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REVIEW

Hydrogen sulfide: physiological properties and therapeutic potential in ischaemia

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Hydrogen sulfide (H_2S) has become a molecule of high interest in recent years, and it is now recognized as the third gasotransmitter in addition to nitric oxide and carbon monoxide. In this review, we discuss the recent literature on the physiology of endogenous and exogenous H_2S, focusing upon the protective effects of hydrogen sulfide in models of hypoxia and ischaemia.

LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

Abbreviations

ALT, alanine transaminase; AOA, amino-oxyacetate; AST, aspartate transaminase; BCA, β-cyano-L-alanine; CA/CPR, cardiac arrest and cardiopulmonary resuscitation; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CDO, cysteine dioxygenase; CO, carbon monoxide; CSE, cystathionine γ-lyase; DADS, diallyl disulfide; DAO, D-amino acid oxidase; DATS, diallyl trisulfide; FXR, farnseoid X receptor; HA, hydroxylamine; HO-1, haem oxygenase 1; H-Ras, GTPase HRas; HSP90, heat shock protein 90; IRI, ischaemia/reperfusion injury; K_{ATP} channels, ATP-dependent K^+ channels; Keap1, Kelch-like ECH-associated protein 1; MI, myocardial infarction; MPST, 3-mercaptopyruvate sulfurtransferase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NSAID, non-steroid anti-inflammatory drug; PAG, DL-propargylglycine; PTP1B, protein-tyrosine phosphatase 1B; ROS, reactive oxygen species; SOD, superoxide dismutase; Sp1, specificity protein 1; Tom20, translocase of the outer membrane 20; WT, wild type

Tables of Links

TARGETS		LIGANDS			
Caspase-3	GSK3β	Aminooxyacetate (AOA)	Dopamine	IL-1β	Naproxen
COX-2	Haem oxygenase 1 (HO-1)	Aspirin	Glutathione (GSH)	Latanoprost	Nitric oxide (NO)
Cysteine aminotransferase (CAT)	Hydrogen sulfide (H_2S)	ATP	Haem	Levodopa	Propargylglycine (PAG)
Cystathionine β-synthase (CBS)	Inducible (i) NOS	cGMP	H_2O_2	L-cysteine	Sildenafil
Cystathionine γ-lyase (CSE)	Keap1	D-cysteine	HSP90	Memantine	TNF-α
Endothelial (e) NOS	K_{ATP} ion channels	Diclofenac	IL-6	NaHS	
ERK	$K_{ir}6.1$ ion channels				
FXR	MPST				
	VEGFR2				

These Tables list key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c,d).

Endogenous production and function

Hydrogen sulfide (H_2S) is considered to be the third gaseous signalling molecule – or gasotransmitter – together with nitric oxide ($^{\bullet}NO$) and carbon monoxide (CO). H_2S is produced enzymatically via three routes: from L-cysteine by cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS); from 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (MPST) with cysteine aminotransferase (CAT); and from D-cysteine via MPST with D-amino acid oxidase (DAO) (Figure 1). Interest in the field of H_2S research has grown markedly in recent years, with much less focus upon toxicity and far more attention to the many functions of endogenously produced and exogenously administered H_2S.

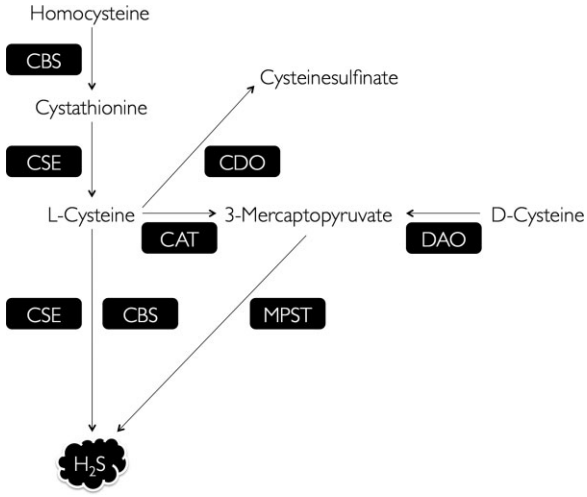

Figure 1
Overview of endogenous H_2S production and the enzymes involved. Abbreviations: CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CDO, cysteine dioxygenase; CSE, cystathionine γ-lyase; DAO, D-amino acid oxidase; MPST, 3-mercaptopyruvate sulfurtransferase.

Physiological function

The most widely studied function of endogenous H_2S relates to its vasodilator effects, of which the mechanism has been partially clarified. The functional properties of H_2S in the vascular bed can best be studied in genetically manipulated mice, such as the $CSE^{-/-}$ mice that have been developed in recent years, which need dietary cysteine to survive (Ishii *et al.*, 2010; Mani *et al.*, 2011). CSE deficiency causes hypertension, as evidenced by $CSE^{-/-}$ mice that show an increase in systolic BP of ~18 mmHg (Yang *et al.*, 2008). This increase in BP is similar in magnitude to that observed in endothelial NOS (eNOS) knockout mice (Huang *et al.*, 1995; Ishii *et al.*, 2010; Mani *et al.*, 2011). $CSE^{-/-}$ mice have diminished endothelium-dependent vasorelaxation, which is probably related to the vasorelaxant effects of H_2S. These effects are predominantly mediated through the opening of ATP-dependent K^+ (K_{ATP}) channels in vascular smooth muscle cells (Zhao *et al.*, 2001; Yang *et al.*, 2008). Recently, it has been shown that the mechanism behind the opening of K_{ATP} channels may involve the sulfhydration of critical cysteine residues within the $K_{ir}6.1$ subunit of this channel by H_2S (or intermediates derived from H_2S), and this effect is absent in $CSE^{-/-}$ mice, indicating an essential role for CSE/H_2S in this vasodilatation (Mustafa *et al.*, 2011). CBS regulates the cerebral microcirculation, and CBS-deficient mice show reduced or absent vasodilatation in precapillary arterioles in response to hypoxia, whereas CSE deficiency has no effect (Morikawa *et al.*, 2012).

CSE and CBS both respond to oxidative stress. $CSE^{-/-}$ animals are more susceptible to ischaemic damage (Bos *et al.*, 2013), and overexpression of CSE in cardiac tissue is protective in myocardial infarction (MI) (Elrod *et al.*, 2007). This protection seems to be related to the reduction of reactive

oxygen species (ROS) by H_2S (Kimura *et al.*, 2010; Bos *et al.*, 2013) and the protection of mitochondrial integrity and function (Elrod *et al.*, 2007). Mice deficient in CBS show increased oxidative modification of protein in their livers (Robert *et al.*, 2005), also indicating an anti-oxidative role for H_2S. Inhibition of CSE using DL-propargylglycine (PAG) in rats increased infarct size and myocardial inflammation after MI (Zhu *et al.*, 2006; Zhuo *et al.*, 2009), and bilateral ischaemia/reperfusion injury (IRI)-induced renal function loss was exacerbated by PAG (Tripatara *et al.*, 2008).

H_2S is essential for VEGF-mediated angiogenesis. Exogenous H_2S increases endothelial cell proliferation and migration, and deficiency of CSE causes impaired microvessel formation in aortic rings (Papapetropoulos *et al.*, 2009). In $CSE^{-/-}$ mice, wound healing is impaired and treatment of rats with NaSH improves wound healing (Papapetropoulos *et al.*, 2009; Coletta *et al.*, 2012). These beneficial effects were related to modulation of angiogenic mechanisms. The actions of endogenous H_2S seem to be dependent upon NO and might act through a cGMP-mediated mechanism (Coletta *et al.*, 2012).

Deficiency of CBS can cause vascular and endothelial dysfunction and cerebral interstitial remodelling (Sen *et al.*, 2008; Kundu *et al.*, 2009), and is associated with increased oxidative stress (Robert *et al.*, 2005). Lack of CBS produces hyperhomocysteinaemia, in humans as well as in mice, and the phenotypic changes in the CBS mouse can be due to either hyperhomocysteinaemia, reduced levels of H_2S or a combination of both. The $CBS^{+/-}$ mouse has been extensively used as a model for this disease. $CBS^{-/-}$ animals die at a very young age (3–4 weeks after birth) and are not frequently used for research purposes (Watanabe *et al.*, 1995). In Drosophila, CBS is necessary for the increased lifespan linked to dietary restriction (Kabil *et al.*, 2011). CBS is encoded on chromosome 21, and patients with Down's syndrome have triple the amount of CBS protein in their brains (Ichinohe *et al.*, 2005). It is still unclear whether this is related to the reduced mental capacity in this syndrome.

MPST is the least studied H_2S-producing enzyme and has been mostly associated with the CNS – with activity in the brain and retina – and the vascular endothelium, at least in rodents (Shibuya *et al.*, 2009a,b; Markand *et al.*, 2013). Intramitochondrial MPST has been shown to produce H_2S, where it is able to maintain energy production by donating electrons to the electron transport chain, facilitating ATP generation (Goubern *et al.*, 2007; Módis *et al.*, 2013b). Oxidative stress has been shown to perturb MPST-derived H_2S generation, an effect reversed by exogenously applied sulfide (Módis *et al.*, 2013a).

The loss of mitochondrial H_2S could account for the detrimental effects of oxidants on the mitochondrial function. H_2S can be used as an inorganic energy substrate for mitochondria (Goubern *et al.*, 2007). Under stress, such as increased intracellular calcium concentrations, or hypoxia, CSE and CBS can translocate to the mitochondria mediated via translocase of the outer membrane 20 (Tom20) (Suzuki *et al.*, 2011; Fu *et al.*, 2012; Szabó *et al.*, 2013; Teng *et al.*, 2013). It is hypothesized that translocation of CSE and CBS to the mitochondria can also increase mitochondrial H_2S levels, which, in turn, can be used as an electron donor in the electron transport chain.

Regulation of H_2S

CSE and CBS expressions are regulated through the specificity protein 1 (Sp1) transcription factor (Figure 2) (Wu *et al.*, 2010; Yang *et al.*, 2011b; Zhang *et al.*, 2011; Yin *et al.*, 2012). Recent research suggests that microRNA 21 (miR21) reduces the expression of Sp1 and CSE by directly targeting Sp1 (Yang *et al.*, 2012). ERK-mediated phosphorylation of Sp1 reduces its activity (Wu *et al.*, 2010). CSE expression through Sp1 can also be activated by TNF-α (Sen *et al.*, 2012). Nrf2 [nuclear factor (erythroid-derived 2)-like 2], a transcription factor that is activated by oxidative stress, can translocate to the nucleus upon activation and bind to an antioxidant responsive element to mediate gene transcription of protective genes such as glutathionine *S*-transferase or haem oxygenase 1 (HO-1) (Kaspar *et al.*, 2009). The expression of CSE can be modulated by Nrf2 (Figure 2) (Hassan *et al.*, 2012). Modulation of Sp1 or Nrf2 might be a strategy to affect CSE expression and endogenous H_2S production. Also, the CSE promotor contains a farnesoid X receptor (FXR, also known as NR1H4) responsive element, activation of which induces expression of CSE while mutation blocks FXR-mediated CSE expression (Renga *et al.*, 2009). It has been shown that CSE and CBS can be post-translationally modified by sumoylation and carbonylation (Carballal *et al.*, 2013), suggesting that there is reversible redox regulation of these enzymes. The activity of CBS is decreased by sumoylation, and it can theoretically be involved in nuclear translocation, although this has not been directly shown for CBS or CSE (Agrawal and Banerjee, 2008).

Recently, a new pathway for the production of H_2S from D-cysteine has been described, mainly by MPST in the cerebellum and kidney (Shibuya *et al.*, 2013), involving the

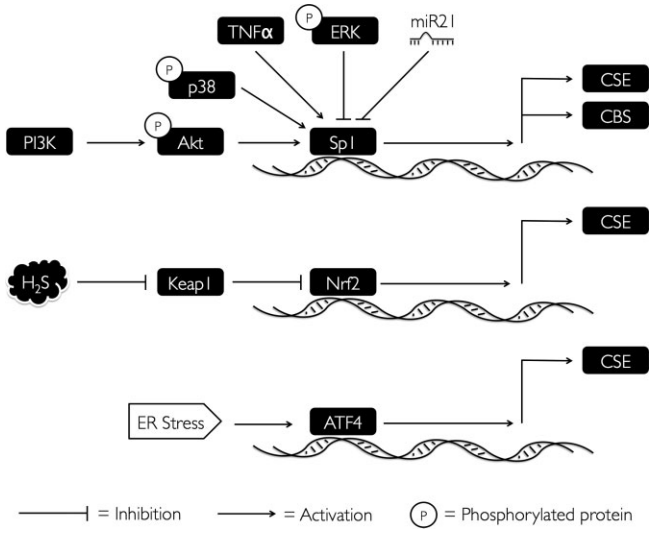

Figure 2

Overview of endogenous CSE and CBS regulation. Abbreviations: ATF4, activating transcription factor 4; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; ER, endoplasmic reticulum; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Sp1, specificity protein 1.

activity of DAO. CAT produces 3-mercaptopyruvate from cysteine and could be involved in the regulation of H_2S production (Mikami *et al.*, 2011b). Thioredoxin or dihydrolipoic acid and possibly other reductants seem necessary for production of H_2S from MPST (Mikami *et al.*, 2011a). CAT activity in retinal neurons is modulated by Ca^{2+} (Mikami *et al.*, 2011b). Cysteine dioxygenase (CDO) is responsible for most of the cysteine catabolism. In models of CDO deficiency, the increase in cysteine metabolism causes excess H_2S production through CSE and CBS and related toxicity (Ueki *et al.*, 2011; Roman *et al.*, 2013). For an overview, see Figure 1.

Another interesting mechanism is intracellular translocation to modify protein function. CSE can be translocated to the mitochondria, which might affect its function. This process is regulated by Tom20 (Fu *et al.*, 2012). When this process could be regulated, it might lead to new therapies to modulate CSE function. Sumoylation of CBS and CSE has also been demonstrated, where CBS decreases in activity when sumoylated (Agrawal and Banerjee, 2008). The effects of sumyolation on CSE have yet to be determined as well as the localized effects of the associated nuclear translocation.

One of the most remarkable properties of H_2S is the sulfhydration of proteins and modification of their activity, as mentioned earlier. This post-translational protein modification, where an SH-group is added to a reactive cysteine residue, can alter the activity of the target protein (Paul and Snyder, 2012). This is analogous to protein phosphorylation, although the mechanism of its reversal is not understood. It was first shown that 10–25% of the abundant GAPDH protein was sulfhydrated *in vivo*, and that sulfhydrated GAPDH had a higher activity than native GADPH. Well-studied proteins such as NF-κB, VEGFR2 (vascular endothelial growth factor receptor 2), PTP1B (protein-tyrosine phosphatase 1B), H-Ras (GTPase HRas), the K_{ATP} channel and Keap1 (Kelch-like ECH-associated protein 1) were all shown to be modified by exogenously applied H_2S, with associated changes in protein activity (Mustafa *et al.*, 2009; 2011; Krishnan *et al.*, 2011; Nishida *et al.*, 2012; Sen *et al.*, 2012; Hourihan *et al.*, 2013; Tao *et al.*, 2013). However, note that the biotin-switch assay used in these experiments is prone to artefacts, and these results therefore should be treated with caution. This is a field still in its infancy, but it seems that it can be a meaningful aspect of intracellular signalling pathways.

Exogenous administration of H_2S

There are currently several options for altering H_2S levels in experimental settings:

- Sulfide-sodium salts (NaSH, Na_2S)
- Exposure to gaseous H_2S
- Slow-releasing H_2S donors
- Hybrids of H_2S donors and known substances
- Cysteine analogues
- Modulating the expression or activity of H_2S-producing enzymes

The physicochemical properties of H_2S cause several problems for application in biomedical experiments. In most published literature, sodium salts that dissociate to produce H_2S in solution have been used (NaSH or Na_2S). The major problem in using sulfide salts as H_2S donors is the very quick loss of sulfide from solution and the rapid reactivity and catabolism of the H_2S (Kimura *et al.*, 2006; Li *et al.*, 2007; Marutani *et al.*, 2012; Predmore *et al.*, 2012). It has become clear that these crude salts have disadvantages, such as the rapid peak and subsequent rapid decrease in H_2S levels when dissolved or injected (Li *et al.*, 2008; Marutani *et al.*, 2012). This makes it difficult to achieve controlled, stable and therapeutic levels *in vitro* and *in vivo*. In addition, it is unclear whether the concentrations that are added in different models are physiological, and it seems likely that many effects of H_2S are observed at concentrations that far exceed endogenous levels.

The development of slow-releasing H_2S donors has improved the ability to achieve stable increases in H_2S (Li *et al.*, 2007; 2008; Zhao *et al.*, 2011; Marutani *et al.*, 2012; Predmore *et al.*, 2012). Addition of NaSH or Na_2S to a pH-neutral solution leads to an increase in HS^- and H_2S levels, with peak levels occurring within minutes, after which the volatile H_2S rapidly escapes from the solution, or quickly reacts with proteins so that the amount of H_2S decreases to normal levels in 30 min to 3 h (Li *et al.*, 2007; Marutani *et al.*, 2012; Predmore *et al.*, 2012). Although some postulate that one of these two common sulfide-sodium salts is superior to the other, with the purity of NaHS solutions being questioned (Doeller *et al.*, 2005), the little comparative work that has been performed in biological settings shows no obvious differences between them (Kai *et al.*, 2012).

The exposure of animals or cells to gaseous H_2S poses some technical problems that mainly concern toxicity and related safety measures that need to be taken when using pressurized H_2S-containing gases. In addition, the corrosive nature of H_2S absolutely requires specialized materials. The use of gaseous H_2S can induce very stable states of hypometabolism in mice (Blackstone *et al.*, 2005; Blackstone and Roth, 2007). Gaseous H_2S can be used for small animal experiments or for the exposure of cells or nematodes (Blackstone *et al.*, 2005; Miller and Roth, 2007; Bos *et al.*, 2009). However, the actual local concentrations in target organs that are concurrently delivered with different concentrations of gaseous H_2S are still very uncertain. In addition, attempts to show similar effects in larger animals have not produced states of reduced metabolism (Haouzi *et al.*, 2008). However, one study has shown that i.v. administration can reduce metabolic parameters in pigs (Simon *et al.*, 2008).

In recent years, two major developments in this field have occurred. Firstly, compounds that slowly generate H_2S have been produced (see Table 1). These have made it possible to study the effects of long-term heightened H_2S levels *in vitro* and *in vivo*. This as opposed to the large transient peaks in H_2S levels produced by daily injection of sulfide-generating sodium salts, where the serum H_2S levels are close to normal most of the day.

The second development is the creation of hybrids from known and widely used drugs with sulfide-releasing compounds. These new conjugates can potentiate effects or diminish side effects of the old drug. A large proportion of the hybrids currently under development are non-steroid anti-inflammatory drugs (NSAIDs). For example, ACS14

Table 1

Overview of H_2S-releasing compounds available or under development

Name	Substance	Producer	Effects	References
Conjugates				
ACS14	H_2S-releasing aspirin	CTG Pharma	Increased H_2S and GSH levels in rats. Reduced cell death and ROS in retinal neurons and increases GSH levels.	Giustarini *et al.* (2010); Osborne *et al.* (2012)
ACS15	H_2S-releasing diclofenac	CTG Pharma	Attenuated ischaemic damage in isolated perfused hearts. Inhibited vascular outgrowth from tumour and muscle. Reduced endothelial proliferation.	Rossoni *et al.* (2008); Isenberg *et al.* (2007)
ACS6	H_2S-releasing sildenafil	CTG Pharma	Antioxidative properties in arterial endothelial cells. Similar effects to sildenafil on corpus cavernosum relaxation and reduces ROS. Attenuated Hcy-induced cell death, apoptosis and ROS.	Muzaffar *et al.* (2008); Shukla *et al.* (2009); Tang *et al.* (2012)
ACS67	H_2S-releasing latanoprost	CTG Pharma	Protected from retinal ischaemia, reduces apoptosis and ROS	Osborne *et al.* (2010)
ACS83/84/85/86	H_2S-releasing Levodopa	CTG Pharma	Increased dopamine and GSH levels in the brain	Lee *et al.* (2010)
S-Memantine	H_2S-relasing memantine	CTG Pharma	Improved neurological outcome and smaller infarct volume in brain ischaemia	Marutani *et al.* (2012)
Singular H_2S donors				
NaHS	Sulfide-sodium salt	Generic	Releases H_2S very rapidly	–
Na_2S	Sulfide-sodium salt	Generic	Releases H_2S very rapidly	–
IK-1001	Dissolved H_2S-gas solution	Ikaria	Iso-osmotic, pH neutral	Elrod *et al.* (2007)
SG-1002	Mixture, mainly sulfide-ring	Sulfagenix	Reduced cardiac hypertrophy after transverse aortic constriction in WT and $CSE^{-/-}$ mice	Kondo *et al.* (2013)
ADTOH	Dithiolethione	Generic	Increased GSH levels in plasma, aorta and heart	Sparatore *et al.* (2009)
DATS	Diallyl trisulfide	Generic	Reduced ischaemic damage in myocardial infarction, protected mitochondria	Predmore *et al.* (2012)
SPC	*S*-Allyl-L-cysteine	Generic	Reduced oxidative stress in rat model of myocardial infarction	Wang *et al.* (2010b)
SAC	*S*-Propyl-L-cysteine	Generic	Reduced oxidative stress in rat model of myocardial infarction	Wang *et al.* (2010b)
SPRC	*S*-Propargyl-cysteine	Generic	Reduced oxidative stress in rat model of myocardial infarction	Wang *et al.* (2010b)
NACET	*N*-Acetyl-L-cysteine ethyl ester	Generic	Increased H_2S level in plasma is rapidly taken up by erythrocytes	Giustarini *et al.* (2010)
N-(Benzoylthio)benzamide	Cysteine-activated H_2S donor	Generic	Increased H_2S levels only in the presence of L-cysteine	Zhao *et al.* (2011)

SPRC, *S*-propargyl cysteine; WT, wild type.

(S-aspirin), a H_2S-releasing form of aspirin, has a broader inhibitory effect on platelet aggregation compared with aspirin, showing an effect on ADP- and thrombin receptor activating peptide (TRAP)-induced aggregation where aspirin has minimal effect (Pircher *et al.*, 2012). ATB-346, a H_2S-releasing form of naproxen, greatly reduces gastrointestinal mucosal damage associated with NSAID usage (Wallace *et al.*, 2010; Blackler *et al.*, 2012). ACS15 (S-diclofenac) inhibits angiogenesis, has strong anti-inflammatory effects in LPS- or amyloid-α-induced inflammation, and attenuated

detrimental effects of ischaemic myocardial damage in rats (Isenberg *et al.*, 2007; Li *et al.*, 2007; Baskar *et al.*, 2008; Rossoni *et al.*, 2008). Other examples are ACS6 (S-sildenafil), which has smooth muscle relaxant effects on the corpus cavernosum, but also showed antioxidative effects in endothelial cells (Muzaffar *et al.*, 2008; Shukla *et al.*, 2009). ACS83 (S-Levodopa) increased dopamine and GSH levels in the brain and reduced the glial inflammatory response (Lee *et al.*, 2010).

An alternative development is the use of naturally occurring cysteine analogues such as diallyl trisulfide (DATS), which can be derived from garlic (Benavides *et al.*, 2007) or *S*-propargyl cysteine. These compounds seem to increase H_2S levels for longer periods of time and have protective effects in models of ischaemia (Q Wang *et al.*, 2009; 2010b; Predmore *et al.*, 2012). However, it is not sure whether these compounds change H_2S levels significantly *in vivo*, and these compounds might have additional effects independent of H_2S.

Another option for H_2S-based treatments is modulation of the expression or activity of H_2S-producing enzymes. There are several compounds that inhibit the activity of CSE or CBS. These are however not very specific. Also, since therapeutic effects of H_2S mostly are related to increased levels, use of these substances has often been shown to be detrimental in model systems. CSE inhibitors are PAG and β-cyano-L-alanine (BCA). CBS inhibitors are amino-oxyacetate (AOA) and hydroxylamine (HA). These compounds are aspecific and need very large concentrations to have an effect on CBS or CSE activity (Whiteman *et al.*, 2011; Asimakopoulou *et al.*, 2013). In addition, HA is a widely used donor of NO. At the moment, no inhibitors are known for MPST. *S*-adenosyl-L-methionine can increase the CBS activity *in vivo* and *in vitro* (Jensen *et al.*, 2011; Hnízda *et al.*, 2012). As mentioned, modulation of Sp1, Nrf2, FXR or miR21 might be targets for modulation of the expression of CSE and CBS.

Therapeutic potential of H_2S in oxidative stress

One of the most prominent and well-studied effects of H_2S is the reduction in damage related to oxidative stress. Many groups have investigated the role of H_2S in IRI or *in vitro* oxidative stress, and as a consequence, many different models and treatment regimens have been used. The mechanisms of H_2S-induced protection remain unclear, although many different approaches have been investigated. It seems that H_2S has many different effects, and at present, it is not possible to formulate a 'one size fits all' hypothesis to a single mechanism that explains all effects in these models.

In vivo models of ischaemia

Heart

MI is one of the main models used for the study of the protective effects of H_2S in ischaemia. Intravenous Na_2S 24 h before cardiac ischaemia attenuated infarct size, serum troponin levels, lipid hydroperoxide levels and apoptosis, while cardiac function improved (Calvert *et al.*, 2009). Injection of Na_2S in the left ventricular lumen at reperfusion attenuated myocardial infarct size, troponin levels, inflammation, apoptosis and loss of cardiac function. Na_2S preserved mitochondrial oxygen consumption and integrity after myocardial ischaemia (Elrod *et al.*, 2007). Other studies have shown similar effects of NaSH or Na_2S in models of MI, using different models, treatment regimens and modes of administration (Pan *et al.*, 2005; Sodha *et al.*, 2008; 2009; Sivarajah *et al.*, 2009; Yao *et al.*, 2010; Nishida *et al.*, 2012). In a mouse model with NaSH in drinking water for 4 weeks after MI, H_2S preserved cardiac function loss, reduced fibrosis, and increased the number of collaterals formed 4 weeks after MI (Qipshidze *et al.*, 2012). Na_2S improved myocardial function after cardiac arrest and cardiopulmonary resuscitation (CA/CPR) (Minamishima *et al.*, 2009). Na_2S also induced a significant improvement in the recovery of myocardial and endothelial function in a canine model of cardiopulmonary bypass with hypothermic cardiac arrest (Szabó *et al.*, 2011) and had anti-apoptotic effects in a porcine model of cardiopulmonary bypass (Osipov *et al.*, 2010). From our own experiments, we have observed cardioprotective effects of gaseous H_2S in hypometabolic as well as non-hypometabolic concentrations. Sub-hypometabolic concentrations of H_2S prior to ischaemia protect hearts from ischaemia-induced fibrosis and inflammation, whereas inducing hypometabolism offers additional protection against short-term myocardial necrosis (Snijder *et al.*, 2013a). A novel oral H_2S donor (SG-1002) has shown promising effects in a model of transverse aortic constriction, reducing parameters of cardiac failure and oxidative stress (Kondo *et al.*, 2013).

Besides exogenous H_2S, modulation of endogenous H_2S production is also a promising approach. Cardiac-specific overexpression of CSE increased H_2S production rate (Elrod *et al.*, 2007), improved survival, and reduced ventricular dilatation and hypertrophy in a model of heart failure induced by permanent left coronary artery occlusion (Calvert *et al.*, 2010). After MI, cardiac-specific CSE overexpression reduced left ventricular dilatation and hypertrophy, and improved left ventricular function (Elrod *et al.*, 2007). $CSE^{-/-}$ mice showed increased amounts of oxidative stress in their hearts and livers, and have exacerbated cardiac injury after MI (King *et al.*, 2014). In rats, inhibition of CSE using PAG increased infarct size. PAG-treated animals had increased inflammation after MI (Zhu *et al.*, 2006; Zhuo *et al.*, 2009).

Kidney

In renal IRI, NaSH at clamping and during reperfusion attenuated the increase in serum creatinine and improved microvascular flow. Necrosis and apoptosis and inflammation are attenuated by NaSH (Xu *et al.*, 2009; Zhu *et al.*, 2012). Renal IRI-associated liver injury (so-called distant dysfunction) is also attenuated by NaSH (Zhu *et al.*, 2012). In another study, bilateral renal ischaemia caused mortality, renal failure and inflammation, which were prevented by a gaseous H_2S-induced hypometabolic state (without hypothermia), but only when H_2S was given before ischaemia (pretreatment) (Bos *et al.*, 2009). Structural damage and apoptosis caused by IRI was nearly abolished by pretreatment and combined pre- and post-treatment, whereas post-treatment alone had less

albeit significant effects (Bos *et al.*, 2009). Transmission electron microscopy showed that H_2S pre-/post-treatment prevented IRI-induced mitochondrial swelling and degeneration (Bos *et al.*, 2009). In a model of porcine aortic occlusion, renal function loss was attenuated by Na_2S, as were renal oxidative DNA base damage, and circulating IL-6 and IL-1β levels (Simon *et al.*, 2011).

In the kidney, production of H_2S by CSE seems to modulate the effects of oxidative stress. In $CSE^{-/-}$ animals, which have low renal H_2S levels, mortality, renal function loss and DNA damage are increased following bilateral IRI. These effects could be rescued by NaSH treatment (Bos *et al.*, 2013). In human transplant biopsies, the level of CSE mRNA pre-transplantation was associated with improved outcome as measured by renal function after kidney transplantation (Bos *et al.*, 2013). After bilateral renal ischaemia, CSE protein, renal H_2S production and plasma H_2S levels are increased. NaSH injection reduces IRI-induced renal function loss (Tripatara *et al.*, 2009). Bilateral IRI-induced renal function loss was attenuated by NaSH. NaSH also reduced acute tubular necrosis and apoptosis (Tripatara *et al.*, 2008). Interestingly, D-cysteine can protect kidneys from ischaemia as well, possibly with increased potency compared with L-cysteine by the production of H_2S through DAO and MPST (Shibuya *et al.*, 2013).

Brain

In middle cerebral artery occlusion in rats, gaseous H_2S for 2 days starting after reperfusion improved neurological outcome and reduced infarct size and inflammation (Florian *et al.*, 2008). NaSH injection increased performance on maze escape and reduced damage in the hippocampal area after bilateral common carotid occlusion (Li *et al.*, 2011). However, not all models of stroke show unequivocal protective effects of H_2S. In global cerebral ischaemia in rats, low-dose NaSH protected neurons, whereas high-dose NaSH exacerbated neuronal damage (Ren *et al.*, 2010). Another study showed that infarct volume was increased in animals treated with NaSH. Both CBS inhibitors (AOA and HA) and CSE inhibitors (BCA and PPG) reduced cortical H_2S production, and all inhibitors reduced infarct volume 24 h after ischaemia (with the CBS inhibitors being more potent) (Qu *et al.*, 2006). Such discrepant observations may occur because at low concentrations, H_2S acts as a vasoconstrictor by inhibiting production and availability of NO (Liu *et al.*, 2011). In a mouse model of CA/CPR, Na_2S improved survival, neurological function and neuronal survival. Serum H_2O_2 levels were reduced by Na_2S shortly after CPR, indicating antioxidative effects. Mice overexpressing CSE in cardiomyocytes had increased survival and neurological outcome after CA/CPR (Minamishima *et al.*, 2009). In another study, short-term neurological outcome was better in the sulfide group, but was similar 1 week after CA/CPR. Here, apoptosis and neuronal death was not affected by Na_2S (Knapp *et al.*, 2011). Interestingly, a gain-of-function polymorphism in CBS has recently been associated with less delayed cerebral ischaemia following aneurysmal subarachnoid haemorrhage in humans (Grobelny *et al.*, 2011).

Liver

In a model of total hepatic IRI in rats, post-treatment with NaSH reduced plasma alanine transaminase (ALT) 6 h after reperfusion. Hepatic IRI-associated renal and cardiac damage was attenuated by NaSH, as were plasma MDA levels (Chen *et al.*, 2010b). Pretreatment with gaseous H_2S in partial hepatic ischaemia reduces necrosis, aspartate transaminase (AST), ALT, apoptosis, granulocyte influx, up-regulation of IL-6, IL-1β and TNF-α, and ROS production. HO-1 expression was not associated with protection (Bos *et al.*, 2012). In partial hepatic ischaemia, low-dose Na_2S treatment (0.3 and 1 $mg \cdot kg^{-1}$) protected from liver damage, whereas 2 $mg \cdot kg^{-1}$ increased damage, as measured by AST and ALT, underlining the narrow therapeutic window of H_2S. H_2S treatment preserved GSH stores and attenuated lipid hydroperoxide and caspase-3 cleavage (Jha *et al.*, 2008). Treatment with NaSH 30 min before and after reperfusion attenuated AST and ALT levels, whereas after PAG, levels were similar to control IRI. Histological damage was reduced by NaSH and increased by PAG. Inflammation and lipid peroxidation were reduced by NaSH, but unaffected by PAG (Kang *et al.*, 2009). A recent study showed that $CSE^{-/-}$ mice have increased hepatic damage compared with controls after IRI, which could be rescued by Na_2S treatment (King *et al.*, 2014).

NaSH suppressed hepatic autophagy *in vitro* and *in vivo*, and pharmacologically reducing autophagy further diminished the protective effect of NaSH, whereas rapamycin reversed the autophagy inhibitory effect and enhanced the protective effect of NaSH in IRI (Wang *et al.*, 2012). Interestingly, $CBS^{-/-}$ mice have 30% increased oxidatively modified proteins in the liver (Robert *et al.*, 2005), indicating an antioxidative function of CBS and/or endogenous H_2S under unstressed circumstances. However, the hyperhomocysteinaemia in these mice might be related to these effects as well.

Shock

In haemorrhagic shock in rats, gaseous H_2S and i.v. Na_2S greatly increased survival (Morrison *et al.*, 2008) and injection of NaSH 10 min before reperfusion reduced superoxide levels in aorta and heart (Ganster *et al.*, 2010). In a porcine model of haemorrhagic shock, Na_2S infusion improved survival compared with controls, and troponin levels were reduced in Na_2S-treated animals. Renal function (plasma creatinine and creatinine clearance) was improved by pre-/post-treatment with Na_2S. Plasma IL-6 levels were reduced by pre-/post-treatment, and lung, liver and kidney histopathological scores were improved (Bracht *et al.*, 2012). However, in another porcine study, Na_2S had no effect on survival or organ injury parameters (Drabek, 2012).

Full body hypoxia

Fascinatingly, in a model of hypoxia in mice (5% O_2), animals survived for a median time of 12 min and a maximum time of 17 min, whereas 20 min of gaseous H_2S pretreatment followed by 1 h of 5% O_2 did not cause any mortality or morbidity (Blackstone and Roth, 2007), with one experiment showing survival for more than 6 h.

Intestine

NaSH reduces oxidative stress and structural damage, whereas it increased intestinal GSH levels and superoxide dismutase (SOD) activity in a model of rat intestinal IRI (Yonezawa *et al.*, 2007; Liu *et al.*, 2009). In a model of jejunal ischaemia, NaSH

treatment preserved villus height after reperfusion (Henderson *et al.*, 2010b). Leukocyte rolling and adhesion are attenuated by NaSH (Yusof *et al.*, 2009).

In vitro *studies*

In models of hypoxia, NaSH attenuated apoptosis in endothelial cells as well as fibroblasts (Henderson *et al.*, 2010a), and reduced ROS in hippocampal neurons (Luo *et al.*, 2012) and cardiomyocytes (Sun *et al.*, 2012). In two different cell lines, $CoCl_2$-induced chemical hypoxia-induced cell death, apoptosis and ROS production were attenuated by NaSH (Chen *et al.*, 2010a; Yang *et al.*, 2011a). NaSH protects neuroblastoma cells from oxygen-glucose deprivation/$Na_2S_2O_4$-induced cell death (Tay *et al.*, 2010). In neuroblastoma cells, NaSH concentration-dependently reduced formation of nitrotyrosine caused by $ONOO^-$ and reduced cell death (Whiteman *et al.*, 2004a). HOCl-induced protein oxidation, lipid peroxidation and loss of cell viability were concentration-dependently inhibited by NaSH (Whiteman *et al.*, 2004b). Overexpression of CBS reduced $Na_2S_2O_4$-induced apoptosis (Tay *et al.*, 2010). NaSH also protected primary rat astrocytes from H_2O_2-induced cell death and ROS production, whereas inhibition of CBS decreased viability (Lu *et al.*, 2008). Furthermore, NaSH protected photoreceptor cells from light-induced degeneration, reducing apoptosis and oxidative stress (Mikami *et al.*, 2011b). Isolated mitochondria exposed to hypoxia had better recovery of respiratory rate when treated with Na_2S (Elrod *et al.*, 2007), and NaSH reduced calcium-induced mitochondrial swelling (Minamishima *et al.*, 2009). In contrast, the addition of NaSH to mouse primary cortical neurons led to significant intracellular calcium mobilization, calpain activation and neuronal cell death. This process could be inhibited by NMDA receptor antagonists, once again indicating that higher concentrations of H_2S are neurotoxic (Cheung *et al.*, 2007).

NaSH protected pancreatic beta cells from H_2O_2 and cytokine induced apoptotic cell death (Taniguchi *et al.*, 2011). In neuronal cells and astrocytes, NaSH protected oxidative stress and increased GSH levels (Lu *et al.*, 2008; Kimura *et al.*, 2010). However, H_2S can react directly with H_2O_2, so these protective effects may simply have been due to direct reactions in the medium (Geng *et al.*, 2004).

In CSE-deficient vascular smooth muscle cells, hypoxia causes reduced viability and increased apoptosis and ROS (Bryan *et al.*, 2011). In HEK293 cells, adenovirus-mediated overexpression of CSE reduced antimycin-induced ROS and mitochondrial superoxide production, which could be further reduced by additional NaSH (Bos *et al.*, 2013). NaSH reduced cell death and LDH release after hypoxia, while inhibition of CSE using PAG increased these parameters (Zhu *et al.*, 2006).

Mechanisms of H_2S-mediated protection in IRI

The major antioxidative mechanism of cellular protection of H_2S in models of oxidative stress is thought to be by the increase in GSH levels, as shown in many studies. Direct scavenging has also been proposed; however, this seems unlikely as the rate constants of reaction are too slow (Carballal *et al.*, 2011). NaSH directly scavenges O_2^- and H_2O_2 (Geng *et al.*, 2004), and it protects cells from the cytotoxic lipid oxidation product 4-hydroxyneonal, possibly by scavenging (Schreier *et al.*, 2010). Also, NaSH activates CuZn-SOD *in vitro* and in a cell-free system (Sun *et al.*, 2012). However, the physiological concentrations of H_2S are probably too low to achieve these effects *in vivo*.

Na_2S preserves mitochondrial integrity and oxygen consumption after myocardial ischaemia (Elrod *et al.*, 2007) and attenuates mitochondrial dysfunction during the development of ischaemia-induced heart failure (Calvert *et al.*, 2010). The effects of NaSH were abolished in cells lacking mitochondria (Kai *et al.*, 2012). Modulation of mitochondrial activity might reduce the amount of mitochondrial superoxide production during stress. Endogenous H_2S protects cells from oxidative stress, loss of mitochondrial function and inhibits cell death (Fox *et al.*, 2012).

Induction of a hypometabolic state is one of the most interesting aspects of H_2S (Blackstone *et al.*, 2005). When given in subtoxic concentrations in gaseous form, mice exhibit a reversible hibernation-like state, showing >90% reduction in metabolic rate and core body temperature declines to near ambient temperature. This state has been shown to be highly protective, independent of the associated hypothermia (Blackstone and Roth, 2007; Bos *et al.*, 2009; 2012; Snijder *et al.*, 2013a). However, up until now, mixed results have been attained when trying to translate these results from rodents to larger mammals that may not have a natural adaptation to hibernation (Haouzi *et al.*, 2008; Simon *et al.*, 2008; Drabek *et al.*, 2011).

Another mechanism might be H_2S acting as an electron donor to the mitochondrial electron transport chain during periods of low oxygen availability (Yong and Searcy, 2001; Módis *et al.*, 2013b), causing at least some production of ATP for maintenance of cellular processes during hypoxia. Interestingly, CSE (and CBS) can translocate to the mitochondria upon cellular stress, where they can affect metabolism (Fu *et al.*, 2012), and MPST can also be localized to the mitochondria (Módis *et al.*, 2013b).

The K_{ATP} channel allows potassium to move to the intracellular compartment. Multiple studies show that the effects of H_2S on vascular tone are at least partly dependent upon activation of K_{ATP} channels (see the extensive review by Wang, 2012). Recent experiments have indicated that H_2S can directly sulfhydrate the K_{ATP} channel and thereby activate it (Mustafa *et al.*, 2011). The reduction in infarct size in a model of rat MI was attenuated by inhibition of mitochondrial K_{ATP} channels (Sivarajah *et al.*, 2009). In neurons exposed to hypoxia, the protective effects of NaSH were inhibited by K_{ATP} channel blockade but not by specific mitochondrial K_{ATP} channel blockade (Tay *et al.*, 2010).

An alternative mechanism is the stimulation of angiogenesis and the formation of collaterals. H_2S increased the number of collaterals formed 4 weeks after MI (Qipshidze *et al.*, 2012), and after rat hind limb ischaemia, NaSH increased regional blood flow and angiographic score after 4 weeks, with increased collateral formation, capillary density and VEGF expression (Wang *et al.*, 2010a). CSE siRNA blocked VEGF-induced endothelial cell migration (Papapetropoulos *et al.*, 2009). In addition, wound healing is impaired in $CSE^{-/-}$ mice, and in aortic rings of $CSE^{-/-}$ animals, microvessel formation was reduced compared with wild type (WT) and

could not be stimulated by VEGF (Papapetropoulos *et al.*, 2009). These structural effects are probably not responsible for short-term effects of H_2S, but might be responsible for late effects.

Similarities between H_2S, NO and CO as gasotransmitters have led to the hypothesis that the effects of H_2S might be mediated through NO and its synthesizing enzymes eNOS and iNOS (Coletta *et al.*, 2012; Altaany *et al.*, 2013; Snijder *et al.*, 2013b). In $eNOS^{-/-}$ mice, Na_2S failed to improve survival after CA/CPR, although $eNOS^{-/-}$ mice are more susceptible to CA/CPR, so small effects might have been overlooked with the larger mortality and the relatively small number of animals used (Minamishima *et al.*, 2009). In their recent paper, King *et al.* (2014) showed reduced eNOS activity in $CSE^{-/-}$ animals, which was associated with increased oxidative stress and MI-related damage. Treatment with Na_2S activated eNOS and rescued $CSE^{-/-}$ animals from cardiac damage, whereas these effects were observed in $eNOS^{-/-}$ mice (King *et al.*, 2014). NaSH induced eNOS in human microvascular endothelial cells. Inhibition of NOS partially attenuated the inhibitory effects of NaSH on leukocyte rolling and adhesion, whereas in $eNOS^{-/-}$ animals, leukocyte rolling, but not adhesion, was unaffected by NaSH (Yusof *et al.*, 2009). In all, there seems to be some interaction between H_2S and NO, as also shown in a recent study (Altaany *et al.*, 2013), but which effects of H_2S are mediated or modulated by NO, or vice versa, has yet to be determined.

HO-1 is an enzyme involved in catabolism of haem, and thereby producing CO. It has well-known protective and antioxidant effects in many models (Gozzelino *et al.*, 2010). Inhibition of HO-1 or deficiency in $HO\text{-}1^{-/-}$ mice abolished the effects of NaSH on leukocyte rolling and adhesion after intestinal IRI, whereas activation of HO-1 had similar effects on NaSH (Zuidema *et al.*, 2011). In $Nrf2^{-/-}$ animals, Na_2S pretreatment (single injection 24 h before ischaemia) failed to increase HO-1 expression as it did in WT animals, and the protective effects of Na_2S in MI were abolished (Calvert *et al.*, 2009). This shows that the effects of H_2S pretreatment could be mediated through Nrf2-mediated expression of protective proteins. Direct effects of H_2S when given either before or after ischaemia might be regulated differently as we have recently shown that H_2S-mediated protection was not associated with increased HO-1 expression (Bos *et al.*, 2012).

Heat shock protein 90 (HSP90) has been implicated as a protective protein that can stabilize intracellular proteins and is involved in apoptosis (Taipale *et al.*, 2010). NaSH protected against $CoCl_2$-induced ROS, cell death and apoptosis, but not when combined with HSP90 inhibition in rat phaeochromocytoma cells (Meng *et al.*, 2011). In a model of neuronal hypoxia, NaSH increased HSP90 expression, whereas inhibition of HSP90 abolishes the protective effects of NaSH (Tay *et al.*, 2010). Thus, the effects of H_2S might be partly mediated by HSP90 activation. An overview of these mechanisms is given in Table 2.

Table 2
Overview of possible mechanisms for the effects of H_2S

Proposed mechanisms for protective effects of H_2S
Increase in GSH levels
Reduction of mitochondrial superoxide production
Activation of ROS scavengers (e.g. MnSOD, CuZnSOD)
Preservation of mitochondrial integrity
Acting as an electron donor in the electron transport chain
Modulation of mitochondrial activity/hypometabolism
Induction of hypothermia
Modulation of vascular proliferation
Modulation of leukocyte adhesion/rolling
Modulation of cytokine production
Activation of K_{ATP} channels
Activation of calcium activated K^+ channels
Modulation of protein activity through sulfhydration
Modulation of platelet function
Modulation of hypoxia inducible factor
Modulation of Nrf2 signalling
Increase in eNOS/iNOS/HO-1
Increase in HSP90
Modulation of COX-2 activity
Modulation of GSK3β activity
Modulation of apoptotic signalling
Modulation of autophagy

Translation to therapeutic applications

Therapeutic window of H_2S

As mentioned, the effects of H_2S are very dose-dependent, with high doses showing no or even detrimental effects. In porcine cardiac arrest and resuscitation, high-dose Na_2S (1 $mg \cdot kg^{-1}$) worsened cerebral damage and levels of troponin T, whereas 0.3 $mg \cdot kg^{-1}$ had no effect (Derwall *et al.*, 2010). In a model of partial hepatic ischaemia, Na_2S treatment (0.3 and 1 $mg \cdot kg^{-1}$) protected from liver damage, whereas 2 $mg \cdot kg^{-1}$ increased damage (Jha *et al.*, 2008). These results infer a narrow window of opportunity. Before clinical translation, meticulous dose-finding studies will be required, and possibly reliable serum or urine level measurement needs to be developed to monitor treatment.

Timing of treatment

Many different treatment protocols have been used, and one of the most variable factors was the timing of treatment with H_2S. For example, NaSH injection 1 day prior to MI in rats decreased infarct size, left ventricle internal diameter and anterior wall thickness. Injection of NaSH 3 days prior to MI also showed protective effects, whereas NaSH 5 days prior to MI had no effect. Post-treatment (after MI, at days 0, 1 and 2) also had therapeutic effects, but these were less when compared to pretreatment, and combination treatment (before and after MI) had no additional effect as compared to pretreatment only (Pan *et al.*, 2009). In another study, single administration of Na_2S did not affect IRI-induced cardiac function loss, whereas daily administration for 1 week was protective (Calvert *et al.*, 2010). In acute joint inflammation,

H_2S donor GYY4173 only had an effect when it was given to animals after inflammation had started, but when it was given before it potentiated adjuvant swelling without a change in neutrophil influx. If given at the same time as the adjuvant, it had no effect (Li *et al.*, 2013). Lastly, NaSH at 24 h pre-ischaemia, but not at 1 h before ischaemia or during reperfusion, reduced leukocyte rolling and adhesion post-reperfusion (Yusof *et al.*, 2009). This might be related to the activation of transcription factors such as Nrf2 as the preconditioning effect of H_2S in cardiac MI is absent in $Nrf2^{-/-}$ mice (Calvert *et al.*, 2009). It is likely that the effects of H_2S when given more than a couple of hours before ischaemia are mediated through increasing the expression of other protective proteins, such as HO-1 in a Nrf2-dependent pathway (Calvert *et al.*, 2009), whereas the more direct effects of H_2S are mediated through a scavenging mechanism as H_2S levels are only transiently elevated after bolus injection. Interestingly, CSE up-regulation can also be dependent upon Nrf2 signalling (Hassan *et al.*, 2012), as are HSP90 and HO-1 (Calvert *et al.*, 2009).

Pretreatment and post-treatment have both been effective in ischaemic models; in some models, pretreatment was more effective (Bos *et al.*, 2009). However, pretreatment is not clinically applicable in many situations, such as acute MI or shock. In the clinical setting, this would mean p.o. or i.v. application, or injection into the organ itself (e.g. intramyocardial injection).

Other situations comprise predictable moments of ischaemia, mainly during surgical interventions such as organ transplantation, momentary aortic occlusion or temporary occlusion of cerebral arteries during the clipping of cerebral aneurysms. In the setting of organ transplantation, the brain dead organ donor can be treated with i.v. or gaseous H_2S. Also, the organ can be exposed to H_2S during machine perfusion, a treatment system that has already shown clinical potential in protecting donor organs (Moers *et al.*, 2009, 2012). Lastly, the organ recipient can be treated with H_2S post-transplantation. In elective transplantation from a living donor, it is theoretically also possible to treat the donor prior to organ donation.

Mode of administration

Experimental studies have shown many methods of administration for H_2S treatment, and each has its advantages and disadvantages for eventual clinical application. Gaseous H_2S is difficult to handle safely because it is corrosive, toxic at higher concentrations and very malodorous. This implies that it can only be applied in well-ventilated rooms or in intubated persons with specialized equipment. It can however cause very long and stable hypometabolic states in mice (Blackstone *et al.*, 2005; Blackstone and Roth, 2007), indicating that theoretically long-term stable levels can be reached with gaseous H_2S.

The sulfide salts (NaSH and Na_2S) have very rapid dynamics, which makes it hard to achieve steady concentrations *in vivo* (Li *et al.*, 2008; Marutani *et al.*, 2012). Continuous i.v. administration might achieve constant levels, but this has a narrow therapeutic window in experimental studies. Slow-releasing donors of H_2S are being developed and could counter some of these problems, but still need to be proven safe. Offering H_2S through the drinking water, as has been carried out in experimental settings, is not recommended in the human situation because of the horrendous taste and smell. A new oral dietary compound named SG-1002 might be a promising development. Another option that has shown potential is the use of naturally occurring cysteine analogues, such as diallyl trisulfide (DATS) and diallyl disulfide (DADS). These can increase H_2S levels over longer periods of time and are suitable for oral use. As DATS and DADS can be derived from garlic, large amounts of garlic, or even garlic concentrates could be used as a food supplement. However, studies that use these compounds do not always perform necessary controls to show that the effects are directly related to their H_2S-donating action, and sometimes fail to show any differences in H_2S levels caused by their administration.

Last is the modulation of endogenous production, which is at the moment problematic, since the best known modulators are inhibitors of CSE and CBS of dubious quality and specificity (Whiteman *et al.*, 2011; Asimakopoulou *et al.*, 2013), whereas most studies show that the activity of these proteins needs to be increased to be beneficial. Adenovirus-mediated overexpression is an option, but to our knowledge, no groups are investigating this for human applications. Modulation of proteins involved in sulfide detoxification is only hypothetical at the moment, and the detrimental effects of the loss of Ethe1 do not bode well for long-term modulation of this protein (Tiranti *et al.*, 2009).

In summary, H_2S is a highly promising molecule that has only allowed us a brief peek into its broad functionality. In this paper, we have tried to provide an overview of the main knowledge on H_2S and its application in the setting of ischaemia and oxidative stress. The protective effects can be strong, but the precise molecular mechanisms underpinning these protective effects remain to be solved. As in many rapidly expanding fields of research, well-characterized drug candidates are lacking. However, the development of H_2S-based therapeutics has considerable potential to greatly improve outcome of surgically induced ischaemia and of ischaemic disease.

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Author contributions

E. M. B. performed literature research, wrote the paper and created tables and figures. H. G., J. A. J., M. W. and H. G. D. L. wrote and revised the paper.

Conflict of interest

None.

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