



## Review Paper

## Exploring mitochondrial hydrogen sulfide signalling for therapeutic interventions in vascular diseases



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## ABSTRACT

Hydrogen sulfide (H<sub>2</sub>S), a gaseous signalling molecule, is important in numerous physiological and pathophysiological processes. Despite its initial identification as an environmental toxin, H<sub>2</sub>S is now well described as an essential physiological molecule that is finely balanced to maintain cellular functions, especially in modulating mitochondrial activity. Mitochondria are responsible for the oxidation of H<sub>2</sub>S and its safe elimination while maintaining mitochondrial biogenesis. H<sub>2</sub>S oxidation in mitochondria generates various reactive sulfur species that could post-translationally modify proteins by sulphydration. Sulphydrated proteins participate in many regulatory activities either by direct interactions in the electron transport chain or indirect regulation by epigenetics. These investigations explain the importance of research of H<sub>2</sub>S as a therapeutic molecule beyond the traditional understanding as a protective role through its anti-inflammatory and antioxidant properties. This review focuses on highlighting the significant involvement of the H<sub>2</sub>S pathway in vascular diseases and current H<sub>2</sub>S donors for therapeutic use under development.

## 1. Introduction

The endothelium is formed by a monolayer of endothelial cells located in the inner layer of the vascular wall and continuously exposed to hemodynamic shearing forces, circulating molecules in the blood and mediators released from underlying smooth muscle cells. Disturbances

to the physiological status of the endothelium lead to activated endothelial cells that culminate in vascular dysfunction [1]. Dysfunctional endothelium contributes to a pro-inflammatory and pro-thrombotic phenotype, which causes disturbances to endothelial homeostasis and impair vasodilation-vasocontraction [1,2]. Consequently, endothelial dysfunction has been described as a hallmark for developing several patho-

**Abbreviations:** AR, androgen receptor; AMPK, 5'-adenosine monophosphate-activated protein kinase; Akt, protein kinase B; ATP5A1,  $\alpha$ -subunit of ATP synthase; ATP, adenosine triphosphate; b.End3 cells, brain-derived microvascular endothelial cells; Bax, apoptosis regulator BAX; Bcl-2, B-cell lymphoma 2; Ca<sup>2+</sup>, calcium; CDKN2A, cyclin-dependent kinase inhibitor 2A; CAT, catalase; CSE KO, cystathionine  $\gamma$ -lyase knockout; COX, cyclooxygenase; Cys, cysteine residues; DADS, diallyl disulfide; DATS, diallyl trisulfide; DNA, deoxyribonucleic acid; DJ-1, known as Parkinson disease protein 7 (PARK7); Drp1, dynamin related protein 1; eNOS, endothelial nitric oxide synthase; EA.hy926 cells, human umbilical vein cell line; Fis1, mitochondrial fission 1 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA3, GATA binding protein 3; GSH, glutathione; GYY4137, morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate; HEK293 cells, human embryonic kidney 293 cells; HUVECs, human umbilical vein endothelial cells; HO-1, heme oxygenase-1; ILs, interleukins; IFN $\gamma$ , interferon gamma; IRF-1, interferon regulatory factor-1; Keap1, Kelch-like ECH-associated protein 1; KATP ATP, sensitive potassium channel; KLF5, kruppel-like factor 5; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese-dependent superoxide dismutase; MEK1, map kinase-1; MitoROS, mitochondrial reactive oxygen species; mPTP, mitochondrial permeability transition pore; Na<sub>2</sub>S, sodium sulfide; NaHS, sodium hydrosulfide; Nrf1, nuclear respiratory factor 1; Nrf2, nuclear factor erythroid 2-related factor 2; NMDAR1 subunit, N-methyl-D-aspartate receptor; NOX4, NADPH oxidase 4; NF-KB, nuclear factor-kappa beta cells; NR, not reported; ox-LDL, oxidised low-density lipoprotein; PARP, Poly (ADP-ribose) polymerase; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; PI3K, phosphoinositide 3-kinase; PINK1, PTEN-induced kinase 1; PGC, peroxisome proliferator-activated receptor- $\gamma$  coactivator; PP1c, protein phosphatase-1; PC, pyruvate carboxylase; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PTP1B, protein tyrosine phosphatase 1B; PP2A, protein phosphatase 2A; PPRC, peroxisome proliferator-activated receptor- $\gamma$  coactivator-related protein; RAGE, receptor for advanced glycation end-products; Runx2, runt-related transcription factor 2; Sp-1, specificity protein 1; VEGFR, receptor of vascular endothelial growth factor; TRPV6, transient receptor potential cation channel subfamily V member 6; BMMSC, bone marrow mesenchymal; SIRT1, sirtuin 1; SIRT3, sirtuin 3; USP8, ubiquitin specific peptidase 8 USP8.

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physiological conditions, including atherosclerosis, diabetes, stroke and ageing-related diseases [1,3,4]. Endothelial dysfunction appears to be a consequence, at least in part, of increased production of highly reactive oxygen and nitrogen species, which are byproducts of normal metabolism of oxygen. Endothelial cells are exposed to the highest oxygen levels in the blood and mainly generate ATP via aerobic glycolysis [5]. Therefore, endothelium is less dependent on mitochondrial oxidative phosphorylation than most cells using the tricarboxylic acid cycle, aiding cells to survive in highly oxygenated environment with a controlled production of mitochondrial reactive oxygen species (ROS). In this regulated environment, endothelium maintains the regulation of vascular relaxation and vascular smooth muscle dilation by sustained nitric oxide (NO) production. In addition to NO, there are two more gases: carbon monoxide (CO) [6] and hydrogen sulfide (H<sub>2</sub>S) that have been subsequently identified as gasotransmitters. Many evidence suggest that the vasoprotective effect of H<sub>2</sub>S either as a direct antioxidant or via indirect mechanisms. The study of H<sub>2</sub>S as a physiological mediator began with discovering its endogenous production and bioactive properties in mammals [7–9]. Further developments in later years led to the re-evaluation of H<sub>2</sub>S from a toxic molecule to a protective gaseous signalling mediator when present at low concentrations [10], putting it on a par with the NO and CO [11,12].

H<sub>2</sub>S can be synthesized by both endothelial cells and vascular smooth muscle cells within the vascular wall. The production of H<sub>2</sub>S has shown to 100-1000 fold higher in smooth muscle (nanomolar) as compared to endothelial cell lines (picomolar) allowing H<sub>2</sub>S to act as a smooth muscle relaxant [13]. In mammals, the desulfhydration of the amino acid cysteine is considered as a major source of H<sub>2</sub>S through the transsulfuration pathway mediated by two cytosolic enzymes, cystathionine- $\beta$ -synthase (CBS), cystathionine  $\gamma$ -lyase (CSE) and the mitochondrial enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST). Once generated, H<sub>2</sub>S is believed to function as a secondary messenger, and its signalling functions have been explored, including its ability to reduce free radicals, interactions with metal centers in the active sites of proteins and post-translational modification of thiol groups in cysteine residues [14,15]. Whilst the involvement of H<sub>2</sub>S has been described in almost every cell and tissue type, its ability to relieve endothelial dysfunction has gained much interest in physiology and pathophysiology [16–18].

## 2. Biogenesis and metabolism of H<sub>2</sub>S

### 2.1. Biogenesis of H<sub>2</sub>S

The intracellular H<sub>2</sub>S concentration is maintained at a low steady-state through a balance between its biogenesis and metabolism [19]. In mammals, endogenous H<sub>2</sub>S production is catalysed primarily by the transsulfuration pathway (Fig. 1A) [19]. While the pyridoxal-5'-phosphate-dependent CBS and CSE are mainly located in the cytoplasm, the pyridoxal-5'-phosphate-independent 3-MST enzyme resides in both mitochondria and cytoplasmic compartments [20], and utilizes a different enzymatic reactions to produce H<sub>2</sub>S. In addition, enzymatic activity of cysteine aminotransferase (CAT) produces 3-mercaptopyruvate (3-MP), which is subsequently used by mitochondrial 3-MST for H<sub>2</sub>S production.

In mammals, the activity of CBS is commonly found in the brain and central nervous system [21,22]; preferentially expressed in the glia and astrocytes [23]. While CSE is the main H<sub>2</sub>S-producing enzyme in the cardiovascular system, H<sub>2</sub>S-generating 3-MST enzyme is ubiquitously expressed in all tissues [24]. Unlike CBS and CSE, 3-MST is regulated in a redox-state dependent-manner rather than by transcriptional regulation [25]. 3-MST contains a redox-sensitive cysteine in its active site (Cys<sup>247</sup>) that is modified to cysteine sulfenate, resulting activity loss under oxidative stress [26]. This also works in favor of restoring cellular redox state by increasing the availability of cysteine to produce cellular antioxidants such as thioredoxin (Trx) and glutathione (GSH), contributing for the cellular redox homeostasis. 3-MST exhibits its activ-

ity in combination with another mitochondrial enzyme, CAT, and there is a growing interest in the CAT: MST axis for H<sub>2</sub>S synthesis and cell metabolic rewiring [14,15]. Especially, H<sub>2</sub>S synthesis through this pathway is shown to highly dependent on GSH levels [16] interconnecting cysteine bioavailability for protein synthesis and cellular redox status. The potential role of 3-MST and CAT on endothelial cell bioenergetics and metabolism has been recently reported through 3-MST inhibition studies [27]. Silencing 3-MST suppressed the angiogenesis by decreasing mitochondrial respiration, mitochondrial adenosine triphosphate (ATP) production and perturbed the entire endothelial cell metabolome [27].

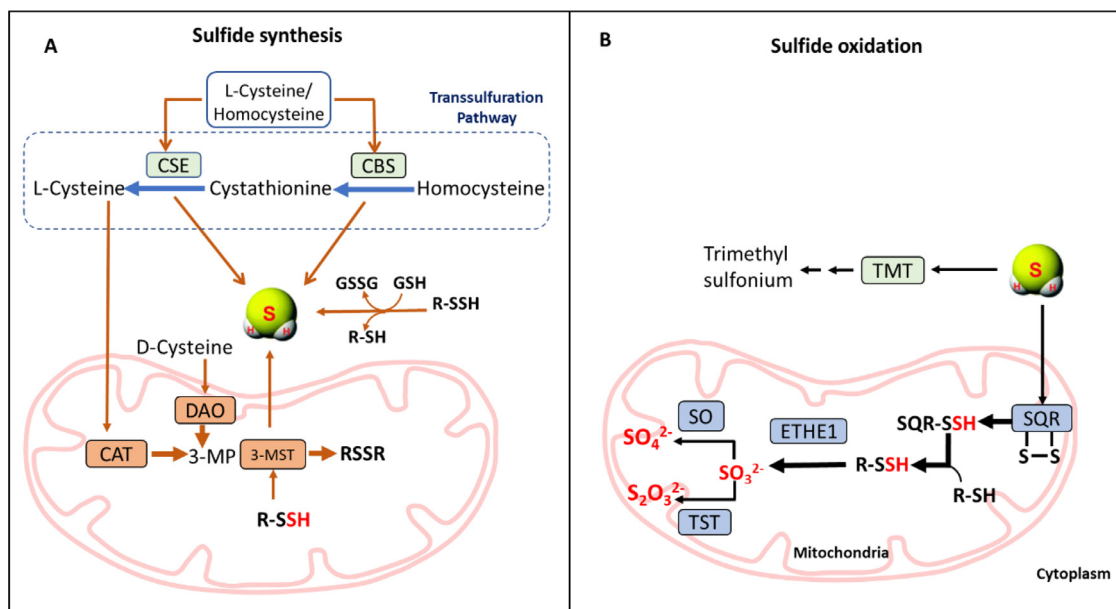
Although there is an apparent overlap in tissue localization, the activity of these enzymes can vary under specific conditions. For instance, CSE can be translocated to the mitochondria under the regulation of Tom20 in mitochondrial membrane [28]. Since the cysteine level inside mitochondria is about three times than in the cytosol, mitochondrial translocation of CSE increases mitochondrial H<sub>2</sub>S production and ATP production [28]. The accumulation of CBS in mitochondria increased H<sub>2</sub>S, which prevented cytochrome c (Cyt c) release from mitochondria and decreased hypoxia-induced ROS generation [29]. Therefore, endogenous H<sub>2</sub>S was identified as an important regulator of energy production in mammalian cells when the oxygen supply is limited, such as in hypoxic conditions. This action of H<sub>2</sub>S was also observed in hibernating animals where metabolic depression is apparent. In addition to being formed enzymatically from the substrate cysteine, hibernating animals regenerate H<sub>2</sub>S from its oxidation products, including thiosulfate and polysulfides [30,31]. This protective mechanism not only facilitated ATP production during hibernation, but also preserved free cysteine for synthesis of a major antioxidant, GSH [30].

In addition to the above-mentioned enzymes, recent reports show a fourth enzymatic pathway regulated by peroxisomal enzyme, D-amino acid oxidase (DAO) that uses D-cysteine instead of L-cysteine as a substrate to produce 3MP, which is a substrate for 3-MST [24]. Since the expression of DAO is organ specific, the usage of D-cystine to produce H<sub>2</sub>S is high in tissues such as the kidney and the cerebellum [24,25]. For example, the production of H<sub>2</sub>S from D-cysteine in the kidney is 60 times higher than that from L-cysteine [25]. Therefore, administration of D-cysteine has been suggested as a therapeutic approach to deliver H<sub>2</sub>S to specific tissues [32].

The level of endogenous H<sub>2</sub>S production is variable depending on both the tissue and experimental conditions used. Endogenous levels of H<sub>2</sub>S have been measured as 10-15 nM in murine brain, but higher concentrations of 1  $\mu$ M were observed in the murine aorta [33]. In vascular tissue, the endogenous production in rat aorta and mesenteric artery are inferior compared to the rat tail artery and ileum [34]. In healthy humans, plasma levels were found to be between 70 -125  $\mu$ M [35]. These variations in the H<sub>2</sub>S content suggest that specific tissues demand higher production, which is associated with effective metabolic machinery to avoid the accumulation of the gaseous molecule and ultimately toxicity [36].

### 2.2. Metabolism and oxidation of H<sub>2</sub>S

Mitochondrial enzymes play a central role in the catabolism and oxidation of H<sub>2</sub>S, which regulates its steady-state levels. The oxidation of H<sub>2</sub>S begins in the mitochondrial matrix and is completed in the inter-mitochondrial membrane space through tightly regulated mitochondrial sulfide oxidation pathway (Fig. 1B) [37]. In the mitochondrial matrix, H<sub>2</sub>S is oxidised by a cluster of mitochondrial enzymes, otherwise known as sulfide oxidation unit (SOU) that consists of sulfide quinone oxidoreductase (SQR), ethylmalonic encephalopathy 1 (ETHE1 or persulfide dioxygenase, PDO), Thiosulfate sulfurtransferase (TST) and sulfite oxidase (SO) [38,39]. H<sub>2</sub>S is oxidised by SQR to generate a sulfane sulfur (S<sup>0</sup>) by forming a persulfide (R-SSH). This reaction causes the release of two electrons, which are transferred to the electron transport chain (ETC) via coenzyme Q (CoQ). Persulfide is then oxidised by ETHE1 to



**Fig. 1.** Cellular pathways leading to H<sub>2</sub>S synthesis and oxidation. A) CBS catalyses the first step in transsulfuration by converting homocysteine to cystathionine, which is subsequently cleaved into cysteine and  $\alpha$ -ketobutyrate by CSE. Mitochondrial 3-MST generates H<sub>2</sub>S via CAT, using L-cysteine and requires the transfer of sulfur from a persulfide (RSSH). Persulfides can also act as a source of H<sub>2</sub>S with the aid of reductants such as GSH. B) H<sub>2</sub>S oxidation occurs in mitochondria. H<sub>2</sub>S-reduced SQR generates persulfide by accepting a thiol (R-SH). This persulfide is then oxidised to short-lived sulfite (SO<sub>3</sub><sup>2-</sup>) via ETHE1 and rapidly convert to thiosulfate (S<sub>2</sub>O<sub>3</sub><sup>2-</sup>) and sulfate (SO<sub>4</sub><sup>2-</sup>) by thiosulfate sulfurtransferase (TST) and SO, respectively.

produce sulfite and further oxidised to SO<sub>4</sub><sup>2-</sup> by SO or to S<sub>2</sub>O<sub>3</sub><sup>2-</sup> by TST [40].

Apart from oxidation, H<sub>2</sub>S can also be catabolized through methylation, a cytosolic process that yields methanethiol that can be further methylated to dimethyl sulfide (non-toxic compound) via thiol S-methyltransferase (TSMT) [41]. Metabolically, dimethyl sulfide serves as a substrate for rhodanese forming thiocyanate (SCN<sup>-</sup>) and sulfate, which is the major end-product of H<sub>2</sub>S clearance [42]. Another H<sub>2</sub>S catabolism pathway includes H<sub>2</sub>S-scavenging by metalloproteins such as methemoglobin, forming sulfhemoglobin or forming disulfides such as oxidised glutathione (GSSG) [43]. H<sub>2</sub>S biosynthesis and oxidation pathways are also connected to other metabolic pathways such as serine biosynthesis, the folate cycle, and the nucleotides metabolism [44]. Therefore, fine-tuned H<sub>2</sub>S synthesis and oxidation is important not only to maintain cellular bioenergetics but also for cellular metabolic profile.

### 3. Implications of H<sub>2</sub>S on redox balance

It is now evident that there are a number of mechanisms whereby H<sub>2</sub>S can potentially influence redox balance. While some reactions occur over a relatively short timescale such as free radical scavenge and interactions with the electron transport chain, other H<sub>2</sub>S induced changes might operate on a longer timescale. Long-term effectors of H<sub>2</sub>S includes oxygen-sensitive regulation of mitochondrial functions by translocating CSE or CBS under hypoxic conditions [29]. Rapid and long term effectors of H<sub>2</sub>S-mediated O<sub>2</sub> sensing in cells and tissue is extensively reviewed in Olson, 2015 [45]. In addition, H<sub>2</sub>S-mediated post translational modification of cysteine thiol groups in target proteins via S-sulfhydration (also termed as persulfidation), will be described below.

#### 3.1. H<sub>2</sub>S as an antioxidant

The regulatory mechanisms of H<sub>2</sub>S on endothelial cells as an antioxidant could be via several pathways; it can quench free radicals as a chemical reductant, increase intracellular antioxidants such as GSH and redoxins or scavenge free radicals by increasing the expression of enzymatic antioxidants such as nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase (SOD), CAT or glutathione peroxidase

(GPx) [16]. Depending on the local concentration, H<sub>2</sub>S can acts as an antioxidant towards ROS and reactive nitrogen species (RNS) such as hypochlorous acid, hydrogen peroxide, lipid hydroperoxides, superoxide and peroxynitrite [46]. Even though the calculated rate constants for the reactions between hydrosulfide (HS<sup>-</sup>) and ROS/ RNS are highly favorable, very low physiological concentrations [47] suggest that H<sub>2</sub>S has little importance as a direct antioxidant in humans.

In addition to be a precursor for H<sub>2</sub>S, cysteine is also the source of GSH production. H<sub>2</sub>S in extracellular space has been shown to increase GSH production by inducing a reduction of cystine into cysteine [48]. H<sub>2</sub>S treatments is suggested to enhance  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -GCS) activity to increase cellular GSH synthesis and augments the localization of GSH to mitochondria. Since GSH is not synthesised in mitochondria, authors suggested an important role of H<sub>2</sub>S by enhancing cystine/cysteine transporters and redistribution of GSH to mitochondria [48]. As described before, H<sub>2</sub>S induced GSH production also observed in other mammals during hibernation as a protective mechanism against oxidative stress [30].

H<sub>2</sub>S administrations has shown to increase other antioxidant proteins such as Trx-1 via Nrf-2 pathway. H<sub>2</sub>S-dependent cardioprotection was observed in a mouse model of ischemia-induced heart failure, where Trx-1 is upregulated at both gene and protein levels [49]. H<sub>2</sub>S is able to regulate the activity of several members of the sirtuin (SIRT) family that catalyses post-translational modifications of both histone and non-histone proteins. H<sub>2</sub>S increased the expression of SIRT1 in an oxidative stress induced cardiomyocytes [50,51] and induced cardio protection in diabetic rat model [52]. Cardiomyocytes treated with the SIRT1 inhibitors reverted the antioxidant protection induced by H<sub>2</sub>S indicating the importance of SIRT pathway in cells [51]. H<sub>2</sub>S also shown to induce other members of SIRT family such as SIRT3; a major regulator of mitochondrial function [53] and nuclear located SIRT6 [54], to exert either physiological or pathophysiological effects.

#### 3.2. H<sub>2</sub>S and mitochondrial respiratory oxidation

The influence on mitochondrial respiratory chain is one of the main pathways that H<sub>2</sub>S helps to maintain the cellular redox balance. The absence or reduced ability of O<sub>2</sub> to act as the terminal electron accep-

tor such as in hypoxic conditions could prevent electron flow along the respiratory chain followed by accumulation of H<sub>2</sub>S in mitochondria. A recent report described a genetic defect in SQR gene in patients with Leigh syndrome. Patients presented with abrogated SQR enzyme activity and decreased mitochondrial complex IV activity with intermittent accumulation of H<sub>2</sub>S [55]. Excess accumulation of H<sub>2</sub>S was also reported in patients with pathogenic variants in ETHE1 [56]. Both CoQ deficient in vitro and in vivo models caused a reduction in SQR levels and activity leading to an impairment of H<sub>2</sub>S oxidation with accumulation of H<sub>2</sub>S and depletion of the GSH system [57]. However, the regulation and interplay between these pathways is still a developing field. Recent studies suggest that CoQ supplementation increases SQR expression, thus inducing the H<sub>2</sub>S oxidation pathway whilst down-regulating CSE and CBS in the transsulfuration pathway [44].

The pathological consequences of H<sub>2</sub>S accumulation are associated with its capacity to exhibit toxicity at high concentrations by binding the copper center and blocking cytochrome oxidase (complex IV), compromising the functioning of the mitochondrial ETC [36]. H<sub>2</sub>S interacts with ETC via two ways: firstly by transferring sulfide-derived electrons at the level of complex III via the reduced quinone and into the complex IV via reduced Cyt c [58]; secondly by transferring electrons to ETC via Cyt c that bypass the complex III to increase ATP synthesis [59]. During the process of H<sub>2</sub>S mediated Cyt c reduction, generation of a mixture of reactive sulfur species (RSS) including SO<sub>3</sub><sup>-</sup>, SO<sub>2</sub><sup>-</sup>, H<sub>2</sub>S<sub>2</sub><sup>-</sup> was observed [58]. Due to their highly reactive nature, these products readily reduce O<sub>2</sub> to form superoxide and then H<sub>2</sub>O<sub>2</sub>. In turn, H<sub>2</sub>O<sub>2</sub> and reactive sulfur species reoxidised Cyt c to its original state for further H<sub>2</sub>S removal [58].

### 3.3. H<sub>2</sub>S-Induced protein sulfhydrylation

Owing to its unique sulfur containing functional -SH, cysteine residues play a ubiquitous role in protein structure and function. Cysteine thiol groups react with ROS, RNS or reactive sulfur species (RSS) to produce various post translational modifications on proteins [60,61]. While oxidative modifications such as S-nitrosylation (-SNO), S-sulfenylation (-SOH) are reversible modifications, higher modifications (sulfonic acids) result protein or enzyme deactivation [62]. Sulfhydrylation is a reversible post-translational modification resulting from the conversion of a thiol group (R-SH) to a persulfides (R-SSH) [63]. However, as reductants, H<sub>2</sub>S or HS<sup>-</sup> cannot directly react with protein thiols. Sulfhydrylation can occur by nucleophilic attack of an HS<sup>-</sup> anion on the sulfur atoms of disulfides, -SNOs or -SOH, as well as by transsulfuration reactions. RSS generated as a result of H<sub>2</sub>S oxidation can react with protein thiols to generate protein sulfhydrates (persulfidates). Sulfhydrylation could change the original function of proteins, serving as important switchers or regulators in many physiological and pathophysiological processes.

#### 3.3.1. Sulfhydrylation of cytosolic proteins

Research now demonstrates that RSS could sulfhydrate proteins under physiological conditions, accounting for 10-25% of liver proteins, including actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [14]. This highly abundant post-translational modification has shown to affect a variety of cellular signalling and biological pathways (Table 1). Sulfhydrylation can modify enzymatic activity of proteins, activity of ion channels, nuclear localization of proteins [10]. For example, endothelial nitric oxide synthase (eNOS), which causes vasodilation, can sulfhydrate the same cystine residue (Cys 443) that undergoes nitrosylation. H<sub>2</sub>S releasing donors increased eNOS activity by eNOS dimerization via sulfhydrylation dependent mechanism [64]. Another protein that loses its nitrosylation site to sulfhydrylation in the presence of H<sub>2</sub>S is the phosphatase and tensin homolog deleted on chromosome ten (PTEN). PTEN is a phosphatase that suppresses the activity of the class I phosphoinositide 3-kinase/AKT signalling pathway. Sulfhydrylation of PTEN

has shown to inhibit its enzymatic activity under physiological conditions [65]. The regulation of ion channels by means of post-translational modification enables cells to respond to changing environments. Sulfhydrylation activated ATP-sensitive potassium (K<sub>ATP</sub>) ion channels that control membrane potential and cellular excitability [66] and Ca<sup>2+</sup> flux via multiple Ca<sup>2+</sup> transient receptor potential (TRP) ion channels [67]. H<sub>2</sub>S-induced sulfhydrylation has been reviewed previously in relations to its diverse roles in pathophysiological processes [68].

#### 3.3.2. Sulfhydrylation of mitochondrial proteins

Change to mitochondrial activity were either observed as direct sulfhydrylation of mitochondrial proteins or through indirect actions of sulfhydrated cytosolic proteins. Módis and collaborators found that sulfhydrylation of alpha subunit of ATP synthase (ATP5A1) at Cys<sup>244</sup> and Cys<sup>294</sup> in response to H<sub>2</sub>S exposure [69], where the modification increased ATP synthase activity and stimulated mitochondrial bioenergetics in HEK293 and HepG2 cells. This observation was also confirmed by reduced levels of sulfhydrated ATP5A1 and ATP synthase activity in the livers of CSE knockout mice [69]. While this is an interesting observation, it remains to be examined whether S-sulfhydrylation of ATP synthase occurs in other cell types and their exact functional role. S-sulfhydrylation of interferon regulatory factor 1 (IRF-1), a mitochondrial transcriptional factor that is involved in mitochondrial biogenesis pathway, enhances mitochondrial DNA replication and cellular bioenergetics via modification of TFAM promoter [70]. Thereby sulfhydrylation mediates epigenetic regulation of TFAM, which leads to promotion of methylation and maintenance of mitochondrial DNA. SIRT3 enzymes and PARPs can utilize NAD<sup>+</sup>, a cofactor required for mitochondrial function that is upregulated by H<sub>2</sub>S in the vascular endothelium [71]. Sulfhydrylation of SIRT3 enhance its catalytic activity leading to reduction of mitochondria dysfunction, improvement of mitochondrial ETC performance and ATP production in the vasculature [72,73]. The Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) is a key regulator of calcium signaling in health and disease. Treatment with H<sub>2</sub>S inducer drugs, S-propyl-L-cysteine (SPRC) or sodium hydrosulfide (NaHS), attenuated the development of heart failure in animals and preserved mitochondrial function via sulfhydrylation of CaMKII at Cys<sup>6</sup> [74]. The intrinsic apoptotic pathway is initiated by the release of Cyt c, a mitochondrial intermembrane space protein, to the cytoplasm. H<sub>2</sub>S in known to regulate Cyt c release by maintaining mitochondrial membrane potential (ΔΨ<sub>m</sub>) [75]. Vitvitsky et al, 2018 showed that reduction of Cyt c by H<sub>2</sub>S increased the formation of RSS allowing the proteins in close proximity, such as procaspase 9 to be sulfhydrated [58].

Nonetheless, it is important to maintain intracellular sulfanesulfur homeostasis. In this context, redox systems such as thioredoxin (Trx)/Trx reductase (TrxR1) and glutaredoxin (Grx)/glutathione reductase (GR)/GSH play a vital role. Elevated levels of protein sulfhydrylation was observed in mouse livers where hepatocytes lack both TrxR1 and GR suggesting the importance of reducing systems [76]. The increased Trx levels is associated with HIV-1 infection and these patients had significantly lower total sulfanesulfur levels compared to patients treated with antiretroviral therapy. This evidence suggests a role for the mitochondrial Trx system as regulator of protein sulfhydrylation [77].

Thus, by enhancing mitochondrial cellular function and bioenergetics via target protein sulfhydrylation, H<sub>2</sub>S helps to protect mitochondria against a variety of stressors. The advent of H<sub>2</sub>S releasing drugs, especially mitochondrial-targeted, should permit considerable insight into this field.

## 4. Therapeutic strategies for H<sub>2</sub>S donors

Considering the mounting evidence of potential therapeutic option in a range of conditions, a wide range of commercially available H<sub>2</sub>S donors including sulfide-containing salts, H<sub>2</sub>S-releasing prodrugs and natural products containing sulfur have been investigated [107]. Com-

**Table 1**

Sulfhydrated proteins involved in a range of cellular functions. Effects of sulfhydration on the activity of identified proteins involved and its cellular and subcellular function. (NR: not reported).

General Function	Protein Sulfhydrated	Modified Cysteine	Cellular Effect	Reference
Cell Signalling	eNOS	443	Promotes eNOS phosphorylation and dimerization	[64]
	GAPDH	150, 156, 152	Inhibition of GAPDH catalytic activity and enhanced nuclear translocation	[14] [78]
	PC	265	Promotes gluconeogenesis	[79]
	PTEN	71, 124	Inhibits S-nitrosylation of PTEN	[65]
	PTP1B	215	Inhibits PTP1B activity and thereby promotes PERK activity under ER stress	[80]
	PP1c	127	Promote eIF $\alpha$ phosphorylation resulting in inhibition of general protein synthesis	[81]
	PPAR $\gamma$	139	Increases PPAR $\gamma$ nuclear accumulation, induce adipogenesis	[82]
	RAGE	259, 301	Prevents RAGE-mediated oxidant pathological effects	[83]
	Sp1	68, 75	Enhances VEGFR2 expression and maintain vascular homeostasis	[84]
	SUR1 subunit of K <sub>ATP</sub>	6, 26	Activates K <sub>ATP</sub> channel	[66]
	SUR2B subunit of K <sub>ATP</sub>	24, 1455	Reduces the tyrosine nitration of Kir6.1	[85]
TRPV6	172, 329	Induces Ca <sup>2+</sup> influx and maintains bone homeostasis	[67]	
Hrd1	115	Regulates VAMP3 ubiquitylation and prevents CD36 translocation	[86]	
Transcription Factors	SIRT1	NR	Increases its deacetylase activity, reduced its degradation and endothelial inflammation	[87]
	GATA3	84/182, 84/248	Inhibits GATA3 nuclear translocation and differentiation of splenocytes	[88]
	Keap1	151	Activates Nrf2-target signalling and ameliorates cellular oxidative stress	[89–91]
Apoptosis and Cell proliferation	Kir6.1 subunit of K <sub>ATP</sub> channel	43	Stimulates vasorelaxation and hyperpolarization	[92]
	MEK1	341	Stimulates nuclear translocation of phosphorylated ERK leading to PARP-1 activation and repair DNA damage	[93]
	AR	611, 614	Inhibits AR-DNA binding activity and AR dimerization, which suppress cancer proliferation	[94]
	Akt	77	Contributes to activation of protein kinase GSK3 $\beta$	[95]
	Caspase 3	163	Inhibits protein activity	[96]
	p65 subunit of NF-KB	38	Anti-apoptotic actions in liver and inhibits ox-LDL-induced macrophage inflammation	[97,98]
Runx2	123, 132	Promotes osteoblast differentiation and maturation	[99]	
Mitochondrial Function	ATP5A1	244, 294	Stimulates ATP synthase activity	[69]
	DJ-1	106	Prevents irreversible oxidation of DJ-1 to maintain mitochondrial redox balance	[100]
	IRF-1	53	Increases TFAM expression to maintain mitochondrial DNA replication	[70]
	LDHA	163	Stimulates LDH activity by increasing NAD <sup>+</sup> generation and enhances mitochondrial bioenergetics	[101]
	PP2A	NR	Inhibits PP2A activity, which leads to AMP kinase activation	[102]
	p66Shc	59	Prevents mitochondrial reactive oxygen species production	[103]
	Parkin	59, 95, 182	Activates E3-ubiquitin ligase activity of parkin, which induces degradation of misfolded proteins and reduces neuron cell death	[104]
	PPRC	NR	Promotes murine mitochondrial biogenesis	[105]
	PGC-1 $\alpha$	NR	Stimulates mitochondrial biogenesis	[105]
	SIRT3	256, 259, 280, 283	Increases deacetylase activity and improves mitochondrial function	[53]
USP8	NR	Increases association of parkin with USP8, which promotes mitophagy	[106]	

mon H<sub>2</sub>S donors and their specific vascular effects at the subcellular level are summarised in Table 2.

#### 4.1. General H<sub>2</sub>S donors

Sulfide-containing salts such as NaHS and disodium sulfide (Na<sub>2</sub>S) are common fast releasing H<sub>2</sub>S donors used in vitro and in vivo. These donors have displayed cytoprotective actions in vascular models through direct modulation of mitochondrial biogenesis and function [105,108,109]. Lawesson's reagent has been widely used to generate H<sub>2</sub>S-releasing compounds. For example, commonly used GYY4137 (morpholin-4-ium-4-methoxyphenyl phosphinodithioate) is synthesised upon reaction with morpholine [110]. GYY4137 is water soluble and is believed to release H<sub>2</sub>S at a controlled rate under physiological conditions. Furthermore, H<sub>2</sub>S release from GYY4137 is pH and temperature

dependent, with a greater release at acidic pH's and higher temperatures [110]. In vascular endothelium, GYY4137 can protect mitochondria and endothelial cells from oxidative stress [111].

Allium vegetables, such as garlic and onion are known to contain H<sub>2</sub>S generating molecules. Garlic contains  $\gamma$ -glutamyl-S-allyl-L-cysteine and S-allyl-L-cysteine sulfoxides able to generate H<sub>2</sub>S by chemical transformation [112]. Diallyl disulfide (DADS) and diallyl trisulfide (DATS) are both derived from garlic and act as H<sub>2</sub>S donors when they react with biological thiols including GSH. However, whereas cell treatment with DADS observed impaired mitochondrial function [113] and DATS reduced mitochondrial ROS production [114].

A novel H<sub>2</sub>S prodrug, SG1002 has been observed to promote H<sub>2</sub>S bioavailability in heart failure patients [115]. In a murine model with induced transverse aortic constriction, SG1002 preserved cardiac function following activation of a VEGF-Akt-eNOS-NO-cGMP signalling path-

**Table 2**

H<sub>2</sub>S donors. Summary of H<sub>2</sub>S donors and their effects on cellular function with a focus on mitochondrial activity. ↑ Denotes activation or increase, ↓ Denotes attenuation or decrease.

Compound	Model	Target	Outcome	References
Na <sub>2</sub> S (10-500 µg kg <sup>-1</sup> ) (1-50 µM)	Mice cardiomyocytes	Mitochondrial ETC, Mitochondrial Complex II	↑ mitochondria function/appearance ↓ myocardial infarct size, inflammation, apoptosis	[108]
GGY4137 (12.5-100 µM)	EA.hy926 cells	Redox steady state, SIRT3 expression, MAPK phosphorylation, Antioxidant enzymes	↑ endothelial function, mitochondria respiratory capacity/membrane potential ↓ oxidative stress, apoptosis	[90]
(100 µM)	HPAEC cells	PI3K pathway and Redox steady state	↑ alveolar network formation, mitochondrial membrane potential ↓ oxidative stress, apoptosis	[133]
(0.25 mg kg <sup>-1</sup> 250 µM)	Mice glomerular endothelial cells	NMDA-R1 subunit, mPTP channel Ca <sup>2+</sup> channel Mitochondrial cyclophilin D	↓ renal injury, oxidative stress Mitigate Ca <sup>2+</sup> channel expression Prevent mPTP opening	[134]
NaHS (100-300 µM)	Rat cardiomyocytes	Redox steady state, Antioxidant enzymes, Mitochondrial Complex IV	↓ ischemia/reperfusion injury, oxidative stress	[135]
(50-300 µM)	b.End3 microvascular endothelial cell diabetic rats	Cellular DNA and PARP pathway, Mitochondrial membrane potential, Redox steady state	↑ mitochondrial and cellular function	[109]
(10 µM)	HEK293 cell CSE KO mice	ATP5A1 sulphydration	↑ mitochondrial bioenergetics	[69]
(30 µM)	CSE KO mice hepatocytes	Mitochondrial DNA and Complex V, Nrf2/1- transcription factors, PPRC, PGC-1β, Tfam expressions/activity	↑ mitochondrial biogenesis ↓ tissue damage	[105]
(100 µM)	Rat aortic endothelial cells	Redox steady state, Antioxidant enzymes, Apoptosis pathway, Mitochondrial fission/fusion pathway, PINK1/Parkin signalling	↑ mitochondrial membrane potential, mitophagy ↓ oxidative stress, apoptosis	[136]
AP39 (30-300 nM)	b.End3 mice microvascular endothelial cells	Mitochondrial ETC activity and complexes II/III, Redox steady state, LDH pathway	↑ mitochondria activity ↓ oxidative stress, hyperglycemic injury	[130,129]
(1 µmol kg <sup>-1</sup> )	Sprague Dawley rats	Akt/ eNOS phosphorylation, PTP channel	↓ infarct size, renal inflammation ↑ post-ischaemic recovery	[137]
(10 ng/ml)	HUVECs	SRSF2 and CDKN2A genetic expression, Cytokines expression	↓ inflammation, senescence	[131]
AP123 (30-300 nM)	b.End3 mice microvascular endothelial cells	Mitochondrial ETC activity and complexes II/III, Redox steady state, LDH pathway	↑ mitochondria activity ↓ oxidative stress, hyperglycemic injury	[130]
DADS (1 mM)	CD-1 mice cells	Redox steady state, Antioxidant enzymes	↑ lipid peroxidation, mitochondria membrane depolarization, oxidative stress	[113]
50 mg kg <sup>-1</sup> 25 µM	Sprague-Dawley rats H9c2 cells	PGC1α/Tfam/Nrf2/eNOS expressions, Apoptosis pathway, Antioxidant enzymes, α-skeleton actin expression, Citric acid cycle	↑ mitochondria function/apoptosis/ biogenesis, NO bioavailability ↓ oxidative stress, cardiac hypertrophy	[138]
DATS (100 µM)	HUVECs	Redox steady state, Apoptosis pathway, AMPK pathway, mitochondrial dynamic and membrane potential	↑ mitochondria function ↓ mitochondrial apoptosis × mitochondrial fission	[114]
SG-1002 20 mg kg <sup>-1</sup>	CSE KO mice	Akt/eNOS phosphorylation, VEGF/NOX4/HO-1 expression	↓ oxidative stress, fibrosis, cardiac enlargement, mitochondria function	[139]
20 mg kg <sup>-1</sup>	CSE KO mice H9c2 cardiomyocytes	AMPK phosphorylation, mitochondrial DNA, PGC-1α pathway	↑ mitochondria respiration/biogenesis/ content	[102]
ATB-337 (10-50 µmol kg <sup>-1</sup> )	Wistar rats	COXs/ TNF-α activity	↓ inflammation. Platelet aggregation, intestinal tissue damage, gastric mucosa injury	[140]
ATB-429 (25-130 mg kg <sup>-1</sup> )	Mice	Cytokine expression	↓ inflammation, severity of colitis	[141]
ATB-346 (16 mg kg <sup>-1</sup> )	Mice	COX-2 activity	↓ intestinal manipulation.	[142]

(continued on next page)

Table 2 (continued)

Compound	Model	Target	Outcome	References
ATB-352 (4.6-46 mg kg <sup>-1</sup> )	Mice	Involvement of the endogenous cannabinoid system	↓ gastrointestinal toxicity and nociceptive response to noxious stimuli	[143]
SG-1002 (20-40 mg kg <sup>-1</sup> day <sup>-1</sup> )	Mice	VEGF-Akt-eNOS-NO-cGMP signalling pathway	↑ cardiac function	[144]
GIC-1001 (30-60 mg kg <sup>-1</sup> )	Mice	Activation of peripheral opioids receptors	↓ nociceptive response to noxious stimuli	[145]
MZe786 (20 mg kg <sup>-1</sup> )	Mice	Rescuing mitochondrial activity	↓ hypertension and renal damage ↑ cardiac mitochondrial biogenesis and antioxidant defence	[121,146,147]
Sodium thiosulphate (0.15 g/250 ml over 15 min)	Humans	Anti-inflammatory and antioxidant effects are linked to its reaction with mitochondrial thiosulfate sulfurtransferase	↓ systolic blood pressure an hour after administration ↑ cardio protection	[148]
ACS94 (20 mg kg <sup>-1</sup> )	Sprague-Dawley male rats	Metabolic pathways relating to Hcys, Cys, GSH (by TSP pathway)	↑ GSH and circulating H <sub>2</sub> S ↓ Homocysteine	[149]
ACS6 (10pM-1μM)	PAECs	Adenylyl cyclase-PKA pathway	↑ NADPH, cAMP ↓ Superoxide formation and phosphodiesterase type 5 activity	[150]
ACS67 (5 μl of 4 nM solution)	RGC-5	Attenuates the process of oxidative-induced RGC-5 cell death	↑ GSH ↓ H <sub>2</sub> O <sub>2</sub> -induced toxicity	[151]
ACS84 (0.25-10 μM)	BV-2, neurons isolated from hippocampus of Sprague Dawley rats	Phosphorylation of p38- and JNK	↑ Mitochondria function ↓ cytotoxicity and inflammation	[152]

way in cardiomyocytes. Furthermore, mitochondrial functions were preserved, oxidative stress was attenuated, and increased myocardial vascular density were observed following the administration of SG1002. Studies demonstrate that H<sub>2</sub>S treatments could stimulates endothelial cell proliferation, migration and tube formation in vitro [116]. H<sub>2</sub>S interventions were associated with an increase in vascular endothelial growth factor (VEGF) expression and activation of its receptor, stimulating angiogenesis in vivo [117].

Previous work by Wang and colleagues demonstrated that the inhibition of CSE results in the increased production of antiangiogenic factors, such as soluble Fms-Like Tyrosine Kinase-1 (sFlt-1) and soluble endoglin (sEng) and the administration of H<sub>2</sub>S donors had been shown to suppress sFlt-1 and sEng in endothelial cells [118]. Antiangiogenic factors such as sFlt-1 could inhibit the mitochondrial respiration and promote mitochondrial-specific superoxide production in endothelial cells [119]. Recently we described how H<sub>2</sub>S produced by CSE pathway maintains endothelial mitochondrial bioenergetics and loss of CSE increases the production of mitochondrial-specific superoxide [120]. MZe786, the ADTOH H<sub>2</sub>S donor linked with aspirin, has been explored and observed to improve outcomes in mice with a compromised CSE pathway and/or an increased expression of sFlt-1. MZe786 was employed in both HO-1 haploid deficient (Hmox1+/-) pregnant mice in a high sFlt-1 environment and a refined reduced uterine perfusion pressure (RUPP) model to mimic preeclampsia in C57Bl/6 J mice [121,122]. MZe786 was found to correct the induced preeclampsia state by reducing blood pressure and renal damage. Additionally, the overexpression of sFlt-1 has been observed to inhibit cardiac mitochondrial activity in the same Hmox1+/- mouse model [123]. Moreover, MZe786 was found to rescue mitochondrial activity by stimulating cardiac mitochondrial biogenesis and antioxidant defence in Hmox1-/- mice and in Hmox1+/- mice exposed to a high sFlt-1 environment thus improving outcomes.

Sodium thiosulfate in its role as a H<sub>2</sub>S producer shows beneficial properties and is currently being investigated in phase I trial for potential benefits in patients with acute coronary syndrome and undergoing coronary angiography [124]. Furthermore, anti-inflammatory and antioxidant effects of thiosulfate are linked to mitochondrial thiosulfate

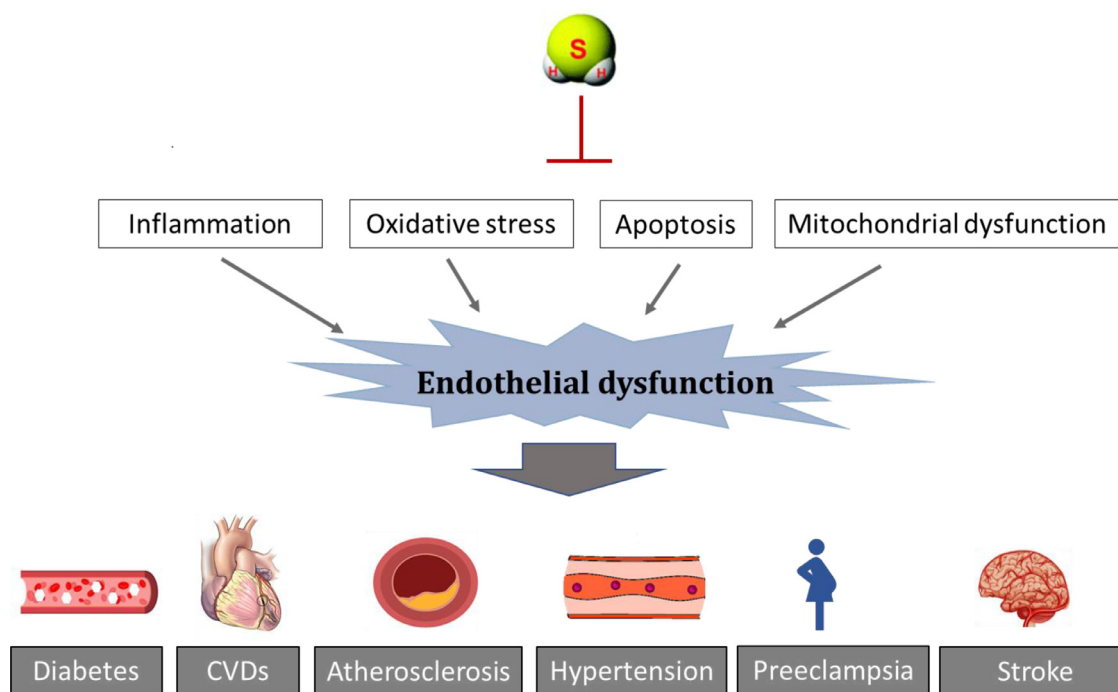
sulfotransferase induced sulfur transfer to GSH and thioredoxin, thus promoting thiol-dependent antioxidative mechanisms [125]. In addition to these H<sub>2</sub>S generators, currently available endogenous inducers of H<sub>2</sub>S-generating enzymes include S-adenosylmethionine (SAM), epidermal growth factors (EGF), S-nitroso-N-acetylpenicillamine (SNAP) and microRNAs-21 [21,23,126,127]. Recently, it was reported that administration of SAM could potentially inhibit vascular endothelial growth factor-A-related diseases induced by adverse effects of long-term treatment of levodopa (L-dopa) [128].

#### 4.2. Mitochondrial targeted H<sub>2</sub>S donors

More recently, special attention has been given to the mitochondrial targeted drugs. Mitochondrial-targeted H<sub>2</sub>S donors, including AP39 and AP123, improved mitochondrial function in glucose oxidase-induced oxidative stress in endothelial cells and restored vascular homeostasis [129]. AP39 treatment attenuated endothelial senescence and protects against oxidative stress in acute renal injury and hyperglycemic injury [130,131]. Both AP39 and AP123 have been observed to decrease hyperpolarisation of the mitochondrial membrane and inhibit mitochondrial oxidant production whilst increasing the electron transport at respiratory complex III thus improving cellular metabolism [132]. Therefore, investigating the role of H<sub>2</sub>S on mitochondrial activity in endothelial cells should increase our understanding of future drug targets.

#### 5. Involvement of H<sub>2</sub>S in vascular diseases

Endothelial dysfunction is ubiquitously observed in both arteries and small vessels in multiple vascular disorders. It is known that H<sub>2</sub>S contributes to vascular protection, specifically, reduced levels of plasma H<sub>2</sub>S have been observed in many pathological conditions such as stroke [153], diabetes [154], pulmonary hypertension [155], portal hypertension [156], chronic obstructive pulmonary disease [157] and age-related diseases [158]. Evidence on the roles of H<sub>2</sub>S in these pathologies is also accumulating (Fig. 2), as discussed below.



**Fig. 2.** The disease association with endothelial dysfunction and H<sub>2</sub>S. Endothelial cell dysfunction is characterized by disturbed redox balance and increased inflammatory reactions within the blood vessel wall. Other contributing factors such as induction of apoptotic pathways and disrupted mitochondrial function further exacerbate changes to vascular tone. Endothelial dysfunction reflects a phenotype that is attributed to many vascular and cardiovascular diseases.

### 5.1. Cardiovascular diseases (CVDs)

CVDs have been the leading cause of mortalities and are accounted for majority of all deaths worldwide [159]. Over the last decade, increasing evidence has demonstrated that H<sub>2</sub>S plays important roles in maintaining cardiovascular homeostasis [4,41]. In vivo studies demonstrated that disruption of CSE pathway in mice, the major H<sub>2</sub>S producing enzyme in cardiovascular system, leads to enhanced atherosclerosis [160] and endothelial dysfunction [2]. Clinical evidence showed that significant changes in CSE/H<sub>2</sub>S pathway are associated with heart failure, atherosclerosis, myocardial ischemia and diabetes [161], suggesting that dysregulation of CSE/H<sub>2</sub>S pathway is implicated in the pathogenesis of CVDs. Furthermore, administration of H<sub>2</sub>S donor or manipulation of endogenous H<sub>2</sub>S production improves cardiovascular function and disease outcomes in a range of animal CVD models, including atherosclerosis, heart failure and myocardial infarction [162,108]. The beneficial effects of H<sub>2</sub>S are mediated through its roles of anti-inflammation, anti-apoptosis, anti-oxidative stress and proangiogenic, highlighting the therapeutic potentials of H<sub>2</sub>S in CVDs.

### 5.2. Atherosclerosis

Atherosclerosis is a leading risk factor for cardiovascular diseases, leading to narrowing and hardening of arteries as a consequence of fatty plaque formation in the artery wall; consequently H<sub>2</sub>S has been investigated as an attractive therapeutic option against atherosclerosis [163]. Mani et al. demonstrated that CSE knockout mice fed with a high-fat diet developed an endothelial dysfunction-related atherosclerosis phenotype which included oxidative stress and excessive expression of adhesion molecules [164]. In an ApoE<sup>-/-</sup> murine atherosclerotic experimental model, lower H<sub>2</sub>S levels and lower CSE expression were reported. These in vivo disturbances were significantly abolished by exogenous H<sub>2</sub>S administration [165]. Administration of H<sub>2</sub>S or overexpression of CSE decreased inflammatory markers through NF- $\kappa$ B downregulation that ame-

liorated the atherosclerotic lesion [166] and reduced pro-inflammatory cytokine production [152]. In addition, oxidative stress-induced mitochondrial dysfunction in vascular cells is considered to play a role in the pathogenesis of atherosclerosis [167]. The excessive superoxide production by mitochondria can trigger a pro-inflammatory response in the vascular wall that ultimately leads to atherosclerosis development [168,169]. H<sub>2</sub>S releasing compound, diallyl trisulfide treatment reduced mitochondrial oxidative stress and improved vasculature offering therapeutic route against atherosclerosis [170]. Alternatively, treatment with H<sub>2</sub>S might protect the vascular wall by increasing NO bioavailability [160].

### 5.3. Diabetes

Endothelial dysfunction plays an important role in the pathogenesis of diabetic complications such as vascular dysfunction, nephropathy, retinopathy, neuropathy, and cardiomyopathy. Plasma levels of H<sub>2</sub>S were found to be significantly decreased in patients with type 2 diabetes (~100  $\mu$ M) when compared with age-matched normal control subjects (~130  $\mu$ M) [154]. The same study also reported a reduced H<sub>2</sub>S levels in streptozotocin (STZ)-treated diabetic rats compared with control Sprague–Dawley rats. Supplementation with H<sub>2</sub>S or L-cysteine prevented secretion of inflammatory cytokines IL-8 and MCP-1 in high-glucose-treated human U937 monocytes [154]. In contrary, some studies reported that the expression levels and the activities of CSE and CBS in the pancreas beta cells, as well as plasma H<sub>2</sub>S concentrations are increased in STZ-treated rats [171]. The local increase of H<sub>2</sub>S levels prolonged pancreatic beta cell survival against high glucose induced cytotoxicity [171]. Therefore, more studies exploring this association are warranted. The potential of exogenous treatment with H<sub>2</sub>S donors has been explored, showing protection against hyperglycemic stress in both in vitro and in vivo models [154,172,173]. In these models, H<sub>2</sub>S improved diabetes-related complications by inhibiting mitochondrial oxidative stress [109].



#### 5.4. Hypertension

Lower circulating levels of H<sub>2</sub>S have also been reported in other forms of hypertensive disorders, such as pulmonary hypertension [155] and portal hypertension [156]. These findings are further supported by evidence showing reduced endogenous H<sub>2</sub>S pathways in hypertensive patients, as demonstrated by Greaney et al, 2017 who reported reduced expression of CSE and 3-MST in cutaneous tissue in hypertensive patients [174]. As CSE is the most active H<sub>2</sub>S-producing enzyme in the vasculature, it is possible that a defective CSE/H<sub>2</sub>S pathway is responsible for reduced H<sub>2</sub>S production and therefore, the key culprit in the onset of endothelial dysfunction leading to hypertension. Many authors have explored the potential for H<sub>2</sub>S donors to prevent/treat hypertension. In this regard, H<sub>2</sub>S donors have effectively shown to ameliorate hypertensive phenotype in many animal models. Intraperitoneal injections of GYY4137 reversed pulmonary hypertension in rats and demonstrated that H<sub>2</sub>S suppresses the oxidative stress by enhancing the activity of the intracellular antioxidants; catalase and mitochondrial SOD [175]. Furthermore, H<sub>2</sub>S protected against the production of mitochondrial reactive oxygen species and apoptosis in pulmonary artery smooth muscle cells exposed to hypoxia [175]. A high-salt diet, a contributing factor to clinical hypertension, results in vascular apoptosis. The molecular events associated with an enhanced mitochondrial protection in the presence of exogenous H<sub>2</sub>S were also demonstrated in HUVEC exposed to a high salt environment. This study showed that exogenous H<sub>2</sub>S inhibited apoptosis, reduced superoxide production, Cyt c release, and caspases expression, as well as restoring mitochondrial membrane potential [176].

#### 5.5. Stroke

Sharing a common defective pathway with other vascular disorders, ischemic stroke has been associated with high blood pressure and endothelial dysfunction [177]. Interestingly, the level of endogenous H<sub>2</sub>S in the brain has been estimated at 50 to 160 μM [178] and some early studies reported correlations with high levels of cysteine in serum and poor clinical outcomes in acute stroke patients [179,180]. Moreover, Sun et al, 2007 demonstrated that plasma H<sub>2</sub>S was significantly lower in patients with stroke, accompanied by elevated blood pressure and hyperhomocysteine [181]. Studies performed in stroke-prone spontaneously hypertensive rats, showed that oxidative stress correlated with lower CBS activity and these events were prevented by the administration of H<sub>2</sub>S [182]. In terms of the potential mechanisms involved, it has been reported that the protective effect of H<sub>2</sub>S links to NO activity (eNOS/ NO signaling pathway) to protect against ischemic brain injury by attenuating oxidative stress and promoting mitochondrial homeostasis [183]. Apart from endothelial dysfunction, several other pathological processes contribute to this complex disease. Therefore, H<sub>2</sub>S may play different roles in the nervous system to the vascular system and further investigations might be needed to get a better understanding of H<sub>2</sub>S interactions [184]. These investigations emphasize the dual role of H<sub>2</sub>S and attention to fine balance of H<sub>2</sub>S for therapeutic use.

#### 5.6. Preeclampsia

Preeclampsia clinically manifests as de novo hypertension often accompanied by proteinuria after the 20<sup>th</sup> week of gestation [185]. While the pathogenesis of preeclampsia is still not clear, some molecular mechanisms such as dysregulation of vascular factors such as sFlt-1 and placental growth factor (PIGF) are considered key events in the development and progression of preeclampsia [186]. It has been proposed that the CO/HO-1 pathway together with H<sub>2</sub>S-CSE pathway are protective routes in pregnancy that when defective may result in preeclampsia pathogenesis [187]. Evidence provided by Wang and colleagues showed that the expression of CSE was reduced in placentas from preeclamptic

women [118]. In addition, the inhibition of CSE resulted in elevated circulating levels of sFlt-1 in mice, while GYY4137 abrogated these effects [118]. Exogenous H<sub>2</sub>S has also shown beneficial effects in protecting against sFlt-1-induced vascular damage. In a study by Holwerda et al., H<sub>2</sub>S restored sFlt-1-induced hypertension and proteinuria via induction of VEGF expression in rats [17]. The potential of H<sub>2</sub>S to modulate the angiogenic balance in models of preeclampsia has also been demonstrated by others [188,189]. A link between preeclampsia, H<sub>2</sub>S and mitochondria was demonstrated by Covarrubias et al, 2019 who observed that the mitochondrial H<sub>2</sub>S donor AP39 prevented antiangiogenic factors and oxidative stress response in trophoblasts exposed to hypoxia. These effects were credited to H<sub>2</sub>S-mediated protection of mitochondrial Cyt c oxidase activity, reduction of superoxide, as well as sFlt-1 levels [190]. Recently, our lab demonstrated that CSE-derived H<sub>2</sub>S sustains mitochondrial bioenergetics and modulates the generation of mitochondrial reactive oxygen species in endothelial cells. In addition, using AP39 as exogenous H<sub>2</sub>S donor, we showed that H<sub>2</sub>S was metabolized at SQR in mitochondria, contributes to the regulation of sFlt-1 levels [120].

## 6. Conclusion

It is evident that as a gasotransmitter, H<sub>2</sub>S plays a fundamental role in vascular function and redox biology. However, the field of H<sub>2</sub>S biology is still a developing area with many unanswered questions from H<sub>2</sub>S biochemistry to its biological functions. H<sub>2</sub>S can act as a substrate of and an inhibitor within biological pathways depending on concentration. Most research reports on the overall effect of H<sub>2</sub>S but overlook many intermediary reactive sulfur species that may result during specific pathways. For example, the exact mechanism of the sulfhydration process (via H<sub>2</sub>S itself, or, more likely, via intermediary persulfide reactions) remains to be characterised. Given the key involvement of mitochondria in H<sub>2</sub>S oxidation pathway, further research is needed to understand the fine balance between H<sub>2</sub>S and ROS. The majority of studies focused on CSE knockout experiments to explore the H<sub>2</sub>S pathway but the roles of other H<sub>2</sub>S generating enzymes: CBS, 3-MST or DAO are less well defined in relation to mitochondrial function.

Recently, there is increasing interest in using H<sub>2</sub>S as a therapeutic molecule and a drive to investigate better H<sub>2</sub>S pro-drugs with some specially designed to target mitochondria. These are very promising research avenues for treating many vascular, cardiac, and neurodegenerative diseases. Without a doubt, H<sub>2</sub>S-mitochondrial research will present interesting and important new findings in near future and these works will benefit understanding of biology and for future therapeutic targets.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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