lesser extent CSE−/+ mice had significantly higher systolic arterial blood pressure than CSE+/+ mice. Furthermore, CSE−/− mice were substantially more sensitive to H2S-mediated vasodilatation than wild-type mice [55].

Plasma or serum levels of H2S in rat, mouse and human adult plasma vary between ~23 and 60 μM (summarized in Table 1). Within these reported plasma concentration ranges, H2S induces the relaxation and transient blood pressure reduction in a variety of vascular tissue such as rat aorta and portal vein [64, 65], rabbit corpus cavernosum [66] as well as perfused rat mesenteric [67] and hepatic [68] vascular beds. H2S has been shown to be produced in myocardial tissue by CSE in isolated cells in vitro and in vivo to protect against hypoxia-reperfusion injury, where it exerts a negative inotropic effect and preserves left ventricular function by lowering left ventricular systolic and end diastolic pressure and reducing infarct size [25, 69–72]. The mechanism for the vasculoprotective effects H2S (summarized in Table 2) may be mediated by a direct stimulation of plasma membrane KATP Channel-dependent vasorelaxation resulting in increased vascular flow. Rodent aortic ring preparations exposed to high concentrations of K+ ions show markedly reduced vasorelaxant response to H2S and KATP channel antagonists such as glibenclamide or 5-hydroxydecanoate (SHD) effectively blocked H2S-mediated vasodilation [65, 70, 71]. Patch clamp studies using isolated rat mesenteric [73] and aortic [74] vascular smooth muscle cells exposed to H2S show increased glibenclamide-inhibitable KATP-dependent current and cellular hyperpolarization [75]. However, H2S-mediated opening of KATP channels does not occur in isolated rat cardiomyocytes [76] suggesting the effects of H2S on KATP may be cell specific.

Additional pathways for H2S-mediated vasodilation have also been proposed. For example, H2S activates adenylate cyclase and elevates cAMP in cultured neurons [30, 32] and isolated human vascular smooth muscle (HVSMS) cells [77], suggesting a vasodilatory effect mediated through cAMP is also possible. On the other hand, the activation of this cAMP pathway was not observed in isolated rat cardiomyocytes where the inhibition of L-type Ca2+ channels was proposed [76] or rat vascular endothelial cells [78]. These findings further suggest cell and perhaps species specific effects of H2S on vascular cells as have been observed with nitric oxide.

H2S is further reported to stimulate charybdoxin/apamin-sensitiv K+ channels in vascular endothelium [73, 75] although other laboratories have not observed this [79]. Nevertheless, it is highly likely that, as with nitric oxide the vascular endothelium represents the major target for H2S [55, 73, 75, 79]. Consistent with this, reduced CSE expression/activity and decreased H2S concentration contributes to the pathophysiology of pulmonary hypertension in rodents [80] whereas CBS deficiency leads to hyperhomocysteinemia, increased blood pressure and endothelial dysfunction [81, 82]. Although CBS is not considered to be the vascular source of H2S [55], tissue deficiencies in CBS are thought to manifest in the vasculature as a consequence of homocysteine accumulation (reviewed in [83, 84]). Homocysteine is directly toxic to vascular endothelial cells (reviewed in [85, 86]) and induces apoptotic cell death [87], up-regulates surface expression of intracellular adhesion molecule (ICAM)-1 [88], augments monocyte adhesion [89] and promotes interleukin (IL)-1β and tumour necrosis factor-α formation [90] and interferes with vascular nitric oxide synthesis and bioavailability [91–94]. Interestingly, homocysteine treatment of Sprague-Dawley rats markedly increased plasma H2S levels via increased expression and activity of CSE [34] and it is tempting to speculate that this response by vascular cells to synthesize vasodilatory H2S in the presence of a detrimental vascular toxin was a protective and/or compensatory response to maintain vascular patency. In rodents, CBS or CSE deficiency induced by genetic deletion or chronic treatment with PAG results in a severe hypertension and severe loss of endothelial function [55, 82]. Interestingly, a close examination of the published literature clearly shows that spontaneously hypertensive rats have lowered H2S concentrations than rats from other species (i.e. WKY, Wistar, Sprague-Dawley) (Table 1). Furthermore, rodent models of pulmonary and hypoxic hypertension also show decreased plasma H2S levels. In each case, the administration of the H2S donor, NaHS, reduced blood pressure. Interestingly, children are reported to have higher plasma levels of H2S than adults [95] and one study has shown that plasma H2S concentrations are decreased with age in adults over 50–80 years old [33]. Together with the observation that H2S-mediated vasodilation is at least partly endothelium dependent [65, 73–75, 79]; this suggests that there is a possible loss of H2S synthesizing capacity or bio-availability over time that parallels age-associated increases in blood pressure in man.

It is possible that part of the vasculoprotective effects may be due to inhibition of platelet aggregation [96]. However, this is unlikely since significant inhibition platelet aggregation by H2S (~30 μM) at physiologically relevant concentrations was only observed with ADP and millimolar concentrations of NaHS were required to inhibit platelet aggregation induced by other mediators such as collagen, arachidonate and thrombin. It is further likely that H2S removes toxic oxidants that are known to be detrimental to endothelial function in the vasculature [11, 23–26]. An additional protective effect, consistent with a potential antioxidant role of H2S, may be in limiting vascular protein modification such as...
### Table 2 Summary of effects consistent with a protective role for H₂S in the vasculature

| Observation | Example | Comment | References |
|-------------|---------|---------|------------|
| **Antioxidant activity** | Scavenging or removal of toxic radical and radical-derived intermediates (i.e. ONOO⁻, HOCl, O₂⁻, H₂O₂, nitric oxide) | Cytoprotective effects observed in neuronal cells but potent in vitro oxidant scavenging also observed so it is possible the interaction with H₂S and these oxidants also occur in the vasculature. Indeed, H₂O₂ / O₂⁻ “scavenging” prevented myocardial injury in vitro. | [11, 23–26] |
| | | 3-nitrotyrosine (a bio-marker for ONOO⁻) and MDA levels (a bio-marker for lipid peroxidation) in rat heart were reduced in a model of myocardial ischemia reperfusion | [99] |
| | Inhibition of oxidative modification to low-density lipoprotein | Oxidation of low-density lipoprotein considered pro-atherogenic | [97] |
| | Inhibition of NADPH oxidase synthesis and O₂⁻ formation in human vascular smooth muscle cells | Down-regulation of NADPH oxidase expression. Possible involvement of cAMP and protein kinase A | [77] |
| | NaHS induces glutathione (GSH) synthesis and decreases plasma levels of oxidized glutathione (GSSG). | Effects observed in neuronal cells. Increased cysteine uptake and increased activity of γ-glutamylcysteine synthetase; GSH is an effective extracellular and intracellular antioxidant | [21, 22, 30, 114] |
| **Effects on vascular cells** | Inhibition of vascular smooth muscle cell proliferation | Activation ERK and p21^[CIP1/WAF1]-mediated pathway; observed in rat and human cells either treated with NaHS or through over-expression of CSE. | [100, 102] |
| | | However, inhibition of ERK has also been reported. | [101] |
| | Induction vascular smooth muscle cell apoptosis | Human artery smooth muscle cells. ERK and p38 MAPK activation, up-regulation of p21^[CIP1/WEAK−1] and down-regulation of cyclin D1 expression. Caspase-mediated cell death. | [103, 104] |
| | Induction of endothelial cell adhesion, migration and proliferation | AKT and PI-3K-dependent pathways | [98–102] |
| | Inhibition of myocardial apoptosis | Rat model of myocardial ischemia-reperfusion injury. NaHS-mediated inhibition of apoptosis was abolished by the putative mitochondrial K<sub>ATP</sub> channel inhibitor 2-HD. JNK, p38 and NF-κB-dependent pathways also activated. | [99] |
| | Promotion of angiogenesis | AKT pathway proposed. | [78] [99] |
| | NaHS inhibited adhesion molecule expression (ICAM-1) in rat model of myocardial ischemia reperfusion | | |
## Table 2 Continued

| Observation                                                                 | Example                                                                 | Comment                                                                                                                                  | References            |
|----------------------------------------------------------------------------|------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| **H₂S required for cardiovascular health**                                   |                                                                       |                                                                                            |                       |
| **Vasodilator effects**                                                    | Vaso-dilation via opening K<sub>A TP</sub> channel in smooth muscle and vascular endothelium; Induces relaxation and transient blood pressure reduction in rat aorta, portal vein and mesenteric and hepatic vascular beds; induces relaxation of rabbit corpus cavernosum; induces relaxation in human internal mammary artery. | H₂S-mediated vasodilation was endothelium dependent and inhibitable by glibenclamide or SHD suggesting K<sub>A TP</sub>-channel-dependent effects | [57, 64–68, 74–76, 79]|
| Inhibition of angiotensin-converting enzyme activity of endothelial cells  | If this reaction occurred in vivo it could potentially lead to vasodilation in vivo since inhibition of angiotensin-converting enzyme would decrease plasma levels of the angiotensin-II and decrease the breakdown of bradykinin. | Presumably the effect of H₂S on angiotensin-converting enzyme was due to H₂S-interaction with the active site Zn²⁺ ions since exogenous Zn²⁺ prevented enzyme inhibition. However, the effect could also be due to direct 'scavenging' effect of Zn²⁺ ions on H₂S as it is the basis of the methylene blue assay | [105]                 |
| Activation of adenyly cyclase and cAMP-mediated vasodilation               | NaHS increased cAMP in human vascular smooth muscle cells;             | NaHS also induces cAMP formation in cultured neurons and macrophages suggesting one additional mechanism for H₂S-mediated vasodilation is mediated through adenyly cyclase / cAMP. | [77]                  |
|                                                                           |                                                                       | However this effect was not observed in isolated rat cardiomyocytes or rat vascular endothelial cells highlighting potential cell-specific and species-dependent effects of H₂S. | [75, 77]              |
| **Low plasma levels implicated in cardiovascular disease**                 | Spontaneously hypertensive rats have lower plasma H₂S levels than normotensive WKY, Wistar or Sprague-Dawley rats | Data consistent with H₂S as a physiological regulator of blood pressure                                                                   | See Table 1 for comparison of levels between different rat strains |
|                                                                           |                                                                       | Data consistent with H₂S as a physiological regulator of blood pressure and cardiac function; low plasma H₂S reflected severity of cardiovascular disease. | [15, 25, 33, 60, 61, 79, 95, 110, 113, 114] [55] |
|                                                                           |                                                                       | Low levels could suggest decreased H₂S synthesis or consumption by toxic intermediates such as nitric oxide, ONOO⁻, O₂⁻, HOCl or H₂O₂. |                       |
|                                                                           | CSE<sup>−/−</sup> mice have higher blood pressure than CSE<sup>+</sup>−<sub>−</sub> and CSE<sup>+</sup>−<sub>+</sub> mice |                                                                       |                       |
Observation | Example | Comment | References
--- | --- | --- | ---
Slow release H<sub>2</sub>S donor decrease systolic blood pressure in spontaneously hypertensive rats but not normotensive WKY rats | Effects of NaHS compared with a novel slow releasing H<sub>2</sub>S donor; biphasic effects of H<sub>2</sub>S | [79]
Plasma H<sub>2</sub>S levels decline with increasing age. | H<sub>2</sub>S-mediated vasodilation is endothelium dependent. Age-dependent increase in blood pressure and endothelial dysfunction are well documented. Data consistent with H<sub>2</sub>S as a physiological regulator of blood pressure. | [33]

**Cardiovascular protective effects of H<sub>2</sub>S**

Protection against myocardial ischemia-reperfusion and myocardial infarction injury | Preserves left ventricular contractility and reduces infarct size | Opening of mitochondrial K<sub>ATP</sub> channels and up-regulation of BCL<sub>2</sub> protein to prevent mitochondrial permeability and release of pro-death factors (i.e. cytochrome c) i.e. H<sub>2</sub>S is cytoprotective. Inhibition of c-Fos signalling. Inhibition of pro-inflammatory signalling (p38, JNK and NF-κB) | [69–72, 99, 115]

Preservation of mitochondrial ultrastructure and respiratory function during ischemia reperfusion | H<sub>2</sub>S is a mediator in ischemic preconditioning | H<sub>2</sub>S activated AKT, PKC<sup>α</sup> and PKC<sup>ε</sup>-dependent signalling | [121]

Inhibition of platelet aggregation | Concentrations of H<sub>2</sub>S within the physiological range (~30 µM) prevented ADP-induced platelet aggregation. However, mM concentrations of H<sub>2</sub>S required to inhibit platelet aggregation via other mediators. Plasma H<sub>2</sub>S unlikely to reach mM concentrations. | [96]

Inhibition of L-type Ca<sup>2+</sup> channels in isolated rat cardiomyocytes | NaHS inhibited electricity-stimulated intracellular Ca<sup>2+</sup> mobilization in a K<sub>ATP</sub>-channel independent manner. Potential to reduced myocardial hypertrophy and inhibit cardiomyocyte apoptosis. | [76]

Inhibiting HOCl-mediated atherogenic modification of low-density lipoprotein and apolipoprotein and inhibit myeloperoxidase activity, presumably by interaction at the heme moiety of myeloperoxidase [97].

Alternatively, the cytoprotective effects of H<sub>2</sub>S may be due to the initiation of ‘protective cellular’ signalling pathways within cardiac and vascular tissues. For example, H<sub>2</sub>S induces extracellular regulated kinase (ERK) and protein kinase B (AKT) signalling pathways in cardiac tissue [78, 98, 99] and preserves mitochondrial ultrastructure and respiratory chain function *in vivo* [72] possibly via a mitochondrial pathway involving the preservation of Bcl-2 signalling and intriguingly, a mitochondrial K<sub>ATP</sub>-channel-dependent mechanism [99]. H<sub>2</sub>S is also reported to induce vascular angiogenesis and induced neovascularization in mice *in vivo* as well as promote proliferation, adhesion and migration in cultured endothelial cells *via* AKT and phosphatidylinositol 3-kinase phosphorylation...
Furthermore, H2S was recently shown to inhibit neointima formation after balloon injury in rat carotid artery in vivo by inhibiting vascular smooth muscle cell proliferation [100, 101], an effect also observed with HSVSM cell proliferation in vitro by either inducing or inhibiting [101] ERK and p21^{cip}/WAF phosphorylation [102]. Furthermore, a pro-apoptotic effect of H2S on HSVSM cells has also been proposed, also mediated through ERK/p21^{cip}/wak^{-1} [103] and p38 pathways involving caspase-3 activation [103, 104] suggesting that the role of H2S in the vasculature is to activate the endothelium and inhibit vascular smooth muscle cell function. Indeed, as with nitric oxide mediated vasorelaxation, the vasodilatory effects of H2S are endothelium dependent [65, 73–75, 79].

An additional effect of H2S may be due to the inhibition of angiotensin-converting enzyme on the surface of vascular endothelial cells [105] possibly resulting from an interaction of H2S with the Zn^{2+} moiety at the enzyme’s active site. Should the corresponding reaction occur in vivo this would result in vasodilatation due to lowered circulating levels of the vasoconstrictor angiotensin-II and consequently decrease the breakdown of the vasodilator bradykinin. Since it is also likely that H2S interacts with NOS3 and endothelial of release of nitric oxide, this suggests that these gases may be reciprocally regulated (see below). Indeed, H2S appears to activate adenylate cyclase/cAMP [30, 32, 77] signalling in certain cell types (but not others [76, 78]) whereas nitric oxide mediated effects are via guanylate cyclase/cGMP perhaps suggestive of complementary or competing pathways for the control of vascular tone.

Although the bulk of these publications to date have suggested that H2S is an endogenous vasodilator, concentrations of H2S towards the lower end of that measured physiologically have also shown that H2S induces endothelium-dependent vasoconstriction in rat [65] and human arteries [57]. In non-mammalian vertebrates, H2S induces vasorelaxation or vasoconstriction (or both) [13, 106]. In isolated ovine pulmonary arteries H2S causes tissue contraction whereas it induces a complex contraction–relaxation–contraction response in rat pulmonary arteries [106]. Indeed closer examination of the pioneering publications which examined the vasodilator effects of H2S on isolated rat aortic rings clearly show that low concentrations of H2S induce contraction [74, 75, 80]. Although this was not stated in the text of these publications, these findings are clearly visible in the published figures. We recently expanded these early studies and showed that in phenylephrine pre-contracted rat aortic ring preparations [65] and isolated human internal mammary artery [57], low concentrations of H2S-induced tissue contraction followed by relaxation. Both of these effects were dependent on intact endothelial cells since denuded tissue showed neither tissue contraction (at low H2S concentrations) or relaxation (at higher H2S concentrations) [65]. Furthermore we also showed that low concentrations of H2S-induced endothelium-dependent vasoconstriction in vitro and in anaesthetized rats this was inhibited by removal of endogenous nitric oxide with L-NAME [65] again suggesting molecular ‘cross-talk’ between H2S and nitric oxide.

Evidence for ‘cross-talk’ between nitric oxide and H2S

H2S-induced vasorelaxation is only partially blocked by glibenclamide when used at a concentration of 10 μM [57, 65, 73–75, 79], which is an appropriate concentration for K_{ATP} specificity. This implies the involvement of additional K_{ATP}–dependent mechanisms for these vascular effects. Together with the studies highlighted above, these findings strongly suggest the possibility of ‘cross-talk’ between vascular nitric oxide and H2S. However, the precise nature of such an interaction has proved difficult to characterize accurately. Recent studies in vascular tissue and cells from several species have shown H2S either acts at the level of NOS, nitric oxide influences CSE activity and that H2S (or species derived from it at physiological pH) interact with vascular-derived nitric oxide and vice versa with apparent formation of a ‘nitrosothiol’–like species. A summary of the present evidence consistent with cross-talk between H2S and nitric oxide is presented in Table 3.

The direct effects of H2S on NOS activity has been investigated with mixed results and as appears common for the emerging area of H2S biology, the mechanisms for these observations have not been fully investigated. For example Kubo et al. [107, 108] showed recombinant NOS1 (rat), NOS2 (mouse) and to a latter extent, NOS3 (bovine) were inhibited by NaHS perhaps through interfering with tetrahydrobiopterin binding. Work by others [63, 109–111] subsequently showed that H2S inhibited NOS3 but not NOS2 activity in isolated rat and mouse aortic rings [107] and in human umbilical vein endothelial (HUVE) cells [109]. However, the lack of effect of NOS2 is not surprising since NOS2 is an inducible protein and was not induced in these experiments. Nevertheless, further experiments showed that intraperitoneal injection of NaHS into rats significantly reduced plasma NO_{2}/NO_{3} levels and suggested inhibition of NOS3 activity [109]. However, in each of these studies, free nitric oxide, H2S or S-nitrosothiols were not measured. It is therefore possible that the lowered NO_{2}/NO_{3} levels in plasma, as an index of nitric oxide, reflected the consumption of nitric oxide by either the added H2S gas, the added H2S donor (NaHS) or endogenously produced H2S via L-cysteine/pyridoxal phosphate in these studies.

It is also possible that H2S exerts its effects on nitric oxide synthesis via modulation of NOS substrate availability. For example in a rat model of pulmonary hypertension, induced by abdominal aorta-inferior cava vein shunt, L-arginine up-regulated CSE expression and H2S synthesis in pulmonary artery smooth muscle cells [111, 112]. However, in rat aortic tissue and HUVE cells H2S gas or H2S generated via L-cysteine/pyridoxal decreased L-arginine uptake and NO_{2}/NO_{3}^{-} formation through an apparently K_{ATP} sensitive pathway [109], suggesting H2S could serve as a negative regulator of nitric oxide synthesis.

The effects of nitric oxide donors on H2S synthesis have also been investigated with species and tissue-dependent effects and contradictory findings reported. For example, the nitric oxide donor, sodium nitroprusside (SNP) up-regulates H2S production in rat vascular tissues and brain by increasing the expression of
Cardiovascular observation

### Involvement in common cardiovascular pathology
- Endogenous H$_2$S and nitric oxide levels negatively correlate with blood pressure and cardiac function
- Haemorrhagic and endotoxic shock induce elevated H$_2$S and nitric oxide synthesis; prophylactic and therapeutic pharmacological inhibition of CSE or NOS2 is protective
- Inflammation and oedema are associated with elevated H$_2$S and nitric oxide levels; inhibition of CSE or NOS decreases inflammation and swelling

### Stimulatory effects of H$_2$S on nitric oxide synthesis and nitric oxide mediated vasorelaxation
- The nitric oxide donor sodium nitroprusside (SNP) enhanced CBS activity and H$_2$S levels in vitro and increased H$_2$S synthesis in rat aortic, liver and ileum [63, 112]
- The vasorelaxant effect of the nitric oxide donors SNP and SIN-1 were enhanced by incubating rat aortic tissue with NaHS [64]
- L-NAME inhibited H$_2$S-mediated vasorelaxation in rat aorta and the conversion of L-cysteine to H$_2$S in rat aortic tissue. This effect was enhanced by treatment with the nitric oxide donor SNP [73]
- NaHS [64, 65] or slow release H$_2$S donor GYY4137 [79] prevented and reversed L-NAME-mediated hypertension in rats
- H$_2$S potentiatively expressed NOS2 following stimulation of cultured rat vascular muscle cells with interleukin (IL)-1β
- H$_2$S inhibited nitric oxide generation in isolated aortic tissues and in vivo [109] H$_2$S down-regulated NOS3 (eNOS) but not NOS2 (iNOS) expression however, NOS2 was not induced [107, 108]
- H$_2$S, NaHS inhibited L-arginine uptake in human umbilical vein endothelial (HUVE) cells [109, 110]
- L-cysteine / pyridoxal phosphate inhibited L-arginine uptake in HUVE cells; inhibited by PAG [109, 110]
- L-Arginine increased CSE mRNA expression in pulmonary vascular endothelial and smooth muscle cells [110]
- L-NAME inhibited CSE expression and H$_2$S synthesis in thoracic artery and superior mesenteric artery in rats [80]

### Inhibitory effects of H$_2$S on nitric oxide synthesis and nitric oxide mediated vasorelaxation
- NaHS inhibited recombinant NOS1 (nNOS) and NOS3 (eNOS) activity through interaction with BH$_4$ and NOS2 through unknown mechanisms [107–109]
- Nitric oxide dependent relaxation of rat aortic rings through either nitric oxide donors (SNP, SNAP or SIN-1) or nitric oxide dependent (acetylcholine, histamine) but not nitric oxide independent (i.e. isoproterenol) mediators was inhibited by exogenous (H$_2$S gas, NaHS) and endogenous (L-cysteine/pyridoxal phosphate) H$_2$S; an effect reversed by PAG [65]
- L-NAME inhibited H$_2$S-induced increase in mean arterial blood pressure in anaesthetized rats [65]
- H$_2$S inhibited vasorelaxation of isolated human internal mammary artery; induced by nitric oxide dependent mechanisms (i.e. acetylcholine) [57]
- PAG inhibited SNP-mediated vasorelaxation of isolated rat aorta [114]

### Other evidence of RNS-H$_2$S interaction
- Scavenging‘ of ONOO$^-$; inhibition of ONOO$^-$-mediated cell death, intracellular protein nitration and tyrosine nitration in vitro [11]
- Inhibition of tyrosine nitration in vivo in animal model of myocardial ischemia reperfusion [99]
- Several nitric oxide donors react with NaHS in vitro to form a species resembling an inert ‘nitrosothiol’ [11, 26, 57, 65]
- H$_2$S-dependent nitrosothiol formation during lipopolysaccharide-induced septic shock in the rat [26]
- NaHS-mediated decrease of O$_2^-$ formation in human vascular smooth muscle cells was inhibited by the nitric oxide donor spermine NONOate [77]
CSE or CBS [110–112] suggesting a possible interaction of these gases at the expression of their synthesizing enzymes. In rat aortic tissue, the vasorelaxant effect of SNP was enhanced in the presence of NaHS. At the level of vascular smooth muscle cells, H2S has been reported to either enhance [63] or to attenuate [65, 75] the relaxant effect of nitric oxide in the rat aorta and human internal mammary artery [57]. Furthermore, NaHS-mediated inhibition of O2− production in HVSM was attenuated by nitric oxide (supplied as spermine NONOate) [64]. However, it is also possible that O2− was consumed by nitric oxide (forming ONOO−) or ‘scavenged’ by H2S [23–26].

Evidence for the formation of a novel intermediate between nitric oxide and H2S at physiological pH

Given the reportedly high concentrations of H2S in the vasculature (Table 1) and the extensive tyrosine nitration (as a bio-marker for ONOO−) and NO2−/NO3− (as bio-markers for nitric oxide) observed in human tissues and fluids, (especially during pathology [4–7]), the reaction between H2S and ONOO− is plausible. Indeed, H2S was recently shown to react with ONOO− (or species derived from it at physiological pH) to inhibit nitrosative stress, cellular 3-nitrotyrosine formation and accompanying cell death [11]. However, since the half life of ONOO− at pH 7.4 and 37°C is <1 sec. [4–6], a much more plausible reaction is between nitric oxide (or intermediates derived from it) with H2S, especially since plasma levels of NO2− (indicative of nitric oxide synthesis) are reported to reach high concentrations during chronic inflammation, oedema and sepsis; the precise conditions in which H2S synthesis is stimulated. Indeed, spectrophotometric, amperometric and electron paramagnetic resonance spectroscopy have recently shown that H2S (or intermediates derived from it at physiological pH) also reacts with nitric oxide (or derived species at physiological pH) to form a novel nitrosothiol-like compound(s) [26]. Further, incubation of RAW264.7 cells with nitric oxide donors (but not NaHS) resulted in cGMP accumulation but in sharp contrast, pre-mixing the nitric oxide donor SNP with NaHS did not result in cGMP formation unless Cu2+ was added to release nitric oxide. This finding suggests that the ‘nitrosothiol’ did not increase cGMP levels itself but only after release of nitric oxide [26] and that this ‘nitrosothiol’ molecule was a relatively inert under these conditions. It is therefore tempting to speculate that the ‘nitrosothiol’-like molecule may act as a sink for regulating nitric oxide and/or H2S bioavailability.

The H2S–nitric oxide interaction has also been investigated ex vivo and in vivo using exogenous and endogenously produced H2S and nitric oxide. In rat aortic preparations the addition several nitric oxide donors (SNP, SIN-1, SNAP) to phenylephrine pre-contracted rat aortic rings resulted in substantial relaxation but the subsequent addition of NaHS or authentic H2S gas resulted in marked inhibition or reversal of nitric oxide donor-mediated relaxation [65]. However, pre-mixing nitric oxide donors with NaHS did not result in aortic ring relaxation unless Cu2+ was added to release nitric oxide from H2S-nitric oxide [52] again suggesting that the ‘nitrosothiol’ molecule formed through the interaction of nitric oxide with H2S at physiological pH was inert. In addition, in isolated rat aortic rings [65] and human internal mammary artery [57], nitric oxide dependent (i.e. using histamine or acetylcholine) but not nitric oxide independent relaxation (i.e. using isoprenaline) was substantially inhibited by low concentrations of NaHS or H2S gas and this effect removed or reversed by the addition of Cu2+ [65]. Nitric oxide dependent relaxation was also inhibited by the addition of L-cysteine/pyridoxal phosphate (as CSE substrate/co-factor), an effect reversed by the CSE inhibitor PAG [65]. Furthermore, we also showed that in liver homogenates from LPS-treated rats, ‘nitrosothiol’ formation was enhanced after the addition of L-cysteine/pyridoxal phosphate but inhibited by inclusion of PAG in the incubate [26]. These results suggest that H2S and nitric oxide or species derived from them at physiological pH, reacted together to form a molecule (presumably a nitrosothiol) which exhibited little or no vasorelaxant activity either in vitro or in vivo. Unfortunately, at present the precise identity, molecular structure and physiological properties of this nitrosothiol-like species are currently unknown.

Concluding remarks

The precise role of H2S under physiological and/or pathophysiological conditions remains unclear. The molecular interaction between NOS–CSE, nitric oxide–CSE, H2S–NOS and nitric oxide–H2S are ill-defined and require further attention. However, from the studies outlined above, it is likely that the physiological role of H2S might be to suppress the vasodilator effect of nitric oxide through two distinct mechanisms; inhibition of NOS3 and ‘scavenging’ of nitric oxide resulting in the enhancement of vascular contractility. Conversely, under pathophysiological conditions whereby nitric oxide and H2S synthesis are greatly increased (i.e. sepsis and shock) the vasculoprotective action of H2S and H2S-mediated nitric oxide ‘quenching’ of nitric oxide resulting in the enhancement of vascular contractility. Therefore, under pathophysiological conditions whereby nitric oxide and H2S synthesis are greatly increased (i.e. sepsis and shock) the vasculoprotective action of H2S and H2S-mediated nitric oxide ‘quenching’ of nitric oxide results in marked inhibition or reversal of nitric oxide donor-mediated relaxation [65]. However, pre-mixing nitric oxide donors with NaHS did not result in aortic ring relaxation unless Cu2+ was added to release nitric oxide from H2S-nitric oxide [52] again suggesting that the ‘nitrosothiol’-like molecule formed through the interaction of nitric oxide with H2S at physiological pH was inert. In addition, in isolated rat aortic rings [65] and human internal mammary artery [57], nitric oxide dependent (i.e. using histamine or acetylcholine) but not nitric oxide independent relaxation (i.e. using isoprenaline) was substantially inhibited by low concentrations of NaHS or H2S gas and this effect removed or reversed by the addition of Cu2+ [65]. Nitric oxide dependent relaxation was also inhibited by the addition of L-cysteine/pyridoxal phosphate (as CSE substrate/co-factor), an effect reversed by the CSE inhibitor PAG [65]. Furthermore, we also showed that in liver homogenates from LPS-treated rats, ‘nitrosothiol’ formation was enhanced after the addition of L-cysteine/pyridoxal phosphate but inhibited by inclusion of PAG in the incubate [26]. These results suggest that H2S and nitric oxide or species derived from them at physiological pH, reacted together to form a molecule (presumably a nitrosothiol) which exhibited little or no vasorelaxant activity either in vitro or in vivo. Unfortunately, at present the precise identity, molecular structure and physiological properties of this nitrosothiol-like species are currently unknown.

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