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

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Abstract

Hydrogen sulfide (H_2S) 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, H_2S has emerged as a novel and increasingly important mediator in the cardiovascular system but delineating the precise physiology and pathophysiology of H_2S 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 H_2S 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 H_2S. We also examine the methods of H_2S detection in biological fluids, processes for H_2S removal and discuss the reported blood levels of H_2S in man and animal models of cardiovascular pathology. We also highlight the complex interaction of H_2S 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, H_2S) 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 H_2S 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 gasesous mediator, nitric oxide. The similarity in physiological and pathophysiological effects between nitric oxide and H_2S 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 H_2S as hydrogen sulfide.

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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 Ca^{2+} calmodulin to produce low nM quantities of nitric oxide over short periods of time. NOS2 is an inducible Ca^{2+}-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 (NO_2^-) and nitrate (NO_3^-) 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 Ca^{2+}/calmodulin-dependent enzymes NOS1 and NOS3 maintains cardiac function and vascular patency through direct endothelial-dependent interaction with soluble guanylate cylcase 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 Ca^{2+}-dependent vascular smooth muscle cell ATPases. $ONOO^-$-mediated oxidation of low-density lipoprotein also leads to the activation of macrophage scavenge 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.

H_2S biosynthesis

H_2S 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, H_2S is a highly lipophilic molecule and freely penetrates cells of all types. Whether or not H_2S 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 H_2S involvement in the regulation of nitric oxide mediated signalling events and/or *vice versa*.

The bulk of endogenous H_2S 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). H_2S may also be formed *in vivo* from the enzymatic desulfuration of β-mercaptopyruvate derived from cysteine transamination [12] although it is currently uncertain how this pathway contributes to the levels of H_2S reported in mammalian tissues (see below). In should be noted at this point that in aqueous solution H_2S 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 (S^{2-}), 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 H_2S are mediated directly by H_2S itself or derived species that will also exist at pH 7.4 (such as S^{2-} or HS^-), we prudently use the term H_2S 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 H_2S 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 H_2S synthesis are not known. However, it has been suggested that the heme component of CBS functions as a cellular redox sensor [19] perhaps increasing H_2S 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 H_2S) in addressing intracellular redox imbalances due to increased oxidant load. In agreement, H_2S or species derived from it at physiological pH, react with several cytotoxic oxidant species such as hypochlorite [23], peroxynitrite [10], hydrogen peroxide, O_2^- [24, 25] and nitric oxide [26] and prevent cell death induced by these oxidants suggesting a cytoprotective function for H_2S.

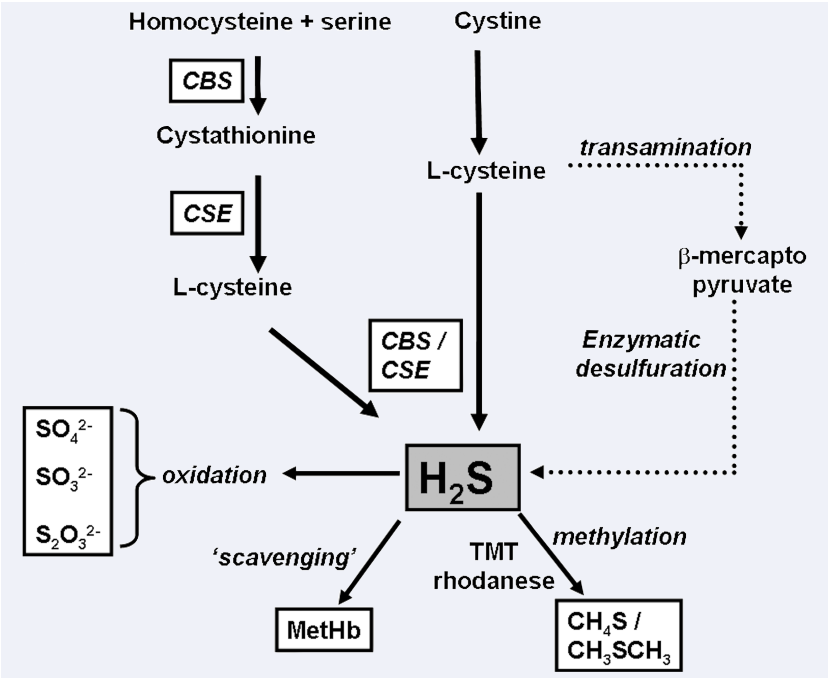

Fig. 1 Major Pathways of H_2S synthesis *in vivo*. H_2S is synthesized from the amino acids L-cysteine and L-cystathionine by one of two enzymes (depending on cell type), cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). Aminooxyacetate and propargylglycine (PAG) are commonly used inhibitors of CBS and CSE activity, respectively. The most prominent source of H_2S in the vasculature is CSE. Removal of H_2S is thought to occur *via* oxidation to sulfate (SO_4^{2-}), sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$), scavenging by methemoglobin (MetHb) or methylation by enzymes such as thiolmethyltransferase (TMT) and rhodanese to form methanethiol (CH_4S) and dimethylsulfide (CH_3SCH_3).

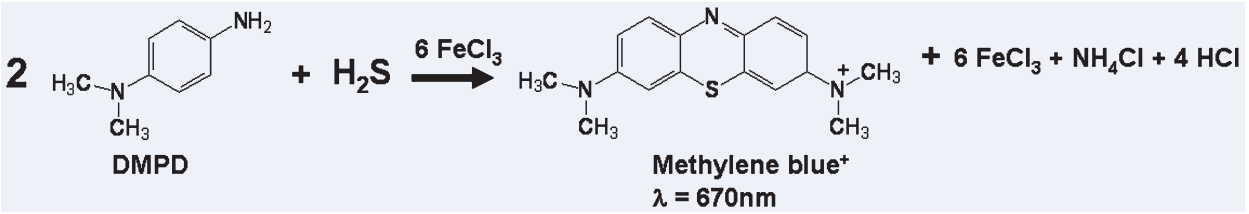

Fig. 2 Methylene blue assay for H_2S in biological fluids. This is the most simple and widely used assay for determining H_2S levels in body fluids. Using this and other methods (see Table 1), healthy human blood has been found to contain ~30-60 μM of H_2S. H_2S levels are reduced in hypertension and increased during endotoxic or haemorrhagic shock. Modified from [31].

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 H_2S synthesis from these enzymes. In neuronal tissue, CBS activity appears Ca^{2+}/calmodulin-dependent suggesting that short-term control H_2S synthesis may be achieved by Ca^{2+} 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 H_2S measured in human, rat and bovine brain (between 50 and 160 μm) has led to the suggestion that like nitric oxide, H_2S may function as an endogenous neuromodulator [29, 30].

H_2S measurement, catabolism and removal

At present there are no known specific and reliable stable end products of H_2S biosynthesis in the same way that NO_2^-/NO_3^- function as a robust index of NOS activity *in vivo*. The majority of studies which have evaluated serum or plasma levels of H_2S 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 H_2S through volatilization or through aerial oxidation resulting in the formation of stable zinc sulfide [31]. Upon subsequent acidification, H_2S 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 H_2S 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 H_2S determined by several commonly used techniques: relevance to the cardiovascular system

Species	Model	Method of H_2S detection	Fluid	H_2S 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. H_2S prevented myocardial injury. CSE expression and activity were lowered by high concentrations of H_2O_2 (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* Isoprotenerol 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 H_2S-mediated 'scavenging' of H_2O_2 and O_2^-.	[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 PGE_2 levels; an effect decreased by the H_2S-releasing drug *S*-diclofenac LPS increased plasma NO_2^- levels; decreased by *S*-diclofenac suggesting H_2S–nitric oxide cross-talk	[113]
Male Sprague-Dawley rats, WKY rats, Spontaneously hypertensive rats (SHR)	Development of a novel slow releasing H_2S donor (GYY4137); 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 H_2S donor.	First demonstration of the biphasic effects of H_2S on cardiac function. Rapid release of H_2S by NaHS reduced cardiac contractility (left ventricular diastolic pressure) by 42% and heart rate by 53%. In contrast, the slow and sustained release of H_2S *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 H_2S 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 H_2S after STZ treatment could reflect consumption of H_2S by nitric oxide and / or derived intermediates	[15]

Continued

Table 1 Continued

Species	Model	Method of H_2S detection	Fluid	H_2S 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 H_2S 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 H_2S 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 H_2S and drop in mean arterial blood pressure. This response was not inhibited by glibenclamide (K_{ATP} 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 H_2S.	[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 H_2S levels to 39.2 ± 5.02 μM whereas treatment with the H_2S donor, NaHS, significantly increased plasma H_2S 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]

Continued

Table 1 Continued

Species	Model	Method of H_2S detection	Fluid	H_2S level reported	Comment	References
Male Wistar rats	Hypoxic pulmonary hypertension	Zinc acetate / DMPD	Plasma	Control group, 299.6 ± 12.4 μM Hypoxia, 187.2 ± 13.1 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 . Administration of NaHS to hypoxia treated rats increased plasma H_2S levels to 309.2 ± 13.6 μM Potential antioxidant action of H_2S: 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		Commercial sulfide-sensitive electrode	Plasma	Healthy rats, 45.6 ± 10.59 μM		[109]
Lobund-Wistar (LW) rats Harlan-Sprague-Dawley (HSD) rats C57 Black/6 (C57) mice	Method comparison study	Polarographic H_2S sensor and Ion-selective electrode Zinc acetate / DMPD	Plasma	H_2S was not detected in LW or HSD rats or C57 mice. H_2S was not detected in LW rats but 4.3 ± 0.5 μM detected in HSD rat plasma.	This study also shows the rapid removal, metabolism or sequestration of H_2S added to vertebrate plasma; half time of decay of 10 μM Na_2S added to plasma at 37°C, 13.0 ± 0.2 sec.	[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. H_2S 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 H_2S levels. PAG treatment lowered plasma H_2S levels, levels pancreatic amylase and myeloperoxidase activity, inhibited acinar cell death and lung injury.	[116]
			Plasma	Control mice, ~30 μM* Caerulein treatment, ~35 μM*		[117]

Continued

Table 1 Continued

Species	Model	Method of H_2S detection	Fluid	H_2S 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 H_2S 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	Plsama	Control mice (NOR/Ljt), ~60 μM* NOD mice stage I, ~60 μM* stage II, ~33 μM* stage III, ~27 μM*	Plasma levels of H_2S decreased markedly as disease progressed. L-cysteine stimulated synthesis of H_2S 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 $CSE^{-/-}$	Commerical 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 H_2S-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:	healthy volunteers; aged 71-80yrs, 35.7 ± 1.2 μM aged 61-70yrs, 34.0 ± 0.9 μM aged 50-60yrs, 36.1 ± 1.1 μM No significant difference between healthy and AECOPD (~35 μM*) but elevated to in stable COPD patients (non-smokers, 51.1 ± 3.0 μM; smokers 49.8 ± 3.8 μM)	Smoking significantly lowered plasma levels of H_2S in healthy controls and AECOPD patients. High levels of nitric oxide (measured as total nitrite/nitrate) correlated to higher H_2S levels. H_2S 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) H_2S levels negatively correlated with sputal neutrophil count and positively with lung function (*predicted* FEV_1).	[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.	H_2S levels positively correlated with percentage of predicted FEV_1, sputum macrophage levels but negatively correlated with sputum neutrophil count. *Sputum levels of H_2S were equivalent to levels of NO_2^-.*	[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 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 levels were also significantly negatively correlated to blood glucose levels and significantly lower in smokers compared to non-smokers.	[60]

Continued

Table 1 Continued

Species	Model	Method of H_2S detection	Fluid	H_2S 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 H_2S correlated to low homocysteine concentrations. Hypertensive children had a lower H_2S: 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 H_2S: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.

H_2S-derived species such as HS^- and S^{2-} that exist at physiological pH rather than H_2S 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 H_2S and closely related species in the cardiovascular system. Levels of H_2S 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 H_2S 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 H_2S 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 chromatrgraphy revealed H_2S levels of 35–80 μM [35] and 25–50 μM [36], respectively. We recently reported comparable serum levels of H_2S 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 H_2S ('free' H_2S, HS^- or S_2^-).

However, it is prudent to note that while the majority of laboratories report plasma H_2S 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 H_2S 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 H_2S 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 H_2S is sequestered or carried in plasma and tissues rather than released as 'free' H_2S. Indeed, it is intriguing to note here that when solutions of H_2S or the commonly used sulfide salt donors (Na_2S and NaHS) are prepared in the laboratory at concentrations reported in plasma, the solutions are characteristically pungent. However, blood, plasma, serum or tissue homogenates, while containing H_2S (Table 1) do not smell, further suggesting that the widely used assays could measure a form of bound or sequestered H_2S rather than 'free' H_2S.

Whatever the absolute level of H_2S in plasma, it is likely that any reported plasma levels could represent an underestimate of the true extent of H_2S synthesis as pathways for H_2S removal exist (summarized in Fig. 1) although it is uncertain how rapid this process occurs in the vasculature. The half-life of H_2S in mammalian plasma has been estimated at $<$30 min. although the mechanism for this has not been identified [38]. A direct reaction with methemoglobin forming sulfhemoglobin has been suggested and it is possible that sulfhemoglobin could act as a 'sink' for circulating H_2S as it releases H_2S upon reduction. Intracellular pathways for H_2S removal also exist. In cells, H_2S is rapidly oxidized to thiosulfate ($S_2O_3^{2-}$) by mitochondria and subsequently converted to sulfite (SO_3^{2-}) and the major and stable product, sulfate (SO_4^{2-}) [40, 41]. Unfortunately, SO_4^{2-} cannot be used as a 'bio-marker' of endogenous H_2S production since it can be derived from the direct oxidation of cysteine by cysteine dioxygenase (E.C. 1.13.11.20) and oxidation of SO_3^{2-} by sulfite oxidase (E.C. 1.8.3.1) [42].

Urinary and blood $S_2O_3^{2-}$ has been used as a marker of environmental H_2S inhalation [43, 44] and elevated urinary $S_2O_3^{2-}$ has been measured in Down syndrome patients [45] with the suggestion that these reflected an overproduction of H_2S in Down syndrome patients although blood $S_2O_3^{2-}$ levels were not determined in this study. However, $S_2O_3^{2-}$ may not be completely reliable as an index of H_2S since $S_2O_3^{2-}$ levels in blood without detection in urine have also been observed after industrial H_2S poisoning [46, 47] and urinary $S_2O_3^{-}$ in otherwise healthy individuals vary greatly and are sensitive to small fluctuations in atmospheric H_2S [47]. In animal studies where SO_3^{2-}, SO_4^{2-} and $S_2O_3^{2-}$ have been measured simultaneously, H_2S exposure resulted in a greater elevation of SO_3^{2-} and SO_4^{2-} compared to $S_2O_3^{2-}$ in lung tissue [48]. Similarly, incubation of plasma, liver, colonic or muscle tissue with H_2S or one of its metabolites methanethiol (see below) also leads to $S_2O_3^{2-}$ and SO_4^{2-} generation [49]. These findings strongly suggest that the metabolism of H_2S *in vivo* is complex and the reliability of $S_2O_3^{2-}$ as an index of endogenous H_2S synthesis requires further attention.

Further cellular H_2S removal processes also exist. For example, in the cytosol, H_2S also undergoes methylation in by thiol-*S*-methyltransferase (E.C. 2.1.1.9) to yield methanethiol (CH_3S) and dimethylsulfide (CH_3SCH_3) [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 thiocyanate (SCN^-) and SO_4^{2-}. H_2S can also be oxidized by activated neutrophils to SO_3^{2-} [52] suggesting H_2S may represent an important source of SO_3^{2-} *in vivo*. Furthermore, H_2S 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.

H_2S in the heart and vasculature

H_2S is emerging as a novel and an important physiological mediator in the cardiovascular system. Much of our current knowledge of the cardiovascular biology of H_2S stems from the use of inhibitors of CSE such as D,L-propargylglycine (PAG), β-cyanoalanine and inhibitors of CBS such as aminooxyacetic 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 H_2S) 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 H_2S synthesizing enzymes in the cardiovascular system [55, 56], where its expression is induced by pro-inflammatory mediators such as lipopolysaccharide and carrageenan but down-regulated during hypoxia, hypertension and myocardial infarction (see Table 1). In sharp contrast, very little is known about the role of H_2S, 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 H_2S levels in human blood have clearly shown perturbed H_2S synthesis in coronary heart disease [60] and myocardial infarction, where lower H_2S 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 H_2S 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 systems and different animal strains. Although PAG reduces H_2S 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 H_2S in the vasculature is from very 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 then $CSE^{+/+}$ mice. Furthermore, $CSE^{-/-}$ mice were substantially more sensitive to H_2S-mediated vasodilatation than wild-type mice [55].

Plasma or serum levels of H_2S in rat, mouse and human adult plasma vary between ~23 and 60 μM (summarized in Table 1). Within these reported plasma concentration ranges, H_2S 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. H_2S has been shown to be produced in myocardial tissue by CSE *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 H_2S (summarized in Table 2) may be mediated by a direct stimulation of plasma membrane K_{ATP} 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 H_2S and K_{ATP} channel antagonists such as glibenclamide or 5-hydroxydecanoate (5HD) effectively blocked H_2S-mediated vasodilatation [65, 70, 71]. Patch clamp studies using isolated rat mesenteric [73] and aortic [74] vascular smooth muscle cells exposed to H_2S show increased glibenclamide-inhibitable K_{ATP}-dependent current and cellular hyperpolarization [75]. However, H_2S-mediated opening of K_{ATP} channels does not occur in isolated rat cardiomyocytes [76] suggesting the effects of H_2S on K_{ATP} may be cell specific.

Additional pathways for H_2S-mediated vasodilation have also been proposed. For example, H_2S activates adenylate cylcase and elevates cAMP in cultured neurons [30, 32] and isolated human vascular smooth muscle (HVSM) 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 Ca^{2+} channels was proposed [76] or rat vascular endothelial cells [78]. These findings further suggest cell and perhaps species specific effects of H_2S on vascular cells as have been observed with nitric oxide.

H_2S is further reported to stimulate charybdotoxin/apamin-sensitive 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 H_2S [55, 73, 75, 79]. Consistent with this, reduced CSE expression/activity and decreased H_2S concentration contributes to the pathophysiology of pulmonary hypertension in rodents [80] whereas CBS deficiency leads to hyperhomocyst(e)inemia, increased blood pressure and endothelial dysfunction [81, 82]. Although CBS is not considered to be the vascular source of H_2S [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 H_2S levels *via* increased expression and activity of CSE [34] and it is tempting to speculate that this response by vascular cells to synthesize vasodilatory H_2S 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 H_2S 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 H_2S levels. In each case, the administration of the H_2S donor, NaHS, reduced blood pressure. Interestingly, children are reported to have higher plasma levels of H_2S than adults [95] and one study has shown that plasma H_2S concentrations are decreased with age in adults over 50–80 years old [33]. Together with the observation that H_2S-mediated vasodilation is at least partly endothelium dependent [65, 73–75, 79]; this suggests that there is a possible loss of H_2S 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 H_2S (~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 H_2S 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 H_2S, may be in limiting vascular protein modification such as

Table 2 Summary of effects consistent with a protective role for H_2S in the vasculature

Observation	Example	Comment	References
Antioxidant activity	Scavenging or removal of toxic radical and radical-derived intermediates (i.e. $ONOO^-$, HOCl, O_2^-, H_2O_2, nitric oxide)	Cytoprotective effects observed in neuronal cells but potent *in vitro* oxidant scavenging also observed so it is possible the interaction with H_2S and these oxidants also occur in the vasculature. Indeed, H_2O_2 / O_2^- '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 O_2^- 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 NaHS increased plasma total antioxidant capacity	[21, 22, 30, 114]
Effects on vascular cells	Inhibition of vascular smooth muscle cell proliferation	Activation ERK and $p21^{cip/WAF}$-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 $p21^{cip/wak-1}$ 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_{ATP} 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	

Continued

Table 2 Continued

Observation	Example	Comment	References
H_2S required for cardiovascular health			
Vasodilator effects	Vasodilation *via* opening K_{ATP} 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.	H_2S-mediated vasodilation was endothelium dependent and inhibitable by glibenclamide or 5HD suggesting K_{ATP}-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 angiontensin-II and decrease the breakdown of bradykinin.	[105]
		Presumably the effect of H_2S on angiotensin-converting enzyme was due to H_2S-interaction with the active site Zn^{2+} ions since exogenous Zn^{2+} prevented enzyme inhibition. However, the effect could also be due to direct 'scavenging' effect of Zn^{2+} ions on H_2S as it is the basis of the methylene blue assay	
	Activation of adenyl cyclase and cAMP-mediated vasodilation	NaHS increased cAMP in human vascular smooth muscle cells;	[77]
		NaHS also induces cAMP formation in cultured neurons and macrophages suggesting one additional mechanism for H_2S-mediated vasodilation is mediated through adenyl cyclase / cAMP.	[30, 32]
		However this effect was not observed in isolated rat cardiomyocytes or rat vascular endothelial cells highlighting potential cell-specific and species-dependent effects of H_2S.	[75, 77]
Low plasma levels implicated in cardiovascular disease	Spontaneously hypertensive rats have lower plasma H_2S levels than normotensive WKY, Wistar or Sprague-Dawley rats	Data consistent with H_2S as a physiological regulator of blood pressure	See Table 1 for comparison of levels between different rat strains
	Low CSE expression, activity and H_2S levels contribute to the pathology of hypertension, coronary heart disease, angina, hypoxia and ischemia-reperfusion injury	Data consistent with H_2S as a physiological regulator of blood pressure and cardiac function; low plasma H_2S reflected severity of cardiovascular disease.	[15, 25, 33, 60, 61, 79, 95, 110, 113, 114] [55]
		Low levels could suggest decreased H_2S synthesis or consumption by toxic intermediates such as nitric oxide, $ONOO^-$, O_2^- HOCl or H_2O_2.	
	$CSE^{-/-}$ mice have higher blood pressure than $CSE^{+/-}$ and $CSE^{+/+}$ mice		

Continued

Table 2 Continued

Observation	Example	Comment	References
	Slow release H_2S donor decrease systolic blood pressure in spontaneously hypertensive rats but not normotensive WKY rats	Effects of NaHS compared with a novel slow releasing H_2S donor; biphasic effects of H_2S	[79]
	Plasma H_2S levels decline with increasing age.	H_2S-mediated vasodilation is endothelium dependent. Age-dependent increase in blood pressure and endothelial dysfunction are well documented. Data consistent with H_2S as a physiological regulator of blood pressure.	[33]
		Interestingly, hypertensive children had lower H_2S than normotensive children. Normotensive children have higher plasma H_2S than adults.	[95]
Cardiovascular protective effects of H_2S			
Protection against myocardial ischemia-reperfusion and myocardial infarction injury	Preserves left ventricular contractility and reduces infarct size	Opening of mitochondrial K_{ATP} channels and up-regulation of BCl_2 protein to prevent mitochondrial permeability and release of pro-death factors (*i.e.* cytochrome *c*) *i.e.* H_2S 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_2S is a mediator in ischemic preconditioning	H_2S activated AKT, PKC·α and PKCξ-dependent signalling	[121]
	Inhibition of platelet aggregation	Concentrations of H_2S within the physiological range (~30 μM) prevented ADP-induced platelet aggregation. However, mM concentrations of H_2S required to inhibit platelet aggregation *via* other mediators. Plasma H_2S unlikely to reach mM concentrations.	[96]
	Inhibition of L-type Ca^{2+} channels in isolated rat cardiomyocytes	NaHS inhibited electricity-stimulated intracellular Ca^{2+} mobilization in a K_{ATP}-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 cytoprotecitve effects of H_2S may be due to the initiation of 'protective cellular' signalling pathways within cardiac and vascular tissues. For example, H_2S 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_{ATP}-channel-dependent mechanism [99]. H_2S 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

[78, 98]. Furthermore, H_2S 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 HVSM cell proliferation *in vitro* by either inducing or inhibiting [101] ERK and $p21^{cip/WAF}$ phosphorylation [102]. Furthermore a pro-apoptotic effect of H_2S on HVSM 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 H_2S in the vasculature is to activate the endothelium and inhibit vascular smooth muscle cell function. Indeed, as with nitric oxide mediated vasorelaxation, the vasodilatatory effects of H_2S are endothelium dependent [65, 73–75, 79].

An additional effect of H_2S may be due to the inhibition of angiotensin-converting enzyme on the surface of vascular endothelial cells [105] possibly resulting from an interaction of H_2S with the Zn^{2+} moiety at the enzyme's active site. Should the corresponding reaction occur *in vivo* this would result in vasodilation 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 H_2S interacts with NOS3 and endothelial of release of nitric oxide, this suggests that these gases may be reciprocally regulated (see below). Indeed, H_2S 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 H_2S is an endogenous vasodilator, concentrations of H_2S towards the lower end of that measured physiologically have also shown that H_2S induces endothelium-dependent vasoconstriction in rat [65] and human arteries [57]. In non-mammalian vertebrates, H_2S induces vasorelaxation or vasoconstriction (or both) [13, 106]. In isolated bovine pulmonary arteries H_2S 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 H_2S on isolated rat aortic rings clearly show that low concentrations of H_2S 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 precontracted rat aortic ring preparations [65] and isolated human internal mammary artery [57], low concentrations of H_2S-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 H_2S concentrations) or relaxation (at higher H_2S concentrations) [65]. Furthermore we also showed that low concentrations of H_2S-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 H_2S and nitric oxide.

Evidence for 'cross-talk' between nitric oxide and H_2S

H_2S-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 H_2S. 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 H_2S either acts at the level of NOS, nitric oxide influences CSE activity and that H_2S (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 H_2S and nitric oxide is presented in Table 3.

The direct effects of H_2S on NOS activity has been investigated with mixed results and as appears common for the emerging area of H_2S 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 H_2S 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, H_2S 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 H_2S gas, the added H_2S donor (NaHS) or endogenously produced H_2S *via* L-cysteine/pyridoxal phosphate in these studies.

It is also possible that H_2S 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 H_2S synthesis in pulmonary artery smooth muscle cells [111, 112]. However, in rat aortic tissue and HUVE cells H_2S gas or H_2S generated *via* L-cysteine/pyridoxal decreased L-arginine uptake and NO_2^-/NO_3^- formation through an apparently K_{ATP} sensitive pathway [109], suggesting H_2S could serve as a negative regulator of nitric oxide synthesis.

The effects of nitric oxide donors on H_2S 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 H_2S production in rat vascular tissues and brain by increasing the expression of

Table 3 Evidence consistent with nitric oxide–H_2S cross-talk in the cardiovascular system

Cardiovascular observation
Involvement in common cardiovascular pathology
Endogenous H_2S and nitric oxide levels negatively correlate with blood pressure and cardiac function
Haemorrhagic and endotoxic shock induce elevated H_2S and nitric oxide synthesis; prophylactic and therapeutic pharmacological inhibition of CSE or NOS2 is protective
Inflammation and oedema are associated with elevated H_2S and nitric oxide levels; inhibition of CSE or NOS decreases inflammation and swelling
Stimulatory effects of H_2S on nitric oxide synthesis and nitric oxide mediated vasorelaxation
The nitric oxide donor sodium nitroprusside (SNP) enhanced CBS activity and H_2S levels *in vitro* and increased H_2S 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_2S-mediated vasorelaxation in rat aorta and the conversion of L-cysteine to H_2S in rat aortic tissue. This effect was enhanced by treatment with the nitric oxide donor SNP [73]
NaHS [64, 65] or slow release H_2S donor GYY4137 [79] prevented and reversed L-NAME-mediated hypertension in rats
H_2S potentiated expression of NOS2 following stimulation of cultured rat vascular muscle cells with interleukin (IL)-1β
H_2S *inhibited* nitric oxide *generation in isolated aortic* tissues and *in vivo* [109] H2S down-regulated NOS3 (eNOS) but not NOS2 (iNOS) expression however, NOS2 was not induced [107, 108]
H_2S, NaHS inhibited L-arginine uptake in human umbilical vein endothelial (HUVE) cells [109, 110]
L-cysteine / pyridoxdal 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]
Nitric oxide donors up-regulated the expression and activity of CSE in vascular tissues and cultured aortic smooth muscle cells [74, 124]
Synergistic effect of nitric oxide and H_2S on stonustoxin-induced relaxation of isolated rat aorta [123]
Synergistic effect of nitric oxide and H_2S on rat pulmonary artery relaxation [64]
H_2S-mediated ischemic after conditioning involved activation of NOS3 [121]
Inhibitory effects of H_2S 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]
L-NAME inhibited CSE expression and H_2S synthesis in thoracic artery and superior mesenteric artery in rats [80]
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.* isoprenaline) mediators was inhibited by exogenous (H_2S gas, NaHS) and endogenous (L-cysteine/pyridoxal phosphate) H_2S; an effect reversed by PAG [65]
L-NAME inhibited H_2S-induced increase in mean arterial blood pressure in anaesthetized rats [65]
H_2S 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_2S 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_2S-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, H_2S 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 O_2^- production in HVSM was attenuated by nitric oxide (supplied as spermine NONOate) [64]. However, it is also possible that O_2^- was consumed by nitric oxide (forming $ONOO^-$) or 'scavenged' by H_2S [23–26].

Evidence for the formation of a novel intermediate between nitric oxide and H_2S at physiological pH

Given the reportedly high concentrations of H_2S in the vasculature (Table 1) and the extensive tyrosine nitration (as a bio-marker for $ONOO^-$) and NO_2^-/NO_3^- (as bio-markers for nitric oxide) observed in human tissues and fluids, (especially during pathology [4–7]), the reaction between H_2S and $ONOO^-$ is plausible. Indeed, H_2S 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 H_2S, especially since plasma levels of NO_2^- (indicative of nitric oxide synthesis) are reported to reach high concentrations during chronic inflammation, oedema and sepsis; the precise conditions in which H_2S synthesis is stimulated. Indeed, spectrophotometric, amperometric and electron paramagnetic resonance spectroscopy have recently shown that H_2S (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 Cu^{2+} 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 H_2S bioavailability.

The H_2S–nitric oxide interaction has also been investigated *ex vivo* and *in vivo* using exogenous and endogenously produced H_2S and nitric oxide. In rat aortic ring preparations the addition several nitric oxide donors (SNP, SIN-1, SNAP) to phenylephirine pre-contracted rat aortic rings resulted in substantial relaxation but the subsequent addition of NaHS or authentic H_2S 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 Cu^{2+} was added to release nitric oxide from H_2S-nitric oxide [52] again suggesting that the 'nitrosothiol'-like molecule formed through the interaction of nitric oxide with H_2S 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 H_2S gas and this effect removed or reversed by the addition of Cu^{2+} [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/pyridoxidal phosphate but inhibited by inclusion of PAG in the incubate [26]. These results suggest that H_2S 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 H_2S under physiological and/or pathophysiological conditions remains unclear. The molecular interaction between NOS–CSE, nitric oxide–CSE, H_2S–NOS and nitric oxide–H_2S are ill-defined and require further attention. However, from the studies outlined above, it is likely that the physiological role of H_2S 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 H_2S synthesis are greatly increased (*i.e.* sepsis and shock) the vasculoprotective action of H_2S and H_2S-mediated nitric oxide 'quenching' are lost resulting in excessive and pathological loss of vascular tone. The isolation and molecular identification of the nitrosothiol-like species derived from H_2S and nitric oxide will greatly facilitate the unravelling of the complex interaction between these gases and could address the varied and contradictory reports on the physiology and pathophysiology of H_2S and nitric oxide.

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