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**Review**

# Hydrogen sulfide in cell signaling, signal transduction, cellular bioenergetics and physiology in *C. elegans*

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**Abstract.** Hydrogen sulfide ($H_2S$), long viewed as a toxic gas and environmental hazard, is emerging as a biological mediator with remarkable physiological and pathophysiological relevance. $H_2S$ is now viewed as the third main gasotransmitter in the mammalian body. Its pharmacological characteristic possesses similarities to the other two gasotransmitters – nitric oxide (NO) and carbon monoxide (CO). Many of the biological effects of $H_2S$ follow a bell-shaped concentration-response; at low concentration or at lower release rates it has beneficial and cytoprotective effects, while at higher concentrations or fast release rates toxicity becomes apparent. Cellular bioenergetics is a prime example for this bell-shaped dose-response, where $H_2S$, at lower concentrations/rates serves as an inorganic substrate and electron donor for mitochondrial ATP generation, while at high concentration it inhibits mitochondrial respiration by blocking the Complex IV in the mitochondrial electron transport chain. The current review is aimed to focus on the following aspects of $H_2S$ biology: 1) a general overview of the general pharmacological characteristics of $H_2S$, 2) a summary of the key $H_2S$-mediated signal transduction pathways, 3) an overview of role of $H_2S$ in regulation of cellular bioenergetics, 4) key aspects of $H_2S$ physiology in *C. elegans* (a model system) and, finally 5) the therapeutic potential of $H_2S$ donating molecules in various disease states.

**Key words:** Signal transduction — Bioenergetics — Oxygen sensing — Nematodes — $H_2S$ donors

**Abbreviations:** CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathioneine γ-lyase; ETHE1, surfur dioxygenase/dioxygenase ethylmalonic encephalopathy; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HIF-1, hypoxia inducible factor; HO-1, heme oxygenase; 3-MP, 3-mercaptopyruvate; 3-MST, 3-mercaptopyruvate sulfurtransferase; Nrf2, nuclear factor E2 related factor 2; PDE, phosphodiesterase; SQR, sulfide:quinone oxidoreductase; Trx1, thioredoxin reductase; TST, thiosulfate sulfure transferase/rhodanese.

## Introduction

In addition to nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide ($H_2S$) is now considered the third gasotransmitter and cellular signaling molecule (Szabo 2007; Wagner et al. 2009; Whiteman et al. 2011; Wang 2012). All these three small molecules freely diffuse through cell membranes to exert their cellular signaling actions.

$H_2S$ is a colorless, flammable, water-soluble gas with the characteristic smell of rotten eggs. Most of the earlier literature focused on its toxicological effects considering as an environmental hazard and as a mitochondrial broad-spectrum poison, affecting the nervous, respiratory, and cardiovascular system (Beauchamp et al. 1984; Reiffenstein et al. 1992). The toxicological effects of $H_2S$ are mainly attributed to the inhi-

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bition of cytochrome *c* oxidize in the mitochondrial electron transport chain (Cooper et al. 2008). This enzyme catalyzes the oxidation of cytochrome *c* molecules and transfers the electrons to the molecular oxygen to produce water. Inhibition of cytochrome *c* oxidase by $H_2S$ results in uncoupling of mitochondria, during which the oxidative phosphorylation is no longer linked to further ATP production and the cell dies by energy depletion. The toxicological effect of $H_2S$ on mitochondrial respiration is manifested at higher concentrations; *in vivo*, *via* the inhaled route, $H_2S$ above 500 ppm can cause rapid unconsciousness and respiratory arrest (Turner et al. 1980). Utilization of nitrite and hyperbaric $O_2$ therapy have been used as antidotes to $H_2S$ toxicity (Reiffenstein et al. 1992).

In sharp contrast to the toxicological effects of $H_2S$, over the last decade, $H_2S$ has attracted increasing attention in cell signaling in physiological and pathophysiological conditions. Emerging findings indicate that physiological amounts of $H_2S$ can stimulate the mitochondrial oxygen consumption and ATP production. The oxidation of $H_2S$ by mitochondria, coupled to the ATP synthesis capability of mammalian cells, has been retained by modern-day organisms from a long-gone oxic and asulfidic world. The first sections of the current review will focus on the signaling and bioenergetic aspects of $H_2S$ biology. Then, we will discuss the role of $H_2S$ in the biology of the nematode *Caenorhabditis elegans* (*C. elegans*), as this model system has been instrumental in recent years in unveiling various $H_2S$-mediated cellular pathways. Finally, since $H_2S$ donation/supplementation may be relevant for future therapeutic approaches, in the last section of our review we will cover the therapeutic potential of novel $H_2S$-releasing donor molecules.

## $H_2S$, the third gasotransmitter

### *$H_2S$ biosynthesis in mammalian cells*

$H_2S$ is generated in mammalian cells *via* enzymatic and, to a lesser degree, non-enzymatic pathways (Figure 1). Among the three key $H_2S$-generating enzymes, cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1) have been investigated extensively. Both enzymes use pyridoxal-5'-phosphate (vitamin B6) as a cofactor and metabolize L-cysteine to produce $H_2S$. For this, L-cysteine, can either be derived from alimentary sources and liberated from endogenous proteins or synthesized endogenously from L-methionine through the reverse transsulfuration pathway using homocysteine as an intermediate in the process (Wang 2002; Fiorucci et al. 2006). The role of a third enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST; EC 2.8.1.2), along with cysteine aminotransferase (CAT; CAT 2.6.1.3) in the process of $H_2S$ biosynthesis has recently been demonstrated as well. Cysteine, along with alpha-ketoglutarate (alpha-KG), is converted into 3-mercaptopyruvate (3-MP) by CAT. 3-MP is then converted by 3-MST to form $H_2S$ and pyruvate. Both CBS and CSE are localized to cytoplasm, while 3-MST is mainly localized to the mitochondria and partly to the cytoplasm (Kuo et al. 1983; Kimura 2011).

### *Free and bound sulfide in the mammalian cells*

The definition of what constitutes "free sulfide", as opposed to "bound sulfide" deserves a brief discussion. Free sulfide is a dissolved $H_2S$ gas, which is a weak acid (pH ~ 4) and in solution exists in the equilibrium $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$. Nearly equal amounts of $H_2S$ and $HS^-$ exist within the cell, and approximately a 20% $H_2S$/80% $HS^-$ ratio in extracellular fluid and plasma at 37°C and pH 7.4. Moreover, the bound sulfide pool, (bound sulfane sulfur pool), is localized to the cytoplasm and generates $H_2S$ under reducing conditions. Glutathione and cysteine are the major cellular reducing compounds. The size of bound sulfane sulfur pool also depends on the $H_2S$-producing activity of the cells (Szabo 2007; Predmore et al. 2012; Wang 2012).

### *The concentration of $H_2S$ in plasma and tissue homogenates*

The plasma concentration of sulfide is regulated at the level of its generation and its consumption. There is no consensus on the actual extracellular (circulating) levels of $H_2S$: various reports describe it in mammalian blood mainly in the micromolar concentration range. In healthy animals and humans the physiological range of $H_2S$ in circulation has been estimated at 10–100 μM (Richardson et al. 2000; Zhao et al. 2001; Hyspler et al. 2002; Hongfang et al. 2006; Whiteman et al. 2009; Wang 2012). Aging appears to have no effect on circulating $H_2S$. A study revealed no change in serum $H_2S$ concentration among three age groups of humans spanning 50–80 years (34–36 μM) (Chen et al. 2005). Rat serum contains 46 μM $H_2S$ (Zhao et al. 2001), and it is 34 μM in mouse serum (Li et al. 2005). In New Zealand rabbits, a quantitative assay detects a plasma $H_2S$ level around 16.5 μM (Srilatha et al. 2009). Plasma $H_2S$ at micromolar ranges has also been reported in many other vertebrates (Olson 2012; Olson et al. 2012). Endogenous levels of $H_2S$ in rat brain homogenates are 50–160 μM (Abe et al. 1996; Hosoki et al. 1997). Similarly, significant degree of $H_2S$ biosynthesis has been documented in the liver, kidney, and pancreas (Goodwin et al. 1989; Warenycia et al. 1989; Yusuf et al. 2005). $H_2S$ production was clearly measured in the cardiovascular system (Hosoki et al. 1997; Zhao et al. 2001; Yang et al. 2008; Coletta et al. 2012). This discrepancy relates to the differences in the various methods to measure the free sulfide concentration: many methods are likely to liberate sulfide from its bound forms, thereby producing concentrations that are likely to

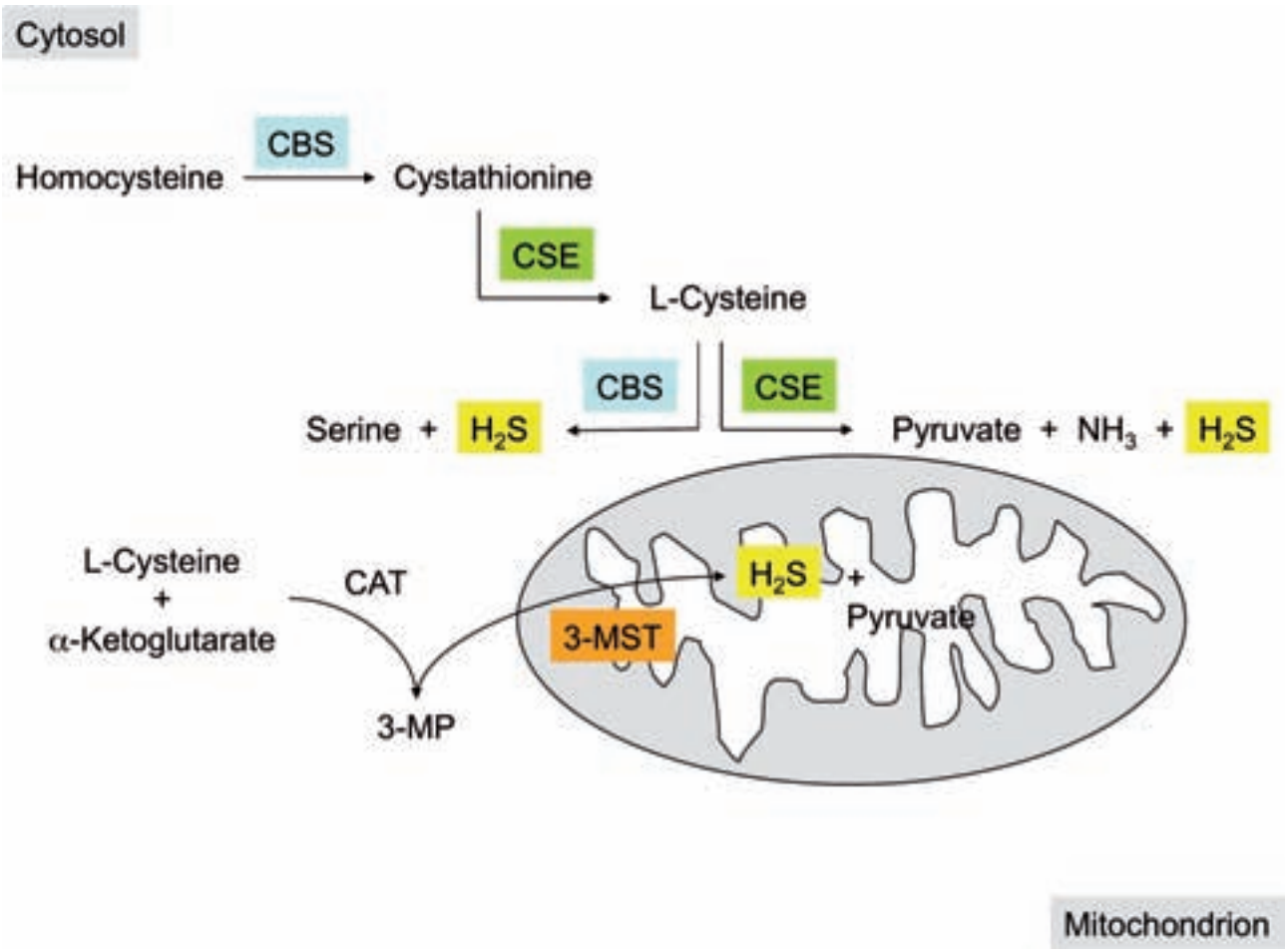


**Figure 1.** Enzymatic pathways of $H_2S$ production in mammalian cells. Cystathionine β-synthase (CBS) catalyses the production of cystathionine from homocysteine. Cystathionine γ-lyase (CSE), subsequently converts cystathionine to L-cysteine. Then CBS and CSE enzymes, utilizing the same substrate, convert L-cysteine to form hydrogen sulphide ($H_2S$). The above reactions predominantly take place in the cytosol. In the mitochondria, L-cysteine in the presents of α-ketoglutarate can be converted to 3-mercaptopyruvate (3-MP) by cysteine aminotransferase (CAT), which can then be converted to $H_2S$ by 3-mercaptopyruvate sulfurtransferase (3-MST). 3-MST is localized both in the mitochondria and in the cytosol.

represent a mixture of free and bound sulfide (Hannestad et al. 1989; Togawa et al. 1992; Ogasawara et al. 1993; Hughes et al. 2009; Wintner et al. 2010).

There are also several reports describing significant changes in the plasma levels of $H_2S$ in various disease states. In many animal models of disease, the plasma/tissue levels of endogenous sulfide/sulfane sulfur and/or $H_2S$-generating enzymes level are reduced; for example in cardiac ischemia (Elrod et al. 2007; Predmore et al. 2011), hypertension (Yang et al. 2008), hyperhomocysteinemia (Gupta et al. 2009), erectile dysfunction (Qiu et al. 2012), chronic kidney disease (Perna et al. 2012), diabetes (Brancaleone et al. 2008; Whiteman et al. 2010; Suzuki et al. 2011) gastrointestinal tract inflammation (Fiorucci et al. 2005), asthma (Tian et al. 2012), wound healing (Coletta et al. 2012). On the other hand, there are certain conditions, including circulatory shock (Hui et al. 2003; Collin et al. 2005; Zhang et al. 2006) or certain forms of cancer (Guo et al. 2012; Lai et al. 2012), in which increased circulating/tissue sulfide levels were reported.

Taken together, an abundance of recent experimental evidence suggests that $H_2S$ plays a prominent role in normal physiology as well as in various pathophysiological conditions.

## $H_2S$-mediated signal transduction pathways

The biological actions of $H_2S$ are mediated by a wide array of molecular mechanisms (Figure 2). It is likely that this list

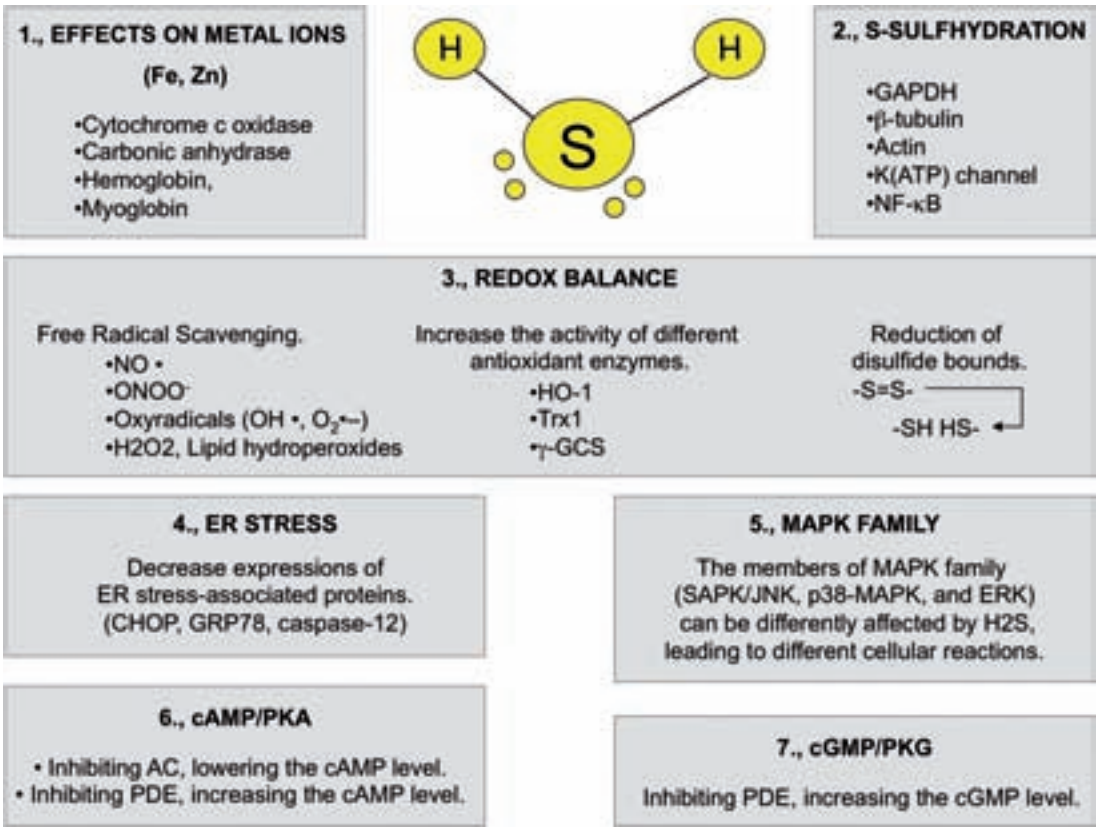


**Figure 2.** Possible biological effects of $H_2S$ in mammalian cells. (**1**) $H_2S$ can target metalloproteins such as cytochrome c oxidase, carbonic anhydrase, hemoglobin and myoglobin. (**2**) $H_2S$ also participates in reactions yielding protein persulfides, protein polysulfides and protein associated sulfur (GAPDH, β-tubulin, $K_{ATP}$ channels, actin, NF-κB). This mode of action is called S-sulphydration and occurs on the cysteine residue of proteins. (**3**) $H_2S$ has a role in regulation of the redox balance. It can reduce disulfide bonds in proteins involved in oxidant sensing; regulate expression of antioxidant genes e.g. heme oxygenase 1 (HO-1), thioredoxin reductase (TrxR), γ-glutamylcysteine synthetase (γ-GCS) and reacts with various reactive oxygen and nitrogen species, resulting in free-radical scavenging. $H_2S$ is involved in the regulation of ER stress (**4**) and the regulation of MAPK superfamily enzymes (**5**). $H_2S$ is also responsible in the alteration of cellular cAMP/cGMP levels *via* the inhibition of phosphodiesterases enzymes (PDEs) (**6, 7**). CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; SAPK/JNK, stress-activated protein kinase/c-JunNH2-terminal kinase; AC, adenylyl cyclase.

is incomplete and will grow further as $H_2S$ biology expands. It is also likely that the relative importance of each of these pathways is different in different cell types, organs, species and in health or disease (for further details, see Szabo 2007; Paul et al. 2012; Wang 2012. $H_2S$ signaling has been shown to involve the following pathways:

1) $H_2S$ is known to interact with metalloproteins such as cytochrome *c* oxidase and carbonic anhydrase interacting with heme moiety and metal ions (Zn, Fe). Such interactions usually results in inhibition of the protein function, as determined for cytochrome *c* oxidase (Hill et al. 1984). The binding of sulfide to hemoglobin or myoglobin results in formation of sulfhemoglobin or sulfmyoglobin that decreases affinity to oxygen and thus diminishes oxygen transport in the body (Pietri et al. 2011).

2) $H_2S$ can induce covalent modification of cysteine residues in proteins through S-sulfhydration (protein persulfides, protein-Cys-S-SH) that is considered to increase and stabilize the protein activity, respectively (GAPDH, β-tubulin, actin, $K_{ATP}$ channel, p65 subunit of the nuclear factor NF-κB). This process may occur at cysteines exposed to surface of proteins or localized in their active sites, and form either disulfide bonds (protein-Cys-S-S-R), or sulfenic acid (protein-Cys-S-OH) (Finkel 2012), or even from cyclic sulfonamide conformation (Krishnan et al. 2012). *S*-sulfhydration is term for persulfide bond Cys-S-SH and commonly is compared with *S*-nitrosation Cys-S-NO, a mechanism of the other signaling molecule and gasotransmitter NO. While S-sulfhydration is considered to increase and stabilize the protein activity, *S*-nitrosation leads to inhibition of the

protein function, as determined for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mustafa et al. 2009), or the transcription factor NF-κB (Sen et al. 2012). In these cases, $H_2S$ and NO may act sequentially by posttranslational modification of the proteins and thus activate and subsequently inactivate protein activity, respectively.

$H_2S$ is an endogenous activator of the ATP-dependent potassium channel ($K_{ATP}$ channel) *via* sulfhydration. This mechanism modulates cell metabolism and vasorelaxation (Zhao et al. 2001; Mustafa et al. 2011). In addition to the activation of $K_{ATP}$ channel other types of potassium, calcium, chloride channels and ion exchangers are regulated by $H_2S$ *via* mechanism, which are different from protein S-sulfhydration (Li et al. 2011; Wang 2012).

3) $H_2S$, as an antioxidant and a reducing agent, participates in regulation of the redox balance: Some of its redox actions are the consequence of the reduction of disulfide bonds, while others may relate to direct reactions with various reactive oxygen and nitrogen species, resulting in free-radical scavenging (e.g. peroxynitrite, superoxide anion, hydroxyl radical, nitric oxide, lipid hydroperoxide), although the rate constants of these reactions appear to be low (Carballal et al. 2011). *Via* these mechanisms, $H_2S$ provides a reducing/antioxidant environment to intracellular compartments, including the mitochondria; loss of this environment – in conditions such as hyperglycemia – can facilitate mitochondrial ROS production and mitochondrial dysfunction (Suzuki et al. 2011; Szabo 2012).

Sulfide can also indirectly upregulate several antioxidant enzymes (e.g. heme oxygenase 1 (HO-1, it produces CO), thioredoxin reductase (Trx1) through increasing the nuclear accumulation of nuclear factor E2-related factor 2 (Nrf2), a key transcription factor that regulates antioxidant genes by its increased accumulation in the nucleus (Calvert et al. 2009). *Via* induction of Nrf2, $H_2S$ may also regulate the levels of Trx and Trx reductase. In conjunction with this finding that Trx enhances the $H_2S$-producing activity of 3MST (as the reduced form of Trx helps 3-MST enzyme release the produced $H_2S$ from its active site) Trx may induce positive feedback that promotes $H_2S$ production through Nrf2 induction (Mikami et al. 2011).

Another indirect effect of $H_2S$ on antioxidant defense involves upregulation of cellular glutathione levels via enhancing the activity of gamma-glutamyl-cysteine synthetase and up-regulating cystine transport. Cystine (cysteine) is the rate-limiting substrate of glutathione synthesis (Kimura et al. 2004). Besides the thioredoxin system the glutathione (GSH) is the other major factor generally responsible for the low redox potential and high free SH level inside the cells.

4) The endoplasmic reticulum (ER) is responsible for lipid synthesis and the maturation and folding of membrane proteins, secretory proteins etc. It also plays an important role in regulating intracellular signaling process, including calcium homeostasis. ER stress refers to altered ER function in general, to liberation of a large amount of calcium, and to accumulation of unfolded protein aggregates or excessive protein traffic specifically. Different research groups have demonstrated that homocysteine provokes ER stress in cardiomyocytes and this is related to reduced CSE expression and $H_2S$ production. Also the expression of different ER stress-associated proteins, including C/EBP homologous protein (CHOP), caspase-12 and glucose-regulated proteins 78 (GRP78) were decreased after the administration of $H_2S$ *in vivo* and/or *in vitro* (Chang et al. 2008; Wei et al. 2010).

5) The MAPK superfamily contains three main members: stress-activated protein kinase/c-JunNH2-terminal kinase (SAPK/JNK), p38-MAPK, and ERK. The activities of MAPK are very important in regulating cell proliferation, apoptosis, differentiation, inflammation, and cycle progression (Wang 2012). The activation of the same MAPK family member can be differently affected by $H_2S$, leading to different cellular reactions. Furthermore, a MAPK family member in different cells may be responsible for the opposite functional consequences. For example, $H_2S$ increased endothelial cell proliferation *via* stimulating a sustained phosphorylation of ERK (Papapetropoulos et al. 2009). ERK activation has also been implicated in the $H_2S$-mediated myocardial protection pathways (Osipov et al. 2009). The same increase in ERK phosphorylation by the $H_2S$ donor NaHS leads to inhibited proliferation of smooth muscle cells (Yang et al. 2004; Yang et al. 2010).

6) $H_2S$ also plays a role in regulation of cAMP/PKA and cGMP/PKG pathways. cAMP and cGMP are signaling molecules playing roles in many cellular functions by activating protein kinase A (PKA) or protein kinase G (PKG), respectively. PKA and PKG phosphorylate various proteins serving as regulators of different cellular signaling pathways. Both metabolites, cAMP and cGMP, are usually hydrolyzed by the enzymatic activity of phosphodiesterase (PDE) to 5'GMP or 5'AMP. $H_2S$ also plays a role in regulation of endogenous levels of these metabolites, because it functions as an endogenous PDE inhibitor such in vasorelaxation and angiogenesis (Papapetropoulos et al. 2009; Coletta et al. 2012; Fusco et al. 2012).

Coletta and colleagues recently have demonstrated that the $H_2S$ donor NaHS concentration-dependently decreases the activity of purified PDE5A. This finding suggests that the $H_2S$-induced increase in endothelial cell cGMP levels and subsequent endothelial proliferation is due to reduced cGMP degradation (Coletta et al. 2012).

In the context of cAMP/PKA signaling, other studies have shown that $H_2S$ can also decrease cAMP production by inhibiting adenylyl cyclase (AC). The decreased cAMP in vascular smooth muscle cells (Lim et al. 2008) and in juxtaglomerular cells (Lu et al. 2010) was observed

and a similar suppression of cAMP levels by $H_2S$ has also been demonstrated in cardiomyocytes (Yong et al. 2008). Whether NaHS directly inhibits AC was not tested in these studies. In the context of these studies it is interesting to mention that a complete cAMP-PKA system is localized in the mitochondrial matrix and the protein kinase A (PKA) regulates ATP production by phosphorylation of mitochondrial proteins, including subunits of cytochrome *c* oxidase (Acin-Perez et al. 2009). Whether $H_2S$ regulates this system remains a subject of further investigations.

## The role of $H_2S$ in cellular bioenergetics

It was well known for several decades that organisms in sulfide-rich environments (in certain swamps and hydrothermal vents) can synthesize ATP from sulfide (Grieshaber et al. 1998; Yong et al. 2001; Searcy 2003; Hildebrandt et al. 2008). Only within the last 6 years did it become apparent that these pathways are also conserved in mammalian cells (Goubern et al. 2007).

To understand the bioenergetic role of $H_2S$ we have to retrace the evolution of eukaryotes. Mitochondria arose from endosymbiosis (a single evolutionary event). This union was highly advantageous to the host cell because it (i) prevents oxygen toxicity, (ii) utilizes a mechanism of hydrogen transfer and /or (iii) enable sulfur cycling (sulfur syntrophy) (Searcy 2003; Olson 2012). Sulfur is a versatile molecule with oxidation states ranging from –2 to +6. Hydrogen sulfide or some sulfur moiety is omnipresent; delivering from extraterrestrial sources, such as meteorites, producing in a pre-biotic atmosphere or generating underwater in the effluent of hydrothermal vents on the deep ocean floor. Sulfide and sulfur species must have been an integral component of early life, irrespective of life's origin. These compounds exist in a variety of oxidation states and because of this ability they can transfer energy and may have had a key role in the great endosymbiotic event. According to this symbiotic relationship a host, sulfur-reducing Archea ($S^0 \rightarrow H_2S$) incorporated a sulfide ($H_2S$, the oxidation state of -2) oxidizing α-protobacteria resulting the eukaryotic cell. Eukaryotes slowly adapted from a sulfidic and anoxic world to one that was highly oxic and asulfidic. It has also become evident that, even in the present-day oxic environment, mammalian cells can utilize the redox chemistry of sulfide as a "source of energy" (Goubern et al. 2007; Lagoutte et al. 2010; Bouillaud et al. 2011; Mimoun et al. 2012; Módis et al. 2012) or a "metabolism as an $O_2$ sensor" (Olson et al. 2010; Olson 2012) to show that these ancient biochemical pathways are still employed by modern-day eukaryotes.

In mammals there are different cellular pathways to eliminate the endogenously produced $H_2S$ as well as $H_2S$ introduced into the body from the environment. $H_2S$ can be exhaled or mainly excreted in urine primarily as sulfate or thiosulfate and in feces as free sulfide. Methylation ($CH_3SH$) is another catabolic pathway for $H_2S$, mainly takes place in the cytosol. The half-life of free $H_2S$ in blood is relatively short as it is being scavenged by methemoglobin to form sulfhemoglobin. Notably, besides its excretion by urine the second most important pathway in the catabolism of $H_2S$ is its inactivation through mitochondrial oxidation (reviewed in Wang 2012).

$H_2S$ is produced by the three enzymes CBS, CSE and 3-MST intracellularly (see above). During normoxia, $H_2S$ is continuously oxidized by mitochondria generating sulfate and thiosulfate (Figure 3.), thereby maintaining low intracellular $H_2S$ levels while being eliminated as sulfate and thiosulfate. This represents a form of detoxification process for $H_2S$.

A mitochondrial membrane-bound enzyme, termed sulfide:quinone oxidoreductase (SQR), oxidizes sulfide to the level of elemental sulfur, simultaneously reducing a cysteine disulfide such that a persulfide group is formed at one of the cysteines (SQR-SSH). The electrons are donated into the respiratory chain *via* the coenzyme Q (Q oxidized = Q reduced), and finally transferred to oxygen by cytochrome oxidase (complex IV). The dioxygenase ethylmalonic encephalopathy (ETHE1, which has a sulfur dioxygenase activity) in the mitochondrial matrix oxidizes persulfides to sulfite ($SO_3^{2-}$), consuming molecular oxygen and water (Tiranti et al. 2009). Subsequently, sulfite is further oxidized to sulfate ($SO_4^{2-}$) by sulfite oxidase (SOX). Thiosulfate sulfur transferase (TST) (also known as one isoenzyme of the rhodanese) produces thiosulfate ($S_2O_3^{2-}$) by transferring the second persulfide from the SQR to sulfite. Taken together, this sulfide oxidizing unit (SOU) acts independently constituting of the sulfide quinone reductase (SQR) and of two other enzymes the sulfur dioxygenase (ETHE1) and TST, acts independently to ensure the final oxidation of the two disulfides (-SSH) bound to SQR into oxidized cysteine linked by a disulfide bond (Bouillaud et al. 2011).

## Sulfide and mitochondria

### *The $O_2$ sensor theory*

Oxidative phosphorylation is the major ATP-producing pathway in most mammalian cells. Molecular oxygen ($O_2$) is the terminal electron acceptor in oxidative phosphorylation, and essential for the ATP generation. Insufficient oxygen supply induces hypoxic conditions that can be serious if it accompanies reduced nutrition supply and accumulation of harmful metabolic waste. A fall in oxygen availability (hypoxia) decreases mitochondrial $H_2S$ oxidation resulting in an increase in biologically active $H_2S$ level and initiation of hypoxic responses.

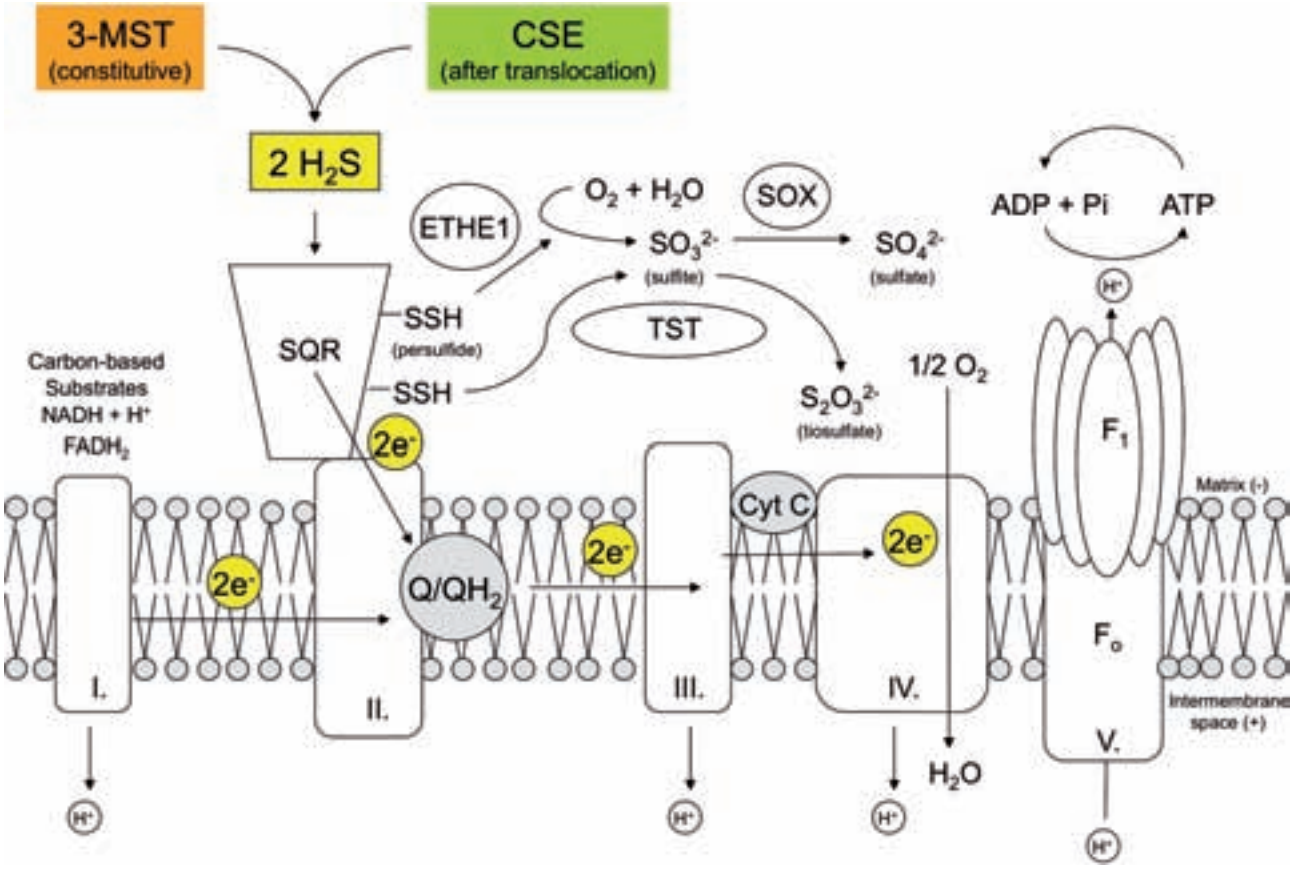


**Figure 3.** $H_2S$ oxidation and consequent bioenergetic effects in the mitochondria of mammalian cells. Oxidation of carbon-based substrates leads to the reduction of the NAD or FAD coenzymes. The reduced forms of these coenzymes (NADH + H, $FADH_2$) yield electrons to coenzyme Q of the mitochondrial respiratory chain. NADH + H is oxidized by mitochondrial complex I. $FADH_2$ coenzymes yield electrons through the function of complex II (succinate dehydrogenase). Both complexes (I and II) donate electrons *via* coenzyme Q of the mitochondrial electron transport chain. The sulfide-oxidizing unit acts independently. It is constituted of the mitochondrial membrane-bound sulfide quinone reductase (SQR) and of two other enzymes the sulfur dioxygenase (ETHE1, also called dioxygenase ethylmalonic encephalopathy) and the thiosulfate sulfur transferase (TST, also known as one isoenzyme of the rhodanese), ensuring the final oxidation of the two disulfides (-SSH) bound to SQR into oxidized cysteine linked by a disulfide bond. The sulfur dioxygenase in the mitochondrial matrix oxidizes persulfides to sulfite ($SO_3^{2-}$), consuming molecular oxygen and water. Sulfite, then, is further oxidized to sulfate ($SO_4^{2-}$) by sulfite oxidase (SOX). The thiosulfate sulfur transferase (TST) produces thiosulfate ($S_2O_3^{2-}$) by transferring the second persulfide from the SQR to sulfite. Taken together, SQR is responsible for the oxidation of $H_2S$ in the mitochondria. While from two $H_2S$ molecules, two disulfides (-SSH) bounds are created on the SQR, two electrons derived from 2 $H_2S$ also enter the mitochondrial electron transport chain, promoting mitochondrial ATP generation. These actions are considered beneficial and physiological, and tend to occur at lower $H_2S$ concentrations/rates. On the other hand, higher concentrations of $H_2S$ can inhibit complex IV, thereby inhibiting mitochondrial respiration. Finally, the utilized H2S in the mitochondria can be produced by the constitutively present 3-mercaptopyruvate sulfurtransferase (3-MST) or by the translocated cystathionine γ -lyase (CSE) enzyme.

In the body there are different oxygen-sensing tissues, like glomus cells of the carotid vasculature, or neuroendocrine cells as chromaffin cells. The glomus cells of the carotid body express both CSE and CBS and produce $H_2S$ (Peng et al. 2010). CSE is also expressed in neonatal adrenal medullary chromaffin cells of rats and mice. The concept that $H_2S$ acts an "$O_2$ sensor" was first demonstrated by Olson and his colleagues in 2006 (Olson et al. 2006). According to their hypothesis the following mechanism exists in oxygen-sensing cells: Blood vessels and chemoreceptors can detect oxygen levels and initiate appropriate physiological responses. In addition, hypoxia stimulates $H_2S$ production from these oxygen-sensing cells while the lack of oxygen also induces a reduced $H_2S$ oxidation by mitochondria. Finally, the resulting $H_2S$ has a role in responding to hypoxia with cardiovascular and respiratory reflexes; $H_2S$ closes calcium-sensitive

potassium (BKCa) channels to depolarize chemoreceptor cells (Telezhkin et al. 2009), triggering catecholamine secretion and hypoxia-induced hyperventilation (Olson et al. 2010). Initially, this mechanism was based on the constitutive sulfide production in the cytoplasm by CSE and CBS enzyme and its reduced oxidization in the mitochondria, although it now appears that sulfide may also be generated within the mitochondria *via* the function of 3-MST (Shibuya et al. 2009).

Many details of the above mechanisms need to be further elucidated in future studies. How does $H_2S$ "sense" the oxygen levels in the carotid body? Does the oxygen level ($PO_2$), *per se*, have a direct effect on the activity of CBS or CSE enzymes? CBS itself may not bind oxygen, but it can be quickly oxidized from the ferrous ($Fe^{2+}$) to the ferric state ($Fe^{3+}$) *via* an outer sphere electron transfer (Banerjee et al. 2005). Furthermore, which one is the real oxygen sensor(s)? CSE/CBS, $H_2S$ (*per se*) or the products during $H_2S$ oxidation, such as sulfite ($SO_3^2$)? Addressing these questions will provide further important insights into the basic physiological regulation of $O_2$ sensing.

*$H_2S$ as a source of energy in mammalian cells*

Based on the pioneering work of Krebs and Szent-Györgyi over half a century ago, the standard model of mammalian cellular bioenergetics was formulated. Krebs cycle products such as NADH and succinate are the obligatory factors that drive mitochondrial electron transport and maintain oxidative phosphorylation and ATP production. The electron transport chain proteins (Complexes I-IV) in the inner mitochondria membrane pass electrons along the chain, and oxygen serves as the terminal electron acceptor at Complex IV (cytochrome *c* oxidase). Mitchell has subsequently recognized that the purpose of this process is to pump protons from the mitochondrial matrix into the intermembrane space, in order to build a chemiosmotic gradient across the membrane. This electrochemical gradient is harnessed by Complex V, thereby generating ATP (Nelson et al. 2008; Voet et al. 2008) (Figure 3).

Data emerging over the last decade has suggested that in bacteria and archaea living in oxygen-deprived environments the electron transport utilizes alternative electron donors (such as nitrate, nitrite, or hydrogen sulfide) (Grieshaber et al. 1998; Searcy 2003; Hildebrandt et al. 2008). However, the generally held view was that these processes are obscure, inefficient, and exclusively reserved to non-mammalian cells. Sulfide has been shown to donate electrons to the mitochondrial respiratory chain in clams, worms, or fishes adapted to environments showing a comparable concentration of sulfide with that measured inside the mammalian gut (Grieshaber et al. 1998). This characteristic is likely to be inherited from the prokaryotic ancestor of mitochondria. These existing evidences show that electrons from sulfide could be given to the mitochondrial respiratory chain at the level of coenzyme Q (Ouml et al. 1997) or cytochrome *c* (Powell et al. 1986).

Hydrogen sulfide has a biphasic effect on mitochondrial function. At lower concentrations (under 5 μM) $H_2S$ has a stimulatory effect on respiration, donating electrons to the electron transport chain while generating ATP. At higher concentration (above 10 μM) has an inhibitory effect on mitochondrial respiration blocking the cytochrome *c* oxidase (Complex IV) and inducing a complete shutdown of the mitochondrial electron transport chain. Taken together, the biological significance of the two opposing effects of $H_2S$ on mitochondrial respiration, i.e. stimulation of ATP generation (Yong et al. 2001; Goubern et al. 2007; Lagoutte et al. 2010; Bouillaud et al. 2011) *vs.* inhibition of mitochondrial electron transport at Complex IV (Nicholls 1975; Khan et al. 1990; Collman et al. 2009) remained to be resolved.

In 2001, Yong and Searcy published a very surprising report: they demonstrated that low concentrations of $H_2S$ (under 5 μM concentration) increased $O_2$ consumption in isolated chicken liver mitochondria. In addition, they reported that the oxidation of $H_2S$ is coupled with measurable ATP production and increased mitochondrial membrane potential (Yong et al. 2001). The same report also considered that higher concentration of $H_2S$ may serve as an uncoupler, however, the mechanism of uncoupling was not explored thoroughly. One possibility is that $H_2S$ might be able to shuttle $H^+$ across the inner mitochondrial membrane in a manner similar to protonophore uncouplers. However, the results of Yong and Searcy showed that the membrane potential varies during constant sulfide input, suggesting that this coupling phenomenon of $H_2S$ may be switched off and on. Thus, uncoupling may involve an actively regulated metabolic switch, such as a gated $H^+$ leakage channel, but the existence of this explanation still needs to be directly confirmed in future studies. Taken together, Yong and Searcy were the first who demonstrated that mitochondria, which are not adapted to sulfidic environments, but nevertheless can oxidize sulfide with coupled ATP synthesis. This type of biochemistry has not been observed previously in non-sulfide-adapted species. Therefore, Searcy and Lee suggested first that coupled respiration on sulfide is a widespread and primitive trait of mitochondria.

In 2007, Goubern and his colleagues suggested that exogenously administered $H_2S$ can act as an electron donor and a potential inorganic source of energy in mammalian cells (Goubern et al. 2007). They used two different human colon adenocarcinoma cell lines (CaCo2 and HT-29 $Glc^{-/+}$). In these permeabilized cells subjected to $H_2S$ an elevated $O_2$ consumption and increase mitochondrial membrane potential were detected. This was the first report

that indicated sulfide can lead to mitochondrial energizing in mammalian cells, and also the first report to conclude that when sulfide is oxidized by mitochondria in colonocytes there are two sites for oxygen consumption: 1) the formation of water at the level of mitochondrial complex IV (cytochrome *c* oxidase), and 2) the formation of sulfur containing oxygenated products (thiosulfate, sulfite, sulfate). In addition, when sulfide reaches a threshold of concentration sufficient to inhibit cytochrome *c* oxidase (complex IV), according to their data electrons are redirected toward mitochondrial complex II (succinate dehydrogenase). In these conditions it acts in reverse mode and reduces fumarate into succinate. This would allow an anaerobic oxidation of sulfide. This group, however, concluded that – in contrast to Yong's and Searcy's assumptions – sulfide *per se* does not lead to increased permeability of the mitochondrial inner membrane for protons, and therefore does not cause "uncoupling" studied in permeabilized cells (Goubern et al. 2007).

In a follow-up study in 2010, Lagoutte and colleagues found that exposure of CHO cells overexpressing human SQR to chemical $H_2S$ donors resulted in an increase in cellular oxygen consumption and ATP production (Goubern et al. 2007; Lagoutte et al. 2010; Bouillaud et al. 2011). This group also demonstrated that the appropriate $H_2S$ concentration, which stimulates the mitochondrial respiration depended on different cell types and related to the various expression levels of SQR protein. In further studies Mimoun and his colleagues have showed that sulfide oxidizing capacity is increased with their level of differentiation in colonocytes (Mimoun et al. 2012). In this study, however, it was concluded that sulfide is not primarily oxidized for increasing the cellular level of ATP but rather for allowing its detoxification. These findings are best conceptualized in the context that in mammals the indigenous sulfate-reducing bacteria in the large intestine represents another source of $H_2S$, which is frequently considered an endogenous "environmental insult" to the colonic epithelium (Magee et al. 2000; Attene-Ramos et al. 2007; Attene-Ramos et al. 2010). Accordingly, the mitochondrial sulfide oxidation in colonocytes proved to be indispensable for maintaining physiological conditions of large intestine.

Although there are now several studies, in a variety of cell types demonstrating the biphasic effect of $H_2S$ on cellular bioenergetics (stimulation at lower concentration, inhibition at higher) (Goubern et al. 2007; Lagoutte et al. 2010; Bouillaud et al. 2011; Groeger et al. 2012). From the above studies it remains unclear whether endogenously produced $H_2S$ plays a role as an inorganic substrate in the physiological regulation of mitochondrial function. However, it has very recently been demonstrated that $H_2S$ produced by 3-MST, rather than CSE or CBS, has a prominent role in the regulation of mitochondrial function in isolated liver mitochondria and in the cultured murine hepatoma cell line Hepa1c1c7 (Módis et al. 2012). According to this finding the endogenous intramitochondrial $H_2S$-producing pathway governed by 3-MST, complements and balances the bioenergetic role of Krebs cycle-derived electron donors. This pathway may serve a physiological role in the maintenance of mitochondrial electron transport and cellular bioenergetics. CSE could play a role since, recent studies by Wang and colleagues showed that under conditions of hypoxia, CSE may translocate to the mitochondria, thereby exerting positive bioenergetic effects (Fu et al. 2012). The possible role of the three $H_2S$ producing enzymes (CSE, CBS, 3-MST) in the regulation of mitochondrial function needs to be thoroughly clarified as $H_2S$ is a gaseous molecule and it can be easily diffusing to the mitochondria. In this case determining whether which $H_2S$ producing enzyme has the main role in the regulation of mitochondrial function is likely to be highly complex.

## $H_2S$ physiology in model systems

Over the last decade, many of the studies aimed to explore the physiology or pathophysiology of $H_2S$ relied on invertebrate model organisms. Using *C. elegans* models, it has been demonstrated by several studies that exposure to $H_2S$ increases the viability and survival of animals protecting them from otherwise lethal conditions. It was also shown in rodents the exogenous $H_2S$ leads to a hibernation-like state (suspended animation), with a decreased metabolic rate and core body temperature (Blackstone et al. 2005). Further studies showed that these regulatory effects induced by $H_2S$ inhalation protect animals from hypoxia, hemorrhage and also diminish ischemia-reperfusion injuries (Blackstone et al. 2007; Ganster et al. 2010). In *Drosophila melanogaster*, also known as the common fruit fly (another common model system), the administration of $H_2S$ was shown to result in elevated desiccation tolerance (Zhong et al. 2010). In *C. elegans* nematode model, as a multicellular organism – that is utilized as a model system to address fundamental questions in developmental biology – $H_2S$ was found to promote thermotolerance and to prolong longevity (Miller et al. 2007).

Studies utilizing roundworm *C. elegans* can be highly useful to explore the molecular alterations mediated by $H_2S$. One of the focus of these studies was hypoxia tolerance; another one was signal transduction during hypoxia. Using such systems, it has been demonstrated that exogenous $H_2S$ leads to translocation of the hypoxia inducible factor HIF-1, worm ortholog of the mammalian HIF-1α subunit (HIF-1) to the nucleus and promotes HIF-1 transcriptional activity in the hypodermis (Budde et al. 2010). Activated HIF-1 and its target genes such as sulfide:quinone oxidore-

ductase (SQRD-1; the mammalian equivalent being SQR) are essential for worm survival in sulfide-rich environment (Budde et al. 2010; Budde et al. 2011). (As discussed above, SQR is known to have a prominent role in oxidizing $H_2S$ in mitochondria in mammals as well).

Exposure to $H_2S$ also elicits a transcriptional response mediated by SKN-1 (Miller et al. 2011), a conserved transcription factor that is encoded by the *Nrf2* gene in humans; both SKN-1 and Nrf2 are indispensable for the resistance of oxidative and xenobiotic stress (An et al. 2003). According to other studies, worms live longer in non-lethal $H_2S$ containing conditions (Miller et al. 2007), and HIF-1 and SKN-1 play a key role in prolonging their lifespan (Kell et al. 2007; Tullet et al. 2008; Hwang et al. 2011). In line of these findings, other studies showed that the garlic constituents, such as diallyl trisulfide (organic $H_2S$ donors) increased the lifespan of *C. elegans via* a mechanism, which included SKN-1 activation (Powolny et al. 2011).

In a separate line of studies it was demonstrated that $H_2S$ exposure induces an elevated activity of SIR-2.1, a homolog of $NAD^+$-dependent deacetylase, that also has a role in prolonged lifespan in *C. elegans* (Miller et al. 2007). Since the mechanism in which $H_2S$ increases the lifespan of *C. elegans* is independent of other common mechanisms related to increase lifespan of *C. elegans*, like the insulin/IGF signaling (IIS) pathway (Miller et al. 2007), or attenuated mitochondrial function or dietary restriction (Miller et al. 2007), it is possible that there are two distinct mechanisms regulating lifespan of *C. elegans* during the $H_2S$ induction – increased activity of SIR2.1 and transcriptional activation of HIF-1 and/or SKN-1.

HIF-1 and SKN-1/Nrf2 activation by the administration of $H_2S$ may be conserved among other animal models; it was shown that cultured rat cells treated with $H_2S$ exhibit HIF-1 activation (Liu et al. 2010), and also the Nrf2 translocation to the nucleus occurs in murine cardiomyocytes (Calvert et al. 2009). These data imply that the regulation of two important transcription factors mediated by $H_2S$ is evolutionarily conserved in distinct organisms. However, the exact signaling pathways mediated by HIF-1 and SKN-1/Nrf2 remains to be clarified thoroughly in future studies.

In *C. elegans*, under normoxic conditions, a conserved proline residue in the worm ortholog of the mammalian HIF-1α subunit (HIF-1) is hydroxylated by EGL-9 (Epstein et al. 2001), a conserved dioxygenase in the mammalian EGLN protein family (Taylor 2001). The hydroxylation of the proline residue permits the binding of VHL-1 (protein von Hipple Lindau) to HIF-1 and thus targets HIF-1 for proteasomal degradation by the ubiquitination pathway (Epstein et al. 2001). Further studies demonstrated that EGL-9 also negatively regulates HIF-1 transcriptional activity through a VHL-1-independent pathway (Shao et al. 2009); negative HIF-1 transcriptional regulation *via* the VHL-1-independent mechanism has also been observed in mammalian cells (To et al. 2005).

What then is the exact mechanism of $H_2S$-mediated HIF-1 activation? Since the levels of $H_2S$ dramatically increase during hypoxia (see above), it is probable that hypoxia and $H_2S$ interact in modulating HIF-1 activity. It is also conceivable that impaired mitochondrial function by $H_2S$ (which will accumulate during hypoxia) will trigger HIF-1 activation; hypoxia or inhibited transport of electrons in the inner mitochondrial membrane results in the generation of reactive oxygen species that *per se* together with hypoxia activate HIF-1 by impairing the hydroxylase activity of EGL-9 in the VHL-1-dependent pathway (Hwang et al. 2011).

Nevertheless, it was demonstrated that $H_2S$ activates HIF-1 by VHL-1-independent signaling (Budde et al. 2010). It was shown that CYSL-1, ortholog of plant/bacterial *O*-acetylserine sulfhydrylase, inhibits EGL-9 in VHL-1-independent manner. Intriguingly, the mechanism of such regulation has evolved from the regulation of cysteine biosynthesis in bacteria and plants: similarly to formation of cysteine synthase complex in these species, the nematode CYSL-1 interacts with the C-terminal peptide of EGL-9 that leads to the sequestration of EGL-9 and the subsequent activation of HIF-1. Such CYSL-1/EGL-9 association is enhanced by the exposure to $H_2S$ in a dose-dependent manner (Ma et al. 2012). However, the exact mechanism by which $H_2S$ enhances CYSL-1/EGL-9 interaction to activate HIF-1 remains to be determined in future studies.

The effects of endogenously produced $H_2S$ on *C. elegans* physiology need to be elucidated thoroughly. It should be noted that these animals possess several genes coding the enzymes of $H_2S$ metabolism, however, only one has been experimentally characterized; CBS-1 (nematode cystathionine beta synthase encoded by ZC373.1 gene. It has been shown that in contrast to mammalian CBS enzymes, CBS-1, does not possess the heme-binding and Bateman domains that are essential for inhibition and activation of CBS activity by carbon monoxide and S-adenosylmethionine, respectively (Vozdek et al. 2012). These data and also the fact that *C. elegans* genome possesses genes coding for the $H_2S$ metabolic enzymes conserved in plants and bacteria, and not in mammals, strongly suggests that some molecular mechanisms of $H_2S$ action may be different in *C. elegans* compared to mammals. Further experimental studies can address this matter directly.

## $H_2S$-releasing molecules and their therapeutic applications

Given the beneficial signaling and bioenergetic effects of $H_2S$, it has been repeatedly postulated that administration (supplementation) of appropriate amounts of $H_2S$ can be of therapeutic potential (Szabo 2007, 2010). However, such efforts must be well-controlled in order to avoid the potential

**Table 1**. Applications of various $H_2S$ donors in different disease models

| Compound | Disease/*in vitro* or animal model | Main effects of the drug and signaling pathways involved | Reference |
|---|---|---|---|
| Classical (fast releasing) $H_2S$ donors | | | |
| $Na_2S$<br>IK-1001 | **Dermal wound healing**<br>Rat wound healing model; chicken chorioallantoic membranes | IK-1001 increased vascular length of chicken chorioallantoic membranes and improved wound re-epithelialization compared to saline. | (Papapetropoulos et al. 2009) |
| Sodium hydrosulfide<br>NaHS | **Type 1 and type 2 diabetes**<br>Renal epithelial cells OVE26 mice T1D model; C57BL/KsJ lepr $^{-}$/$^{-}$ (db/db mice) T2D model | NaHS inhibited high glucose-induced activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1). NaHS attenuated high glucose-induced matrix protein synthesis via activating AMP-activated protein kinase. | (Lee et al. 2012) |
| NaHS,<br>$Na_2S$,<br>Lawesson's reagent,<br>N-acetylcysteine (NAC) | **Leukocyte-mediated inflammation**<br>Aspirin-induced inflammation in rat; rat air pouch model; carrageenan-induced hindpaw edema in rat | NaHS and $Na_2S$ inhibited aspirin-induced leukocyte adhesion in mesenteric venules likely via activation of $K_{ATP}$ channels.<br>NaHS, Lawesson's reagent, and NAC inhibited leukocyte infiltration in a rat air pouch model. NaHS and $Na_2S$ significantly reduced carrageenan-induced paw edema most likely via $K_{ATP}$ channel activation, as similar effect was observed with pinacidil ($K_{ATP}$ channel agonist) and opposite with glibenclamide ($K_{ATP}$ channel antagonist). | (Zanardo et al. 2006) |
| Slow-releasing $H_2S$ donors (comparison with fast releasing donor if stated) | | | |
| GYY4137<br>NaHS | **Sepsis**<br>mouse RAW 264.7 macrophage cell line | GYY4137 inhibited LPS-induced synthesis of the pro-inflammatory mediators (TNF-$\alpha$, IL-1$\beta$, IL-6, NO, PGE2), and stimulated the synthesis of the anti-inflammatory cytokine IL-10. GYY4137 significantly inhibited LPS-induced phosphorylation of HSP27 and ATF-2 and caused inhibition of the activation of NF-$\kappa$B. Conversely, NaHS had no effect on IL-10 production and was not effective as GYY4137 in reducing LPS-induced synthesis of pro-inflammatory mediators. | (Whiteman et al. 2010) |
| GYY4137 | **Sepsis**<br>LPS-induced endotoxic shock in rats | GYY4137 inhibited neutrophil infiltration and activity, and decreased tissue damage via inhibition of LPS-induced activation of NF-$\kappa$B and STAT-3, reduction of the synthesis of the pro-inflammatory mediators (TNF-$\alpha$, IL-1$\beta$, IL-6, CRP, L-selectin, NO, PGE2), and induction of the synthesis of the anti-inflammatory cytokine IL-10. GYY4137 decreased development of hypotension in rats. | (Li et al. 2009) |
| GYY4137 | **Hypertension**<br>Isolated rat aortic rings; perfused kidney; spontaneously hypertensive rats (SHR) and L-NAME-induced hypertension | GYY4137 induced relaxation of rat aortic rings and renal vasculature of perfused kidneys via opening of $K_{ATP}$ channels. It also reduced systemic blood pressure in SHR and mitigated vasoconstrictor response to angiotensin II and noradrenaline in perfused kidney. | (Li et al. 2008) |
| GYY4137<br>NaHS | **COPD, asthma and pulmonary hypertension**<br>Human primary pulmonary smooth muscle cells | Both GYY4137 and NaHS inhibited serum-induced proliferation of pulmonary smooth muscle cells. NaHS decreased IL-8 release, via reduction of phosphorylation of ERK-1/2 and p38. | (Perry et al. 2011) |
| GYY4137<br>NaHS | **Cancer**<br>Human cancer cell lines: cervical cancer (HeLa); colon cancer (HCT-116); hepatic cancer (Hep G2), osteosarcoma (U2OS) breast cancer MCF-7; promyelocytic leukemia (HL-60); acute myelocytic leukemia (MV4-11) and normal human lung fibroblasts (IMR90, WI-38).<br>HL-60 and MV4-11 xenografts in SCID mice | GYY4137, but not NaHS, significantly inhibited cell viability of all cancer cells tested. Viability of normal human lung fibroblasts was not affected by GYY4137 treatment. In MCF7 cells GYY4137 induced cleavage of PARP and pro-caspase 9 and partially arrested cell cycle in G2/M phase. HL-60 and MV4-11 xenografts in SCID mice were significantly inhibited with GYY4137 treatment. | (Lee et al. 2011) |
| S-allylcysteine (SAC) | **Myocardial infarction**<br>Acute myocardial infarction in rat | Pretreatment with SAC significantly reduced mortality and infarct size without affecting BP. SAC increased left ventricular CSE expression, activity and plasma $H_2S$ levels in AMI rats compared to control, PAG or SAC+PAG groups. | (Chuah et al. 2007) |

**Table 1**. Continued

| Compound | Disease/*in vitro* or animal model | Main effects of the drug and signaling pathways involved | Reference |
|---|---|---|---|
| S-allylcysteine (SAC)<br>S-propyl-l-cysteine (SPC)<br>S-propargyl-cysteine (SPRC) | **Myocardial infarction**<br>Acute myocardial infarction in rat, primary rat cardiomyocytes | SPRC protected effects both adult rat hearts and neonatal cardiomyocytes through upregulation of CSE expression.<br><br>SAC, SPC and SPRC reduced oxidative stress in rat acute myocardial infarction via preservation of SOD and GPx activities and tissue GSH levels. They also reduced MDA formation. | (Wang et al. 2009; Wang et al. 2010) |
| S-propargyl-cysteine (SPRC), a structural analogue of S-allylcysteine (SAC). | **Cancer**<br>Human gastric carcinoma (SGC-7901) cell line. Gastric cancer xenografts in nude mice | SPRC reduced cell viability, proliferation and migration of SPRC-7901 cells. SPRC induced apoptosis and cell cycle arrest in the G1/S phase. In nude mice SPRC significantly reduced tumor volume and stimulated apoptosis via of p53 and Bax. SPRC treatment up-regulated the expression of CSE in tumors and elevated plasma $H_2S$ levels. | (Ma et al. 2011) |
| S-propargyl-cysteine (SPRC), | **Pancreatitis**<br>Caerulein-induced acute pancreatitis (AP) in mice. | SPRC administered 3h, but not 12 h before AP induction, significantly reduced inflammation in pancreas and lungs. SPRC treatment was associated with increase of anti-inflammatory cytokine (IL-10) and decrease of pro-inflammatory cytokines (IL-6, IL-1β). | (Sidhapuriwala et al. 2012) |
| Dithiolethione-modified drugs of known structure | | | |
| S-Latanoprost (ACS-67) | **Retinal ischaemia**<br>Rat retina and retinal ganglion cells (RGC-5) | Intravitreal injection of ACS67 decreased ischemia-mediated damage to the retina and optic nerve, and increased intracellular GSH levels. ACS67, but not latanoprost, ameliorated $H_2O_2$-induced toxicity in RGC-5 cells. | (Osborne et al. 2010) |
| S-Naproxen (ATB-346) | **Gastric ulcers**<br>Healthy rats and rats with compromised gastric mucosal defence; mouse airpouch model | ATB-346 showed improved gastric and intestinal safety compared to parent drug and accelerated healing of preexisting gastric ulcers. ATB-346 was more effective than naproxen in suppressing COX-2 activity and leukocyte infiltration. | (Wallace et al. 2010) |
| S-Mesalamine (ATB-429) | **Ulcerative Colitis**<br>TNBS induced colitis in mice | ATB-429 modulated nociception to colorectal distension (CRD) via reduction of colonic COX-2 and IL-1β mRNA and spinal c-Fos mRNA expression. This effect was mediated by activation of $K_{ATP}$ channels, as ATB-429-induced antinociception was abolished by glibenclamide. ATB-429 significantly reduced the severity of colitis in mice, colonic granulocyte infiltration and levels of inflammatory cytokines (TNF-*α*, IFN-*γ*, IL-1, IL-2, IL-12 and RANTES). | (Distrutti et al. 2006; Fiorucci et al. 2007) |
| $H_2S$-releasing L-DOPA hybrids *ACS83, ACS84, ACS85, ACS86* | **Parkinson's disease**<br>Human monocyte THP-1; human astrocytoma U373 cell line; human neuroblastoma SH-SY5Y cell line; primary human microglial and astrocyte cells | $H_2S$ releasing L-DOPA hybrids decreased the release of TNFα, NO and IL-6 from stimulated microglia, astrocytes and from THP-1 and U373 cell lines. They also showed a neuroprotective effect to SH-SY5Y cells via reduction of the toxicity of supernatants from these stimulated cells. S-DOPAs increased intracellular $H_2S$ and GSH levels. | (Lee et al. 2010) |
| S-Diclofenac (ATB-337/ACS-5) | **NSAID - induced gastrointestinal injury in rats**<br>Carrageenan-induced hindpaw edema in rats; carrageenan-induced hindpaw edema in rats | S –diclofenac significantly decreased gastrointestinal toxicity compared with parent counterpart. S-diclofenac had no effect on hematocrit, whereas diclofenac caused 50% reduction. Compared to diclofenac S –diclofenac did not increase gastric granulocyte infiltration, expression of TNF-*α*, lymphocyte function associated antigen-1 and ICAM-1. S-diclofenac –mediated inhibition of COX-1 and COX-2 was equally effective as for parent drug. S –diclofenac did not induce leukocyte adhesion and significantly reduced paw edema.<br>S –diclofenac was more potent than parent drug at reducing both hindpaw edema and neutrophil infiltration measured with myeloperoxidase activity. S-diclofenac was also more effective in reduced hindpaw nitrite/nitrate concentration. Both drugs were similar in terms of reduction of carrageenan-induced rise in $PGE_2$ and $H_2S$ levels. | (Wallace et al. 2010)<br><br>(Sidhapuriwala et al. 2007) |
| S-Diclofenac (ATB-337/ACS-5) | **Atherosclerosis and restenosis after angioplasty**<br>Normal rat aortic smooth muscle cells (A-10) and SV40 immortalized smooth muscle cells (CRL-2018) | S –Diclofenac induced smooth muscle cell apoptosis via stabilization of p53 along with induction of downstream proteins (p21, p53AIP1 and Bax). | (Baskar et al. 2008) |

**Table 1**. Continued

| Compound | Disease/*in vitro* or animal model | Main effects of the drug and signaling pathways involved | Reference |
|---|---|---|---|
| S-Diclofenac (ACS-5)<br><br>S-sulindac (ACS-18) | **Cancer**<br>Hydrocarbon receptor signaling pathway mediated carcinogenesis; human hepatoma cell line-HepG2, human colorectal adenocarcinoma cell line - LS180 | S –NSAIDs inhibited both expression and activity of the carcinogen activating enzymes cytochromes P-450 (CYP) CYP1A1, CYP1B1, and CYP1A2 via down-regulation of the aryl hydrocarbon receptor (AhR) pathway. S-sulindac stimulated expression of carcinogen detoxification enzymes e.g., glutathione reductase, glutathione S-transferase A2, glutamate cysteine ligase. | (Bass et al. 2009) |
| S-diclofenac (ACS-5),<br>S-sulindac (ACS-18)<br>S-valproate<br>NaHS | **Cancer angiogenesis**<br>HUVECs; human adenocarcinoma HT-29 cell line; Wt C57B16 mice, athymic nude mice [Cr:(NCr)-nu fBR] and EGFP transgenic zebrafish embryos | S-NSAIDs, S-valproate and dithiolethiones inhibited HUVECs proliferation, angiogenesis in muscle and HT29 tumor explants independently of COX inhibition. NaHS at similar concentrations had no inhibitory effect on cell proliferation, suggesting that the observed results for S-NSAIDs were due to ADT-OH moiety not $H_2S$ per se. S-NSAIDs and S-valproate induced HSP27 phosphorylation. Developmental angiogenesis in zebrafish embryos was inhibited by valproic acid and dithiolethiones, but S-NSAIDs failed to show the same effect. | (Isenberg et al. 2007) |
| S-valproate<br>S-diclofenac<br>S-sulindac | **Cancer**<br>Human non-small cell lung cancer cell lines A549 and NCI-H1299. A549 or NCI-H1299 xenografts in nude mice | S –valproate, S-sulindac and S -diclofenac decreased PGE(2) levels (reduction of COX-2 activity) and inhibited cancer cell proliferation in vitro. Xenograft proliferation in nude mice was inhibited only by S-valproate and S-diclofenac which was associated with increased expression of E-cadherin and decreased expression of vimentin and ZEB1 (a transcriptional E-cadhedrin suppressor). | (Moody et al. 2010) |
| S-sildenafil (ACS-6)<br><br>NaHS | **Acute respiratory distress syndrome**<br>Porcine pulmonary arterial endothelial cells | Both S-sildenafil and NaHS ameliorated TNF-$\alpha$ - induced superoxide formation with IC(50) ~10 nM and <1 nM, respectively.<br>Both NaHS and S-sildenafil inhibited TNF$\alpha$ - induced gp91(phox) expression (a catalytic subunit of NADPH oxidase) via PKA or PKG and PKA dependent pathways, respectively. | (Muzaffar et al. 2008) |
| Hydrogen sulfide-releasing aspirin (HS-ASA),<br>ADT-OH | **Cancer**<br>Human breast cancer MDA-MB-231 ($ER^-$, $PR^-$, not over expressing Her2 receptor) cell line; normal human mammary epithelial (HMEpC) cell line; tumor xenografts in athymic SCID mice | HS-ASA inhibited the growth of triple negative MDA-MB-231 cells via induction of apoptosis and G0/G1 cell cycle arrest, increased production of ROS, reduction of thioredoxin reductase activity and inhibition of NF-$\kappa$B signaling due to decreased translocation of p65 into the nucleus. HS-ASA treatment significantly reduced growth of tumor xenografts via down-regulation of NF-$\kappa$B, inhibition of cell proliferation and induction of apoptosis. | (Chattopadhyay et al. 2012; Chattopadhyay et al. 2012) |
| Hydrogen sulfide-releasing aspirin<br><br>ACS14 and its deacetylated metabolite ACS21 | **Metabolic syndrome/ hypertension**<br>Buthionine sulfoximine (BSO) – induced metabolic syndrome in rat (associated with GSH depletion); isolated heart and aortic rings | Both ACS14 and ACS21 did not cause gastric lesions; sustained the GSH levels, ameliorated BSO-induced hypertension, lowered plasma $TXB_2$ levels, 8-isoprostane and insulin. ACS14 and ACS21 protected the heart from ischemia/reperfusion, limited vascular endothelial dysfunction and hypertension.<br>ACS14 decreased plasma $TXB_2$ levels comparable to aspirin, but lacked adverse effects to gastric mucosa. ACS14 increased $H_2S$ and GSH production and increased HO-1 promoter activity. | (Rossoni et al. 2010) (Sparatore et al. 2009) |
| S-ASA<br>S-sulindac,<br>S-iburofen,<br>S-naproxen | **Cancer**<br>Human cancer cell lines: colon adenocarcinoma (HT-29, SW-480 and HCT-15); pancreatic cancer (MIA PaCa-2 and BxPC-3), prostate cancer (LNCAP) lung cancer (A549) breast cancer (MCF-7, MDA-MB-231 and SK-BR-3), T cell leukemia (Jurkat ) | HS-NSAIDs were 28 to 3000-fold more potent than their conventional counterparts in inhibiting growth of all cancer cell lines studied. The most potent HS-ASA decreased cancer cell proliferation, stimulated apoptosis and induced G0/G1 cell cycle arrest. The effects were most likely due to additive effects of both ASA and ADT-OH as a mixture of the two components gave similar, however, 95-fold decreased growth inhibition comparing to intact HS-ASA. ASA and ADT-OH alone were less active than the intact HS-ASA. | (Chattopadhyay et al. 2012; Chattopadhyay et al. 2012) |

ASA, acetylsalicylic acid (aspirin); BP, blood pressure; COPD, chronic obstructive pulmonary disease; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; GPx, glutathione peroxidase; GSH, glutathione; HO-1, heme oxygenase-1; HSP, heat shock protein; HUVECs, human umbilical vein endothelial cells; IL, Interleukin; $K_{ATP}$ channels, ATP-sensitive potassium channels; MDA, malon dialdehyde; NF-$\kappa$B, nuclear factor-kappa B; NSAIDs, non-steroidal anti-inflammatory drugs; $PGE_2$, prostaglandin $E_2$; SCID,severe combined immunodeficiency; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; T2D, type 2 diabetes; $TBX_2$, thromboxane B $X_2$.

of cytotoxicity that is associated with high concentrations or high release rates of this mediator. Currently, several classes of slow-releasing $H_2S$ pro-drugs are in various stages of research and development. The biological effects of several classes of such donors are summarized in Table 1.

Many $H_2S$ donors have been used over the years by many research groups to examine the potential role of $H_2S$ in physiological and pathophysiological conditions, utilizing most of the time the sodium salts ($Na_2S$ and NaHS). However, the problem with these donors is that they deliver an instantaneous bolus of $H_2S$ in seconds once added into solution (Li et al. 2008; Whiteman et al. 2010). It is highly unlikely that cells, tissues or organs will ever be exposed to such a high concentrations of $H_2S$ under physiological conditions, since CSE CBS and 3-MST all produce $H_2S$ in a slow and sustained manner (Singh et al. 2009; Huang et al. 2010; Whiteman et al. 2010, 2011; Le Trionairre et al. 2012). Although there are multiple lines of data generated with fast-releasing $H_2S$ donors, showing benefits in various pathophysiological conditions, the therapeutic index of these compounds is rather narrow (Elrod et al. 2007; Esechie et al. 2008; Simon et al. 2008, 2011; Baumgart et al. 2009; Esechie et al. 2009; Osipov et al. 2009; Szabo et al. 2011; Wagner et al. 2011; Bracht et al. 2012). Given the physiological concentrations of $H_2S$ detected in humans (from nanomolar to micromolar, depending on the method used), this raises more questions about the role of physiological $H_2S$ levels and highlights the need for any therapeutically relevant $H_2S$ donor to deliver the "right amount" of $H_2S$, in order to mimic the effects of endogenously synthesized $H_2S$ (or to substitute for it in $H_2S$-deficient states).

From the various $H_2S$ donors, it is worth mentioning the $H_2S$ releasing derivatives of anti-inflammatory drugs (S-diclofenac) (Li et al. 2007), the ophthalmic solution used to control intraocular pressure (S-Latanoprost) (Osborne et al. 2010), as well as certain garlic compounds (diallyl sulfide, diallyl disulfide) (Benavides et al. 2007) and GYY4137 (Whiteman et al. 2010) which are characterized by moderate and slow release of $H_2S$ compared to sulfide salts. The *in vivo* $H_2S$ releasing ability of diallyl disulfide has subsequently been demonstrated in rodents (Insko et al. 2009). L-thioglycine and L-thiovaline represent another novel examples of $H_2S$ donors (Zhou et al. 2012). In addition, other methods have also been proposed to increase biological $H_2S$ levels, such as approaches attempting to increase endogenous $H_2S$ level by providing substrates of $H_2S$-generating enzymes. Selected examples include L-cysteine and N-acetylcysteine.

## Conclusions and future directions

$H_2S$, since its 'rebirth' as a biological mediator and the third gasotransmitter, attracts considerable attention both in physiological and pathophysiological contexts. Clearly, one area of active inquiry and considerable future exploration will be its biosynthesis. Although much is known about the biochemistry of its two PLP-dependent enzymes CSE and CBS, as well as the third enzyme system, 3-MST/CAT, further studies are needed to explore the exact molecular mechanisms of $H_2S$ biosynthesis in health and disease. Up- or downregulation of this enzyme may occur in various pathophysiological conditions; there may be post-translational modifications in these enzymes, as well as processes affecting its substrate or co-factor availability. Much future work is needed to explore them. These enzymes also likely to contribute to the regulation of mitochondrial function, which remains to be studied in the future. As mentioned above, during the last 6 years inquiring reports have demonstrated that $H_2S$ is not only an inhibitor of mitochondrial function, but mitochondria can also use it as an inorganic electron donor, that supports electrons to the mitochondrial respiratory chain. It remains to be determined in future studies whether these finding is a biochemical curiosity, or whether it also has (patho)physiological relevance; can it act as a source of energy in conditions when cellular energy supply are limited (such as in hypoxic and stress conditions)?

Similarly, the absolute levels of endogenous $H_2S$ and distinguishing the different sulfur pools (Wintner et al. 2010) in health and disease remain to be elucidated; as mentioned above, there is a considerable debate even with respect to the actual basal/physiological levels of $H_2S$. One of the tools that needs to be improved/refined is the selective targeting of each of the $H_2S$-producing enzymes. Investigators working in the field would do much better if the specificity and selectivity of the currently available inhibitors would be improved (Whiteman et al. 2011).

Due to utilizing the nematode *Caenorhabditis elegans* as an experimental model system, many possibilities have opened that will help us elucidate certain key regulating mechanisms in $H_2S$ biology. We have no doubt that further utilization of *C. elegans* model system in the field of $H_2S$ biology will yield new insights in many areas, including the regulation of HIF-1, in the regulation of signal transduction pathways and possibly in cellular bioenergetics as well.

Finally, the intensive research of $H_2S$-releasing donors is expected to produce tangible benefits in the near future. As overviewed in the current article, several classes of slow-releasing $H_2S$ pro-drugs are under various stages of research and development. Further chemical modifications should yield compounds with improved specificity and potency; hopefully well fine-tailored to their respective clinical applications. The administration routes of $H_2S$ donor molecules could include compounds delivered *via* inhalation, injection, skin patch, or oral administration, in order to maximize their potency and safety and to decrease side effects. It may also be possible in the future to design

tissue-type or cell-type targeted strategies and approaches to alter local $H_2S$ levels. Toxicological evaluation and early-stage clinical trials with some of the new $H_2S$ donors is expected to take some of these compounds to the "next level", which is the clinical stage (safety and efficacy studies). Short-term $H_2S$ infusion has been demonstrated to be safe in healthy human volunteers (Toombs et al. 2010), providing a yardstick for future human studies as far as dosing, as well as identifying exhaled $H_2S$ as a potential human $H_2S$ biomarker.

The current short review only highlighted a few, selected, rapidly developing areas of $H_2S$ biology. Although it is clear that $H_2S$ does not work in isolation, but, rather, it works in cooperation and interaction with other gasotransmitters (Szabo 2010; Szabo et al. 2011; Coletta et al. 2012) in the current review we did not have space to explore this aspect of $H_2S$ biology in detail. Similarly, we did not review any of the results obtained with pharmacological inhibitors of $H_2S$ biosynthesis or studies using genetic models such as animals lacking CSE. We also did not mention several other novel areas of $H_2S$ biology, such as its role in the regulation of immune functions, or its role in various pathophysiological conditions. Clearly, there are a great number of emerging areas of $H_2S$ biology, and we are certain that continuing research will continue to unveil many additional novel roles of this fascinating biological mediator.

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