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Molecular Characterization of Hydrogen Sulfide Role in Vascular System and Method of Endogenous Production Detections with Common Ion Channels Used to Produce Its Biological Effect

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Abstract

In addition to nitric oxide and carbon monoxide, hydrogen sulfide (H_2S) is the third gasotransmitter in mammals. It is synthesized from L-cysteine by cystathionine β-synthase, cystathionine γ-lyase or by sequential action of alanine aminotransferase and 3-mercaptopyruvate sulfur transferase. Although initially it was suggested that in the vascular wall H_2S is synthesized only by smooth muscle cells and relaxes them by activating ATP-sensitive potassium channels, more recent studies indicate that H_2S is synthesized in endothelial cells as well. The physiological functions of H_2S are mediated by different molecular targets, such as different ion channels and signaling proteins. Endogenous H_2S is involved in the regulation of many physiological processes in the cardiovascular system including the regulation of vascular tone, blood pressure and inhibits atherogenesis. Many new technologies have been developed to detect endogenous H_2S production, and novel H_2S-delivery compounds have been invented to aid therapeutic intervention of diseases related to abnormal H_2S metabolism. The primary purpose of this review was to provide an overview of the role of H_2S in the blood vessel, methods of endogenous production detections and common ion channels used to produce its biological effect describe its beneficial effects.

Keywords: Hydrogen sulfide; Blood vessel; Ion channels

Introduction

Until the last two decades of the 20th century, all known chemical transmitters were liquids that are solids in their pure form [1]. Furchgott and Zawadzki demonstrated that the relaxation of rabbit aorta following acetylcholine administration is dependent on the endothelium, and the substance responsible for the vascular relaxation was determined to be an endothelium derived relaxing factor [2]. Palmer et al. proved that this substance is pharmacologically identical to nitric oxide (NO) [3]. NO was then determined to be one of the most important signaling molecules in biological control systems. Moreover, NO was the first gaseous molecule that fulfilled the criteria of a transmitter [4]. Specifically, gaseous transmitters must be 1) freely membrane permeable; 2) endogenously and enzymatically generated and regulated; 3) have defined functions at physiological concentrations; and 4) have specific cellular and molecular targets, although second messengers are not needed [1-4]. Marks et al. discovered that another simple gaseous molecule, carbon monoxide (CO), operates as a transmitter in the mediation of vasoactivity [5]. Abe and Kimura, who studied neuronal activity, identified a third gaseous transmitter, namely, hydrogen sulfide (H_2S), which is the endogenous mediator in mammals [6] and the vasoactivity of this compound was revealed by Hosoki et al. [7]. Since that time this hypothesis was confirmed by many studies and the "H_2S field" in biology and medicine is now growing rapidly [8]. H_2S thus joined two older counterparts, nitric oxide (NO) and carbon monoxide (CO), to form the family of "gasotransmitters" [4]. Other gasotransmitters, such as ammonia (NH_3), methane (CH_4) and hydrogen (H_2) are suggested to exist as well [8,9]. Epidemiological studies report that a diet rich in organosulfur species is associated with longevity and decreased morbidity [10]. Members of the Allium genus (garlic and onions), which contain organosulfur compounds have a well-documented history of health benefits [11]. Indeed, garlic-derived compounds such as diallyl trisulfide release H_2S in the presence of cellular reductants like glutathione (GSH) [12]. Populations that consume garlic regularly have low blood pressure, low cholesterol, and less vascular disease [13]. Additionally, the ancient Greeks, Egyptians, and Romans regularly bathed in natural sulfur springs as treatments for disease [14]. Depending on the microbiota and oxygen content, sulfur springs typically contain H_2S concentrations ranging from 1 to 500 mM [15,16].

Hydrogen sulfide (H_2S), which is endogenously produced, contributes to numerous physiological functions in mammalian systems [17]. Typically, it participates as a transmitter in the regulation of the cardiovascular system, inflammatory and immune response, gastrointestinal tract, kidney and nervous system functions [7,8,18,19]. Data on the concentration of H_2S in the cardiovascular system varies between 10 nmol/l and 300 μmol/l [1]. Interest in the cytoprotective actions of H_2S has grown since the discovery that it can induce a hypometabolic state characterized by decreased O_2 consumption, heart rate, and body temperature in non-hibernating rodents [20]. The main aim of this review article is to describe the molecular physiology of hydrogen sulfide in the cardiovascular system and method of detection of H_2S with common ion channels used to produce its biological effect within human being.

Chemical properties, synthesis and metabolism of H_2S

H_2S is the colorless flammable gas with a strong odor of rotten eggs, soluble in both water and organic solvents [21]. Like NO and CO, H_2S is also toxic at high concentrations and shares with them

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the main mechanism of toxicity - inhibition of cytochrome c oxidase (mitochondrial complex IV) [21-23]. Some studies state that the concentration of free H_2S in blood and tissues is only 14-15 nmol/L [8,24,25]. In aqueous solutions, H_2S dissociates into H^+, HS^- and S^{2-} with a pKa of 6.76 [21]. At physiological pH (7.4), such as in the blood and other physiological solutions, approximately 14% of the free sulphides are present as undissociated (gaseous) H_2S, more than 80% is present as HS^-, and the rest is S^{2-} (1). Similarly to O_2 and CO_2, undissociated H_2S is lipophilic and easily permeates plasma membranes [26,27]. The main sources of endogenous H_2S are the amino acids cysteine and methionine, which are present in food [1] and there are three pathways for its endogenous production [8,21,28]:

a. Desulfhydration of L-cysteine by cystathionine β-synthase (CBS, EC 4.2.1.22),

b. Desulfhydration of L-cysteine by cystathionine γ-lyase (CSE, EC 4.4.1.1), and

c. Transamination reaction between L-cysteine and α-ketobutyrate catalyzed in mitochondria by cysteine aminotransferase (identical with aspartate aminotransferase) to form aspartate and 3-mercaptopyruvate, followed by decomposition of the latter to pyruvate and H_2S by 3-mercaptopyruvate sulfurtransferase (3-MST).

The first two enzymes, CBS and CSE, determine amount H_2S production than 3-mercaptopyruvate sulfur transferase and are pyridoxal 5'-phosphate (vitamin B_6)-dependent enzymes. Additionally, they are found in the cytosol and act sequentially in the transsulfuration pathway to convert L-homocysteine to L-cysteine with L-cystathionine as the intermediate [8,29,30]. CBS catalyzes the reaction between homocysteine and serine to form cystathionine and H_2O, whereas CSE breaks down cystathionine to cysteine, ammonia and 2-ketobutyrate [8]. H_2S may be synthesized by these enzymes in alternative reactions [30,31]. In particular, in the reaction catalyzed by CBS serine may be replaced by cysteine with cystathionine and H_2S being the products. CSE may catalyze β-elimination of cysteine to pyruvate, H_2S and NH_4^+, γ-elimination of homocysteine to 2-ketobutyrate, H_2S and NH_4^+ and β- or γ-replacement reaction between two cysteine or two homocysteine molecules, with lanthionine or homolanthionine, respectively, as the co-products [30,31]. At physiological concentrations of these amino acids, about 70% of H_2S is synthesized from cysteine and the remaining 30% from homocysteine; the contribution of homocysteine increases in hyperhomocysteinemia [30]. The third, 3-MST-dependent pathway, was until now observed only *in vitro* in the nervous system [32] and in endothelial cells of some species (e.g. rat and human but not mouse) [33] and its contribution to overall H_2S formation is unknown [21]. The enzymatic mechanisms of H_2S production are shown in Figure 1.

The gene expression of CBS and CSE has been detected in various cell types, including the liver, kidney, lymphatic system, vascular wall, cardiomyocytes and fibroblasts. These enzymes contribute equally to the local production of H_2S in the liver and kidney [34]; however, one of the enzymes could be dominant in other organs [1]. The key enzyme for H_2S synthesis in the central and peripheral nervous system is CBS [6]. The source of H_2S in brain could also be the CAT/3-MST complex [35]. In contrast, there is a prevalence of CSE in cardiovascular system, although CSE expression is 24% higher in the myocardium in comparison to the thoracic aorta [36]. Relatively high concentration of CSE is observed in arteries, and H_2S is produced by both endothelial cells [37] and smooth muscle cells of the vessel wall [38]. The expression of CAT and 3-MST was also observed in the endothelium [33]. Although the concentration of free sulphides in the blood and other tissues/physiological solutions of mammals is very low (<100 nmol/l), it can be increased in the parts of the body where increased concentrations of H_2S synthesizing enzymes are present [39]. In specific intracellular spaces (microspaces), the concentration of free H_2S can be increased several fold, whereupon it immediately diffuses, binds or oxidizes. For example, a much higher concentration of H_2S (1 μmol/l) is observed in the aorta of mice. This concentration is 20-200 times higher in comparison with other tissues [40]. It is suggested that endogenously produced H_2S is rapidly oxidized to sulphates or incorporated into proteins [41]. In order to maintain *in vivo* H_2S concentrations, most likely, in the nM to low μM range, there are several enzymatic and non-enzymatic processes participate in H_2S catabolism (Figures 2-4) [17]. Even though all cell are able to oxidize H_2S, it is primarily degraded in liver [25,42] and mitochondria are very active site in sulphide oxidation [25]. Rhodanese, a mitochondrial sulfur transferase enzyme, catalyzes the oxidation of H_2S [43]. It is one part of three enzymatic activities characterized as a major pathway for H_2S catabolism. This pathway consists of a sulfide quinone oxido-reductase (SQR), a sulfur dioxygenase, and the sulfur transferase enzyme rhodanese (Figures 2 and 3) [17]. H_2S reduces the external disulfide on the SQR to form a thiol (RSH) and a perthiol (RSSH) [8,44]. This two electron oxidation of H_2S reduces the FAD prosthetic group, which uses ubiquinone (Q) as an electron acceptor [44].

The second sulphur atom on the perthiol is a reactive sulfane (S^0), which is oxidized by a sulfur dioxygenase enzyme (persulfide dioxygenase) encoded by the gene ETHE1, consuming O_2 and H_2O to form sulfite (SO_3^{2-}) [17]. While the protein responsible for this enzymatic activity is not known, the ETHE1 gene encoding the protein has been identified. Mutations in this gene cause a buildup of H_2S leading to ethylmalonic encephalopathy [45,46]. Rhodanese then transfers sulfane sulfur to sulfite to form thiosulfate ($S_2O_3^{2-}$) [47]. This proposed oxidation pathway, in close proximity to CcO, functions as a major clearance pathway of cellular H_2S.

In addition to liver and kidney, even though in healthy conditions, the amount of H_2S excreted by expiration is negligible, lung is also involved in oxidization of H_2S (Figure 4) [48]. H_2S can also be oxidized by non-mitochondrial heme proteins such as hemoglobin (Hb) and myoglobin [49]. H_2S will reduce the ferric iron in met-Hb, restoring the oxygen binding abilities of the protein [50]. At high concentrations of H_2S, sulf-Hb can also be formed from oxy-Hb [51]. While displaying very weak affinity for O_2, sulf-Hb can still deliver O_2, albeit with no cooperativity [52]. As a result, the bioavailability of H_2S, whether in the context of steady state *in vivo* concentrations or exogenously administered, is dictated by the O_2 concentration.

Therefore, O_2 can be considered an H_2S antagonist, accelerating its oxidation and attenuating its biological actions [17]. The effect of O_2 on H_2S concentration is both direct and indirect. The spontaneous reaction of H_2S with O_2, while slow, can cause an appreciable decrease in the H_2S concentration. Thus, tissues with relatively high O_2 concentrations (e.g. alveolar epithelium) may have less H_2S compared to tissues that are in a lower O_2 environment (e.g. centrilobular region of liver). This has implications in pathological states of hypoxia such as ischemia reperfusion, where the availability, and thus the signaling effects of H_2S may be augmented. Furthermore, O_2 concentration can indirectly affect H_2S concentration through changes in the redox state of heme proteins. Proteins such as Hb will react with H_2S at different rates depending on the redox status of the hemes. For example, H_2S will react more rapidly with met-Hb (Fe^{3+}) than with deoxy-Hb (Fe^{2+}) [50]. Because

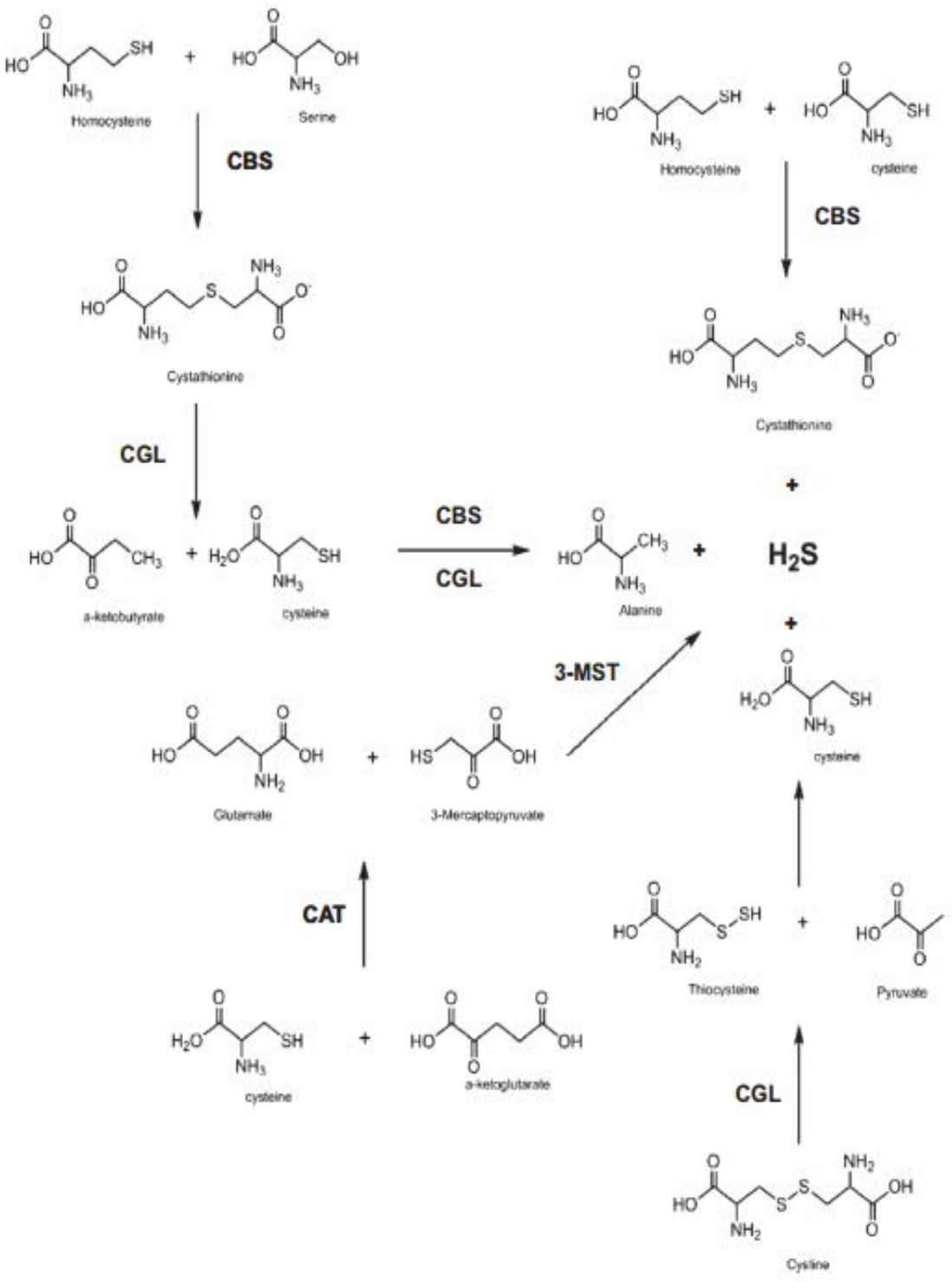

Figure 1: The enzymatic production of H_2S.
CBS catalyzes the first step in H_2S production through the transsulfuration of homocysteine to cystathionine. CGL in an elimination reaction catalyzes the formation of cysteine and α-ketobutyrate. Cysteine is the substrate from which H_2S is directly produced either through elimination (CGL) or β-replacement (CBS). Cysteine amino transferase (CAT) catalyzes the formation of 3-mercaptopyruvate, a substrate for the mitochondrial enzyme 3-mercaptopyruvate-S-transferase (3-MST). 3-MST can directly produce H_2S, albeit at lower levels than CBS and CGL, in mitochondria

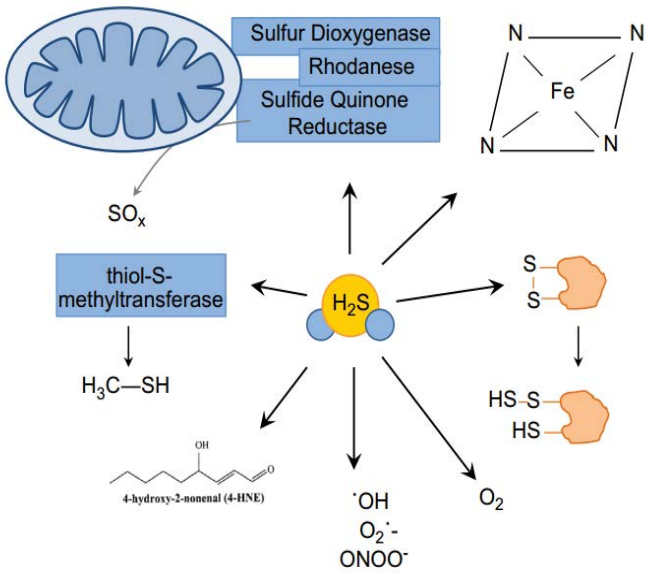

Figure 2: Proposed pathways of H_2S removal in mammalian cells.
The physiological steady-state concentration of H_2S *in vivo* is believed to be maintained in the submicromolar range. H_2S will react non-enzymatically with many biomolecules such as reactive oxygen and nitrogen species, electrophilic lipids like 4-hydroxy-2-nonenal, free heme and disulfide bonds to form a thiol and perthiol. The catabolism of H_2S can also be catalyzed enzymatically by the sulfide quinone oxido-reductase system (SQR) comprised by sulfur dioxygenase, rhodanese and sulfur quinone reductase

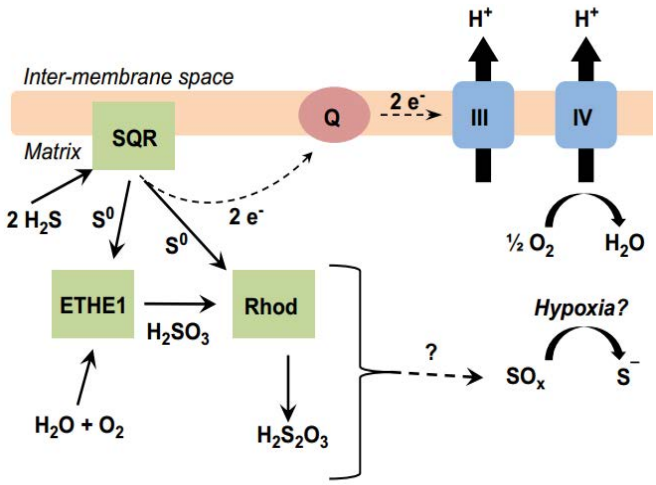

Figure 3: The oxidation of H_2S by the sulfide quinone oxido-reductase system in mitochondria.
H_2S reduces the disulfide composed of the vicinal thiols on the sulfide quinone reductase (SQR) forming a thiol and a perthiol. The second sulfur atom on the perthiol, the sulfane sulfur (S^0), is the substrate for both the sulfur transferase enzyme, rhodanese and the sulfur dioxygenase enzyme encoded by the gene ETHE1. Rhodanese catalyzes the formation of thiosulfate ($S_2O_3^{2-}$) from sulfite (SO_3^{2-}) and S^0. ETHE1 catalyzes the formation of SO_3^{2-}. The reduced SQR can then transfer electrons into the ubiquinone (Q) pool, thus coupling the oxidation of H_2S to electron transfer, H^+ pumping and ultimately ATP synthesis

H_2S is a nucleophile, it can also react with electrophilic lipids [53]; and the thiolate anion, HS^-, can also reduce disulfide bonds (Figure 2) [54]. Indeed, the exfoliation of skin cells in hot sulfur springs is due to H_2S reducing the structural disulfide bonds of cellular junctions in keratinocytes [16]. While this can be harmful at high concentrations, the reduction of external disulfide bonds by H_2S may, in some instances, reverse a deleterious posttranslational protein modification. Although still contentious, the S-sulfhydration of cysteine residues may represent an important sink for free H_2S [55]. In theory, H_2S can also reduce higher thiol oxidation states such as S-nitrosothiols and sulfenic acids [56]. H_2S can also be methylated by the cytosolic enzyme thiol-S methyltransferase to form methane thiol [17]. As with virtually all molecules, H_2S can react with other free radical species, as well as, a number of non-radical reactive oxygen (ROS) and nitrogen (RNS) species (Figure 2) [57]. Many of the oxidized sulfur species as well as sulfur-centered radicals formed are less reactive than their oxygen-containing counterparts [58]. One of the most important oxidants responsible for the catabolism of H_2S is O_2. In the presence of molecular O_2 and redox active metals, H_2S will spontaneously oxidize [59]. In an oxygenated biological medium, metalloproteins catalyze H_2S oxidation. This makes O_2 tension a critical methodological consideration when conducting biologically relevant experiments.

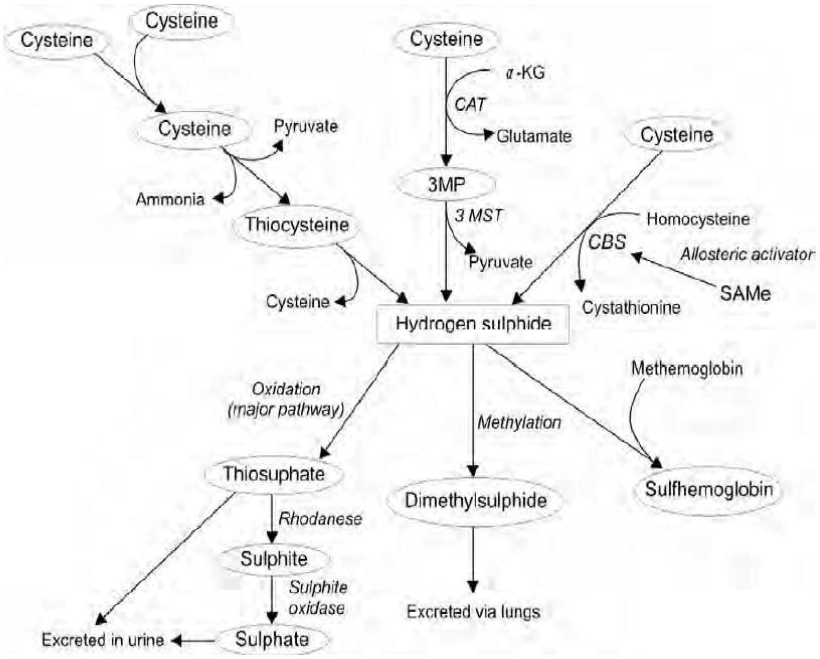

Figure 4: Metabolism of hydrogen sulfide.

Method of Detection of Endogenous H_2S Production

Through determination of physiological level of endogenous H_2S

The determination of the physiological concentrations of H_2S in circulation and in specific tissues is pivotal for determining the impact of H_2S on a given physiological function; correlating H_2S levels with the specific pathophysiological changes; examining physiological roles of H_2S under *in vitro* conditions at organ, tissue, and cellular levels; and guiding pharmacological and therapeutic administrations of H_2S donors not always the measurement of H_2S gives the consistent values [27]. The physiological range of H_2S in circulation has been estimated at 10-100 μM in health animals and humans [38,60-62]. 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) [63].

Rat serum contains 46 μM H_2S [38] and it is 34 μM in mouse serum [64]. In New Zealand rabbits, a quantitative assay detects a plasma H_2S level around 16.5 μM [65]. Plasma H_2S at micromolar ranges has also been reported in many other vertebrates [66]. Endogenous levels of H_2S in rat brain homogenates are 50-160 μM [6,67-69]. Similar H_2S levels were reported in the liver, kidney and pancreas [68-70]. H_2S production was clearly measured in the cardiovascular system [7,38]. Not always the measurement of H_2S gives the consistent values [27]. Using HPLC analysis, Sparatore et al. [71] reported a plasma sulfide level below 0.55 μM. Another study could not detect H_2S levels in lamprey, trout, mouse, rat, pig, and cow blood samples using a special house-made polarographic H_2S sensor that can detect 14 nM H_2S [39]. One explanation for these low values of H_2S is the rapid decay of H_2S concentration from micromolar concentration to undetectable level within 30 min *in vitro*. Whether H_2S would disappear that fast *in vivo* is unknown. Regardless, even 30 min would be far more than sufficient to regulate a specific physiological function [24]. A quick decay may actually indicate a homeostatic mechanism to trigger and to end H_2S signaling. Another related concern is the measurement technologies themselves. The real-time polarographic sensor was initially developed by Doeller et al. in 2005 [24]. Using the same kind of sensor, Benavides et al. [12] demonstrated that red blood cells produced H_2S. In two other studies using polarographic sensors, free H_2S concentrations in whole rat blood have been detected at ≥ 5 μM [72]. As the polarographic sensors are housemade in the study by Whitfield et al. [39], whether the failure to detect H_2S in animal blood was due to some intrinsic factors with the sensor itself cannot be commented on. Availability of these house-made sensors to other research teams would have helped replicate these results or allowed for a better comparison. The simultaneous employment of the polarographic sensor and other detection methods for H_2S detection would also help validate the actual blood levels of H_2S. Finally, in contrast to the sulfur ion selective electrode which detects total sulfur in the blood including its acid labile, bound or free H_2S forms, the polarographic sensor is sensitive only to freely dissolved H_2S gas [27]. It is possible that a significant amount of H_2S in circulation may not be in a free form as a dissolved gas, offering the rationale for the fact that our blood is not so smelly and the possibility that a polarographic sensor may potentially report a low value. A sensitive "nose" can smell "rotten eggs" in the blood if these eggs are broken, releasing free H_2S gas.

Whereas whether H_2S is a circulating gasotransmitter for both its generation and transportation is still being debated, the paracrine or autocrine effects of H_2S may nevertheless be more critical for regulating the functions of the cells, tissues, and organs where H_2S is produced in the proximity [27]. Using gas chromatography technique, Furne et al. [25] found very low tissue production of H_2S at nanomolar range in homogenized mouse brain and liver. An interesting comparison for this observation is that Hyspler et al. [61] also used gas chromatography-mass spectrometry (GC-MS) analysis and detected human whole blood H_2S levels at 35-80 μM. Even using a polarographic sensor, others have

detected significant tissue production of H_2S from the brain and liver [27]. The detection of the volatile gasotransmitter is already difficult to ascertain and what adds to the challenge is the fact that the safety zone to separate toxicological level and physiological level of H_2S is very narrow [27]. The toxic level of H_2S reported by Warenycia et al. [69] is less than twofold higher than its endogenous level in rat brain tissues. At the time of death of mice who exposed to NaHS (60 μg/g), the sulfide concentration in brain, liver, and kidney only elevated from the baseline by 57, 18 and 64%, respectively [73]. Comparison between healthy human subjects and age matched patients with COPD only told a 49.4% increase in serum levels of H_2S with stable COPD [63]. This percentage change translates to a H_2S concentration difference of <20 μM. This narrowness of the transition zone between physiological/biological and toxicological levels of H_2S can also be found in pharmacological studies where the dose-response relationship of H_2S is relatively steep before a given function change occurred and can quickly cause the opposite effect when H_2S concentration further increased [38]. As such, an ideal measurement method for detecting H_2S in mammalians should be sensitive, specific, accurate, noninvasive, on real-time and require a small quantity of samples. Many of the current H_2S measurement techniques, such as spectrophotometry, chromatography, and ion-selective electrode, were originally invented to meet the industrial demand for monitoring H_2S pollution in the environment [27]. These techniques are usually invasive and require a bulky quantity of samples. They also do not take account of the conditions for biological studies, such as the existence of H_2S scavenging molecules, interference of hemoglobins or other pigment compounds, redox balance, pH changes, etc.

Through usage of spectrophotometry

The use of spectrophotometry, also known as the methylene-blue method, to measure trace amounts of H_2S can be traced back to Fischer's study in 1883 for its principle [74] and to the work by Fogo and Popowsky in 1949 for the refining of the technique with the adaption of spectrophotometry [75]. This assay is based on the formation of the dye methylene blue when H_2S reacts with ferric chloride ($FeCl_3$) and *N*,*N*-dimethyl-*p*-phenylenediamine (NDPA). Absorbance of the dye in the reaction milieu can be detected by the spectrophotometer. The quantitative relationship (Beer's law) between H_2S concentration and the intensity of the transmitted monochromatic light can then be determined. The minimum detectable concentration of H_2S is determined by the sensitivity of the spectrophotometer to the optical density changes. Photoacoustic spectroscopy of H_2S converted to methylene blue has greater sensitivity than standard spectrophotometric methods. As the acidification is an important component of the methylene blue method, the incorporation of acid-labile sulfide may impact on the interpretation of the actual H_2S level [66]. For animal tissue samples or cells, the methylene blue method has been used often but usually is for detecting the H_2S generation capacity of the samples. In other words, the activity of H_2S-generation enzymes in term of H_2S production rate is assayed, rather than the absolute H_2S concentration. All variations in this application of the methylene blue method are derived from the original 1982 method of Stipanuk and Beck [76]. Tissue or cell samples are homogenized and incubated in a reaction mixture. The contents of the mixture are important because including L-cysteine is critical should CSE activity be assayed, but homocysteine should be a component if CBS activity is the goal to examine [27]. This first step is to generate H_2S from samples. Step 2 is to transform H_2S to methylene blue. The generated H_2S at 37°C is trapped with an alkaline zinc acetate solution in an apparatus. Zinc sulfide is formed, precipitated, and subsequently dissolved in a hydrochloric acid solution of *p*-aminodimethylaniline (*N*,*N*-dimethyl-*p*-phenylenediamine). In the presence of ferric chloride, methylene blue is formed. The emitted blue color can be stable for hours and measured at 670 or 650 nm [38,77]. This method can also be adapted to detect sulfate level in water or biological solutions by first reducing sulfate to H_2S with hydriodic and hypophosphorous acids [78].

The application of the methylene blue method to cell-free plasma or other cell-free biological fluids will detect the H_2S already existent, rather than to be generated, since H_2S generating enzymes are not in the fluid. Therefore, step 1 as described above to maximally activate H_2S-generating enzymes is no longer needed [27]. The fluid sample can be agitated by adding acid to release H_2S into the gas phase, which then interacts with zinc acetate and NPDA to form methylene blue [76]. Alternatively, the acid release of H_2S gas and trapping processes are omitted by directly adding NPDA and trichloracetic acid (TCA) to the plasma to directly form methylene blue [79]. ForH_2S in air samples, the methylene blue method can be modified to use an alkaline solution of cadmium hydroxide to absorb H_2S [80].

Through usage of nanotube-based sensors

Electrochemical detection is the most commonly used technology incorporated in compact and portable H_2S gas monitors [81]. The principle behind it is the conductivity changes of thin films upon exposure to H_2S gas. Relying on solid state sensors made of semiconducting metal oxides or metals, these portable apparatuses are expensive and suitable for industry utilization. Their drawbacks include high power consumption as found in metal oxide sensors that require high operating temperatures, low sensitivity, short lifetime of often less than 1 year and interference by other gases, such as NH_3 and NOx [82]. More popular electrochemical sensors nowadays are based on one-dimensional nanostructures such as bare or functionalized semiconducting single-walled carbon nanotubes (SWNTs) [83,84], metal oxides, and conducting polymer nanowires [85,86]. Potentially, these sensors may be used to monitor gases with high sensitivity, low sample volume requirement, low power consumption and low cost [82]. A catalytic chemiluminescence sensor made of R-Fe_2O_3 nanotubes has been developed, which can specifically detect H_2S gas as low as at 10 ppm. The problem with this sensor is that high temperature over 110°C is required for catalytic oxidation of H_2S to occur. It is also not suitable for measuring H_2S in liquid [87]. Other sensors based on SnO_2 nanowire [88], In_2O_3 nanowire [89] and ZnO nanowires [90] with increased sensitivity have been reported. The challenges with these one-dimension structures are the difficulties in making the nanostructures and in obtaining large quantities as well as their application under *in vivo* physiological conditions. CuO-SnO_2 and $ZnSb_2O_6$ have been shown to detect H_2S at concentrations below 1 ppm at 300°C [91]. Using single-wall carbon nanotubes (SWNT) [92] as an H_2S sensor as well as an H_2S carrier has attracted a great attention in recent years. This is because of the adsorption of H_2S by activated carbon and the realization of the structural advantages of the carbon nanotubes, which are the uniform pore size distribution, high surface area, and excellent electronic properties. High surface area will result in an increased amount of irreversibly adsorbed H_2S. The activated carbon facilitates H_2S reaction with oxygen at low temperatures, leading to the production of sulfur and water [93]. SWNT-based H_2S biosensor will also potentially reduce the sample volume to nanoscale. The initial attempt of using multi-wall carbon nanotubes to measure H_2S in solution was made by Wu et al. [94]. After carbon nanotubes are immersed in a H_2S solution, on the contact interface between carbon nanotubes and H_2S solution formed is a thin water film. Oxygen molecule is also dissolved in the film and

adsorbed by the carbon nanotubes. Carbon nanotubes also absorb H_2S by the van der Waals force. The interaction of H_2S (hydrosulfide ions and protons) and oxygen on the nanotubes forms hydroxyl ions and sulfur. The protons neutralize the hydroxyl ions and produce water. But the spectra of fluorescence of sulfur on carbon nanotubes can be assayed with either a Raman or a confocal laser scanning microscope [95]. It was found that fluorescence intensity was increased, closely correlated with the increased concentrations of H_2S in the solution. In this preliminary study, 10 μM H_2S in water was successfully measured [94]. To take one step further toward the biological application of the carbon nanotube-based H_2S biosensor, Wu et al. [96] applied this carbon nanotube fluorescence technique to measure H_2S level in serum and reported that the binding of H_2S to nanotubes was not affected by the presence of proteins in rat serum. After removing endogenous H_2S in the serum with hemoglobin, exogenous H_2S added to the serum was successfully detected with a linear relationship between H_2S concentrations (20, 50 and 100 μM) and fluorescence intensities. The mechanism for using carbon nanotubes to detect H_2S, even in the presence of proteins, is believed to be due to a continuous serum albumin film formed on the surface of carbon nanotubes. Other proteins or large molecules cannot pass the albumin film, but H_2S can easily move and pass through this film to the surface of carbon nanotubes. What Wu and co-workers [94-96] did is the combination of carbon nanotube adsorption with the fluorescence emission detection, a chemical approach. A different strategy by detecting the conductance change of carbon nanotubes after binding with H_2S was taken, an electrical approach [82].

The principle for this strategy is to conduct site-specific electrodeposition of gold nanoparticles on SWNT networks. The adsorption of H_2S molecules at different concentrations onto the gold nanoparticle surface can change the carbon nanotube conductivity to different degrees. The researcher reported superior sensitivity of these nanostructures toward H_2S at room temperature with a detection limit of 3 ppb. The application of these nanostructures for detecting H_2S in liquid preparation and biological samples has not been reported.

Through usage of sulfur ion-specific electrodes

Sulfur ion-specific electrodes have been frequently used in detecting H_2S level in blood and cell culture media. The method is easy to operate, and the initial setup is of low cost. Typically, the ion-specific electrode has a linear response range of between 0.1M and 10 μM and a detection limit on the order of 1-10μM. The observed detection limit is often affected by the presence of other interfering ions or impurities. With a modified sulfide-specific electrode, Searcy and Peterson [97] reported measurement of very low free sulfide concentration (0.5 μM). This measurement was done with continuous injection of Na_2S solution into the sample chamber to maintain a constant concentration. Its application to biological fluids close to physiological conditions is not clear. Sulfur ion-specific electrodes are sensitive only to S^{2-} and as such, free H_2S needs to be fully dissociated. This can be achieved under a strong alkali conditions and with a complete lack of oxidation [98]. For both blood (whole blood, serum or plasma) and cell culture media, this alkali and antioxidant condition might cause protein desulfuration and the electrodes may detect S^{2-} dissociated from H_2S and released from proteins. Furthermore, using the electrodes still requires bulky samples and is an off-line measurement [27].

Through usage of polarographic H_2S sensors

A novel polarographic H_2S sensor (PHSS) was developed in 2005 as a voltammetry, which is a method of determining the chemical makeup of an H_2S permeable polymer membrane by measuring electrical activity, or the accumulation of chemicals, on electrodes placed in the substance [24]. The application of PHSS has been reported at cellular, tissue and organ levels with the claimed high sensitivity at the nanomolar range and rapid response time to H_2S. Real-time measurement of the levels of H_2S and O_2 in respirometry and vessel tension experiments with PHSS has been achieved [72].

Most of PHSS have the dimensions similar to that of the polarographic oxygen sensor. Recent advance sees the availability of the miniature PHSS for real-time measurement of H_2S production in biological samples. It was reported that the miniature PHSS detected H_2S production by brain supernatants at ~10.6 pmol·s^{-1}·mg protein^{-1} [99], which is significantly higher than that in vascular tissues (0.5-1.1 pmol·s^{-1}·mg protein^{-1}) [7,38]. Just like the real-time polarographic sensors for other gas molecules (O_2, NO or CO), however, to have consistent and reliable reading of H_2S level with commercially available PHSS is more often than not a daunting challenge and a frustrating experience [27]. Because the polarographic sensor only measures H_2S gas, sulfide (HS^- and S^{2-}) is estimated indirectly from pH.

Through chromatography analysis

Chromatography includes gas chromatography, liquid chromatography, ion-exchange chromatography, affinity chromatography, and their variations such as HPLC (high-performance liquid chromatography or high-pressure liquid chromatography). The readers are referred to a thorough review by Ubuka [100]

, which detailed the application of chromatograph technology in H_2S detection. In short, liquid chromatographic determination of sulfide with or without derivatization and ion chromatography of sulfide have been conducted. HPLC analyses of sulfide after conversion to methylene blue, to thionine, or to the monobromobimane derivative or after labeling with *o*-phthalaldehyde (OPA) have been reported. Gas chromatography has also been employed to analyze sulphur compounds in air, aqueous, and biological samples [100]. For example, the measurement of H_2S in air by ion chromatography has the working range of 20-500 μM for a 20 l air sample [101]. Gas chromotography-mass spectrometry has been used to detect H_2S in animal tissues based on the amount of trapped S^{2-} after acidification of H_2S [25,61]. Reverse-phase (RP)-HPLC for the determination of H_2S-derived methylene blue was used in measuring the sulfide content in brain, liver, and kidney from sulfide-treated mice. After exposure of mice to 60 μg/g Na_2S, tissue contents of H_2S were all significantly increased [73]. Shen et al. [102] reported in 2011 a novel and sensitive method to detect physiological levels of free H_2S in cell lysates, tissue homogenates, and body fluids.

This method is built on the rapid reaction of monobromobimane with H_2S under basic conditions at room temperature to produce sulfide-dibimane (SDB). SDB is stable, in which it favors over the unstable H_2S for biological assays. SDB is also more hydrophobic than most physiological thiols. RP-HPLC can separate SDB with a gradient elution and then analyze it by fluorescent detection. The sensitivity of this SDB-based RPHPLC analysis reaches the H_2S level as low as 5 nM, which is in sharp contrast to the methylene blue-based spectrophotometry method which has a low limit of 2 μM [102]. When the SDB-based method was applied to wild-type mice, heterozygous CSE knock-out (CSE HT) mice, and homozygous CSE KO mice, clear differentiation in plasma level of H_2S was achieved. CSE HT mice have lower plasma level of H_2S than that of wild-type mice, but higher level than that of CSE KO mice [102]. Sensitive and selective detection of H_2S has been one of the hot spots as well as one of the bottlenecks in H_2S study. New methodologies are being continuously devised and reported

and the existing methods improved and adapted to new applications [27]. The quick oxidation and scavenging of H_2S in biological samples are the biggest challenges for accurate and rapid measurement of H_2S levels. At this moment, the spectrophotometry- based method is still of the choice to determine tissue or cell production of H_2S, whereas sulfur ion-specific electrodes and polarographic H_2S sensors hold potential for real time measurement of H_2S net levels in blood or other body fluids. For analyzing H_2S in air samples, such as exhaled air from lungs, chromatography analysis of H_2S would be more suitable. Furthermore, the fluorescence-based quantitative or semi-quantitative methods would be useful for detecting H_2S production in specific cellular organelles.

Biological Roles of H_2S and Its Effect on Ion Channels

Ion channels are pore-forming membrane proteins that help establish and control the small voltage gradient across plasma membrane of cell or intracellular organelle membranes. These channels, individually or collectively, participate in the regulation of cell differentiation, muscle contractility, neurotransmitter release, or hormone secretion. Like NO and CO, H_2S can easily diffuse without the need for transporters and has diverse biological actions by interacting with various channels (Figure 5).

Important factors that determine the biological actions of H_2S include, but are not limited to, differences in the solubility of H_2S in aqueous vs. lipid phases, proximity of the target to H_2S detoxifying enzymes, heme redox state, and inter- and intra-cellular differences in O_2 tension [17]. H_2S plays a role in many physiological processes. However, high O_2 can reverse many of the beneficial roles of H_2S seen at lower O_2 concentrations, resulting in, for example, vasoconstriction rather than vasodilation. Additionally, under hypoxic and normoxic conditions, H_2S promotes angiogenesis. However, at higher concentrations of both O_2 and H_2S, an inhibition of cellular proliferation is seen. H_2S has a narrow therapeutic window within which it is cytoprotective. At high concentrations it can be pro-apoptotic and pro-inflammatory. Finally, the larger doses of H_2S necessary to induce a hypometabolic effect, can, if pushed further, result in cardiac and respiratory toxicity.

H_2S and ATP-sensitive k^+ (k_{ATP}) channels

ATP-sensitive K^+ (K_{ATP}) channels are composed of pore forming subunits (Kir6.x) and sulfonylurea receptor (SUR) subunits that couple cellular electrical activity to metabolism in a variety of tissues. Hydrogen sulfide is an endogenous opener of K_{ATP} channels in many different types of cells. However, the molecular mechanism for an interaction between H_2S and K_{ATP} channel proteins remains unclear. The whole-cell patch-clamp technique and mutagenesis approach were used to examine the effects of H_2S on different K_{ATP} channel subunits, rvKir6.1 and rvSUR1, heterologous expressed in HEK-293 cells.

H_2S stimulated co-expressed rvKir6.1/rvSUR1 K_{ATP} channels, but had no effect on K_{ATP} currents generated by rvKir6.1 expression alone. Intracellularly applied sulfhydryl alkylating agent (N-ethylmaleimide, NEM), oxidizing agent (chloramine T, CLT) and a disulfide bond-oxidizing enzyme (protein disulfide isomerase) did not alter H_2S effects on this recombinant channels. CLT, but not NEM, inhibited basal rvKir6.1/rvSUR1 currents, and both abolished the stimulatory effects of H_2S on K_{ATP} currents, when applied extracellularly. After selective cysteine residues (C6S and C26S but not C1051S and C1057S) in the extracellular loop of rvSUR1 subunits were point-mutated, H_2S lost its stimulatory effects on rvKir6.1/rvSUR1 currents [103]. By targeting K_{ATP} channels, H_2S regulates the processes of inflammation, nociception, pain, and cell death and exerts its beneficial protective effects against ischemia damage, hypertension, inflammation, nociceptiveness and apoptosis, etc. [27]. Extensive experiments on vascular tissues strongly suggest that H_2S-induced vasorelaxation is mainly caused by opening ATP-sensitive potassium channels (K_{ATP}) on the vascular smooth muscle

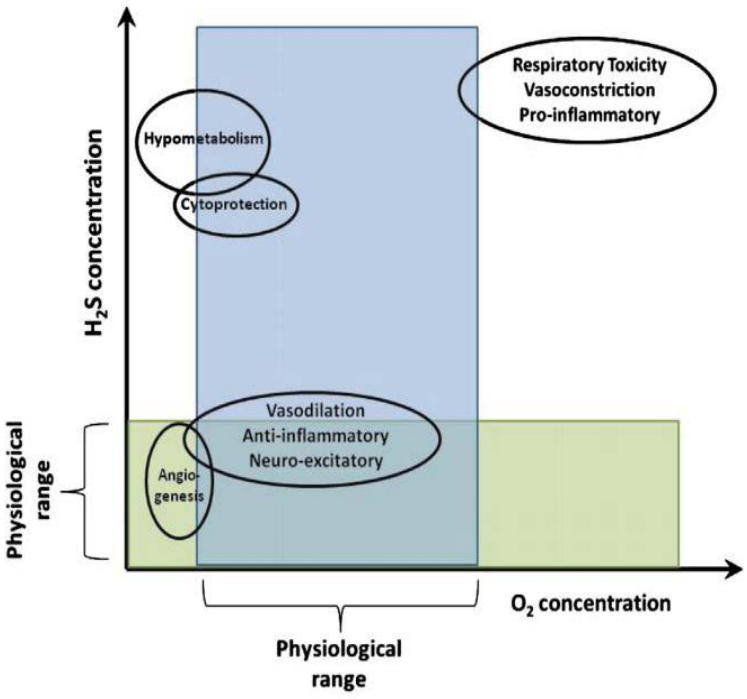

Figure 5: Interaction of O_2 and H_2S on physiological outcomes.

cells [38,104,105]. In isolated piglet cerebral arteriole SMCs, a recent study showed that H_2S activated K_{ATP} channels at physiological steady state voltage (-50 mV), which was antagonized by glibenclamide [106]. Electrophysiological study provides direct evidence that exogenous H_2S increases macroscopic or unitary K_{ATP} currents, which is blocked by glibenclamide in isolated rat aortic and mesenteric SMCs [38,107]. Zhao et al. confirmed an important role of K_{ATP} channels in high-dose H_2S-induced vasorelaxation in isolated rat aortas [38]. Consistent with the role of K_{ATP} channels in mediating the effects of H_2S, reduced endogenous synthesis of H_2S decreased K_{ATP} channel activity [1]. Moreover, exogenous H_2S administration activated K_{ATP} channels and hyperpolarized the membrane of vascular smooth muscle cells isolated from rat mesenteric arteries [107]. H_2S-induced hyperpolarization of SMC membrane is also abolished by glibenclamide. The opening of K_{ATP} channels in myocardium has been seen to play a pivotal role in cardioprotection during I/R injury, which is specifically seen in cardiac ischemic preconditioning [108]. It was observed that in the perfused rat heart preparation, NaHS concentration-dependently limited the size of infarction induced by left coronary artery ligation, and this protective effect was abolished by K_{ATP} channel blockers glibenclamide and 5-hydroxydecanoate [106]. Reperfusion of the isolated Langendorff-perfused heart with NaHS after ischemia attenuated arrhythmias and improved cardiac function during I/R.

These effects of NaHS were blocked by glibenclamide, which suggests that H_2S produces a cardioprotective effect against I/R injury during reperfusion, at least in part by opening K_{ATP} channels [109]. The patch-clamp data provide additional electrophysiological evidence that convincingly shows the effect of H_2S on K_{ATP} channels. Exposure of single cardiac myocytes to NaHS increased single-channel activity of K_{ATP} channels by increasing the open probability of these channels without altering single-channel conductance [110]. This increase in the open probability can be blocked by glibenclamide. In the heart, H_2S and its donors cause the negative inotropic and chronotropic action through activating sarcK_{ATP} and mitoK_{ATP} channels. The cardioprotective effect of H_2S involves not only by the opening of K_{ATP} channels, but also though activation of cardiac ERK and/or Akt pathways in addition to preserving mitochondrial structure and function [111,112]. H_2S-induced neuroprotection and suppression of glutamate toxicity was also partially mediated by the activation of K_{ATP} channels. Glibenclamide and glipizide dose-dependently suppress H_2S-induced protection of HT22 cells from oxidative stress. Neuroprotection was increased by the simultaneous application of H_2S and pinacidil or the combined application of cysteine and pinacidil. While all these results support the involvement of plasma membrane K_{ATP} channels in the effects of H_2S, opening (with diazoxide) or blocking (with 5-hydroxydecanate, 5-HT) of mitochondrial K_{ATP} (mitoK_{ATP}) channels did not modulate protection by H_2S [113,114]. Distrutti et al. [115] have demonstrated that the systemic administration of different H_2S donors inhibits visceral nociception by opening K_{ATP} channels. The activation of K_{ATP} channels in the peripheral nociceptive system has been seen to be involved in the modulation of nociception [116]. For instance, peripheral antinociceptive drugs that directly block ongoing hypernociception induced by PGE_2, such as morphine and dipyrone, exert their effects by opening K_{ATP} channels stimulated by the NO-cGMP anti-nociceptive pathway [116]. Cunha et al. [117] tested the hypothesis that the anti-nociceptive effect of H_2S on direct hypernociception induced by PGE_2 is dependent on K_{ATP} channels in the periphery. Supporting this hypothesis, glibenclamide prevented the anti-nociceptive effect of exogenous H_2S in rodents. A possible direct hypernociceptive effect of glibenclamide was excluded, as glibenclamide administration alone in the rat paw did not produce mechanical hypernociception [118]. Local administration of a K_{ATP} channel opener also directly blocks hypernociception induced by PGE_2, which further supports the findings.

Electrophysiologically, it has been shown that K_{ATP} channel activation reduces the enhanced excitability of rat nociceptive sensory neurons induced by PGE_2 [117]. A key event in inflammation is the recruitment of circulating leukocytes into the damaged tissue. Andruski et al. [119] used intravital fluorescence microscopy to look at leukocyte behavior in an intact rodent knee joint and later surmised that local treatment of acutely inflamed knee joints with an H_2S donor limited leukocyte recruitment and trafficking and decreased synovial blood flow. These anti-inflammatory effects of H_2S were mediated via the K_{ATP} channel because responses could be blocked by glibenclamide treatment. Intra-articular administration of NaHS had no effect on joint pain sensation or secondary allodynia in the rat, although this observation needs to be corroborated in other animal species. Thus it is conceivable that H_2S may function as an endogenous regulator of joint function and that its action is distinctly anti-inflammatory [119]. However, exogenously administered H_2S acts on sensitive neurons and promotes the opening of K_{ATP} channels and subsequent antinociception [117]. The effects of H_2S on K_{ATP} channels also exert influence on pain cognizance. Research has clarified that parenteral administration of either NaHS or an H_2S-releasing derivative of mesalamine inhibited dose-dependently visceral nociception in a colorectal distension (CRD) model in the rat. Administration of L-cysteine also reduced rectal sensitivity to CRD. The inhibitory effect of NaHS on CRD-induced pain or antinociception was completely reversed by pretreating rats with glibenclamide [115]. Also, glibenclamide inhibited colonic smooth muscle relaxation induced by the highest dose of NaHS. The antinociceptive and muscle relaxant effects of NaHS were mimicked by pinacidil. These results show that H_2S functions as a negative regulator of visceral nociception by activating K_{ATP} channels and attenuating pain. NaHS-induced antinociceptive effects are not dependent on the activity of capsaicin-sensitive pathways that can induce smooth muscle contraction [120], although CRD-induced pain is closely related to increased contractility of colorectal smooth muscles. NaHS induced antinociception only at relatively low doses, but caused intestinal smooth muscle relaxation at high doses. Due to the crucial role of K_{ATP} channels in the regulation of pancreatic insulin secretion, multiple studies have examined the effect of H_2S on-cells. K_{ATP} currents were limited after lowering endogenous H_2S level in INS-1E cells, derived from rat insulinoma cell line, by CSE-targeted short interfering mRNA transfection, which was blocked by gliclazide and stimulated by diazoxide [121].

Endogenously produced H_2S by overexpression of the CSE gene significantly aggrandized whole cell K_{ATP} currents in INS-1E cells. Exogenous H_2S markedly increased the open probability of single K_{ATP} channels by twofold in inside-out patches, but single-channel conductance and ATP sensitivity of K_{ATP} channels were not changed by H_2S [121].

H_2S and Ca^{2+}-sensitive K (K_{ca}) channels

Other than K_{ATP}, small, intermediate, and large conductance calcium-dependent potassium channels (SK_{Ca}, IK_{Ca} and BK_{Ca}) have also been demonstrated as possible mediators of H_2S vasodilator effects in resistance vessels [122,123]. It has been observed that H_2S-induced vasorelaxation of rat aortic ring was not affected by iberiotoxin or charybdotoxin. This observation suggests that big-conductance Ca^{2+}-sensitive K (BK_{Ca}) channels might not be responsible for the H_2S-induced vasorelaxation in conduit vessels [124]. Both H_2S and NaHS evoked concentration-dependent relaxation of *in vitro* perfused

rat mesenteric artery beds (MAB) [125]. The vascular effects of H_2S on MAB were related to the stimulation of charybdotoxin/apamin sensitive K^+ channels in the vascular endothelium, in addition to the activation of K_{ATP} channels in vascular SMCs. Similarly, a combination of charybdotoxin and apamin abrogates the vasorelaxant effect of H_2S in the endothelium intact rat aorta. These data suggest that small to medium conductance K_{Ca} channel (SK_{Ca} and IK_{Ca}) in MAB and aorta is activated by H_2S. Therefore, H_2S might fulfill the role of EDHF [115]. The stimulation of SK_{Ca} and IK_{Ca} channels by H_2S was also indirectly demonstrated in isolated rat mesenteric arteries as well as in isolated vascular endothelial cells, based on the changes in membrane potential [122]. One recent patch-clamp study showed that NaHS arrested heterologously expressed BK_{Ca} channels in HEK-293 cells transfected stably with human BK_{Ca} channel –subunits [126,127]. NaHS decreased the open probability and shifted the BK_{Ca}-channel activation curve rightward without altering its conductance, suggesting that the inhibitory action of H_2S on BK_{Ca}-channel. The same conclusion of H_2S-induced inhibition of BK_{Ca} channels was drawn in type I glomus cells of mouse carotid body [128]. In sharp contrast, a recent report showed that NaHS augments whole cell BK_{Ca} currents and enhances single-channel BK_{Ca} activity in rat pituitary tumor cells (GH3) by increasing channel open probability [129]. The above three patch-clamp studies used NaHS at the same concentration range (~300μM), but the conclusions are opposite.

No explanation has been given, but it might be related to specific BK_{Ca} channel subtypes in different types of cells [130]. Another study by Jackson-Weaver et al. [131] examined the myogenic tone of rat mesenteric arteries and cerebral arteries as well as the membrane potential of vascular SMCs. Although the authors did not directly record changes in K_{Ca} channel currents, their results nevertheless showed that exogenous H_2S dilated and hyperpolarized rat arteries and that these effects of H_2S were blocked by iberiotoxin and paxillin. Thus the stimulation of iberiotoxin sensitive BK_{Ca} channels by H_2S is suggested [131].

H_2S and chloride (Cl^-) channels

The ATP-binding cassette superfamily includes cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels and sulfonylurea receptors, which are components of K_{ATP} channels. Both subunits also share key sequence homologies [27]. The Cl^- channel blockers 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and indyanyl oxyacetic acid (IAA-9) suppress protection by H_2S, while levamisole, which is an opener of Cl^- channels, competently stops glutamate toxicity [113]. This research purports that CFTR Cl^- channels may also be involved in protection by H_2S against oxidative stress. The recent findings that a decrease in transmembrane Cl^- gradients causes cell death in hippocampal pyramidal neurons and that the expression of CFTR gene is reduced in the hypothalamus of patients with AD [132] suggest that homeostasis of transmembrane Cl^- gradients is required for normal cell survival. Subsequently, the effect of H_2S on Cl^- channels in the CNS has been studied. In the research, H_2S was seen to activate CFTR Cl^- channels in HT22 neuronal cell lines which led to neuroprotection during oxytosis. This was demonstrated through dose-dependent suppression of neuroprotection due to H_2S using specific CFTR blockers, NPPB and IAA-94, and confirmed using CFTR activator levamisole [113]. Together with the recent observation of H_2S activating Cl^-/HCO_3^- transporters in smooth muscle cells [133], the results suggest possible regulation of Cl^- fluxes by H_2S in the CNS with neuroprotective consequences. The regulation of inhibitory Cl^- currents coincides with the regulation of inhibitory K^+ channels and therefore strongly purports a key role for H_2S in modulating excitability [130].

H_2S and calcium (Ca^{2+}) channels

It is well recognized that voltage-activated Ca^{2+} channels (VDCC) regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and consequently impact Ca^{2+} signaling in excitable cells. Ca^{2+} channels are classified, based on their electrophysiological features, as high voltage-activated (HVA) and low voltage activated (LVA) types. The former include L-, N-, P-/Q- and R-type channels, and the latter are actually T-type channels [27]. In addition to Ca^{2+} channels in the membrane, $[Ca^{2+}]_i$ is controlled by intracellular Ca^{2+} stores. $[Ca^{2+}]_i$ changes due to extracellular Ca^{2+} entry may be facilitated by VDCC, transmitter-gated Ca^{2+}-permeant ion channels, transient receptor potential (TRP) ion channels, and Ca^{2+} pumps located in the plasma membrane [134]. Channels that affect intracellular Ca^{2+} stores include ryanodine receptor (RyR) channels, inositol trisphosphate receptor (IP_3R) channels and sarcoendoplasmic reticular Ca^{2+} ATPases (SERCA) [134].

H_2S and l-type voltage-activated ca^{2+} channels (L-type VDCC)

Voltage-activated Ca^{2+} channels (Cav) are expressed at high density in excitable cells, mostly in neurons, cardiac conduction system and smooth muscles. H_2S modulates cardiovascular homeostasis and exerts cardioprotective effects in different models of *in vitro*, *ex vivo* and *in vivo* ischemia/reperfusion [135-140]. Indeed, whole patch clamp experiments in rat cardiomyocytes revealed that NaHS negatively modulates L-type Ca^{2+} channels composed by the $Ca_V1.2$ subunits [141,142]. More specifically, NaHS (up to 1 mM) causes a dose dependent reduction in the Ca^{2+} current peak. This effect is only partial: the current density diminishes by 50% at 1 mM NaHS [143]. The mechanism could involve a direct modification of Ca_v free sulfhydryl groups (143). The H_2S donor also affects the recovery from depolarization induced inactivation, without altering the steady state activation and inactivation curves. Accordingly, the shortening of single cardiomyocytes and contraction of isolated rat papillary muscles are depressed. Electric field-induced Ca_i transients in single cardiomyocytes are also reduced by 100 M NaHS [141,142]. Consistently, H_2S exerts a negative inotropic effect in isolated perfused rat and papillary muscles when NaHS is administrated at concentrations ranging from 1 μM up to 1 mM [36,144]. More recently, it has been reported its negative chronotropic action in human atrial fibers by blocking L-type Ca^{2+} channels and an enhancement in the repolarization phase by opening KATP channels (50–200 M μNaHS) [145].

Interestingly, according to a recent study, H_2S can reverse the negative inotropic effect induced by NO by causing an increase in the peak amplitude of the electrically stimulated Ca_i transients [140]. These apparently discrepant data may be reconciled when considering that, under such conditions; the modulation of the Ca_i toolkit responsible for the positive inotropic effect is not accomplished by H_2S, but by a new thiol-sensitive endogenous modulator deriving from the interaction between the two gasotransmitters [140]. Interestingly, in this report, H_2S was provided by NaHS at low micromolar doses (10 μM). The negative effect of H_2S on Ca^{2+} influx is not limited to the cardiovascular system. Similarly to rat cardiomyocytes, 100 μM NaHS suppresses voltage-gated Ca^{2+} currents in INS-1E cells (rat insulinoma cell line) and native pancreatic beta-cells: these currents are sensitive to both nifedipine and Bay K-8664, a pharmacological profile consistent with L-type Ca^{2+} channels [130]. On the other hand the effects of NaHS on neurons, that can express both $Ca_V1.2$ and $Ca_V1.3$ subtypes, seem to be opposite [146]. In cultured rat cerebellar granule neurons (CGN), NaHS (50-300 M) induces cell death as well as Cai signals sensitive to nifedipine and nimodipine, L-type Ca^{2+} channel blockers [130].

However, no electrophysiological recordings were conducted and a direct activation of L-type Ca^{2+} channels by NaHS remains to be demonstrated yet. Moreover, there is no evidence about the molecular nature (i.e. $Ca_V1.2$ or $Ca_V1.3$) of L-type channels in these cells. Taken together, these evidences suggest that L-type Ca^{2+} channels are inhibited by H_2S in the myocardium, whereas they are enhanced by the same H_2S doses in the CNS. Future investigations will unveil whether this feature depends on the different molecular make-up of L-type channels, i.e., $Ca_V1.2$ in ventricular cardiomyocytes vs. $Ca_V1.3$ in the cerebellum, or on their associated subunits. Alternatively, an intermediate sensor coupled to the channel complex, whose nature varies between the heart and the CNS, might mediate the regulation of L-type Ca^{2+} channels by H_2S. NaHS increases Ca_i also in astrocytes, hippocampal slices and microglia, through currents sensitive to Ca^{2+} channel inhibitors (La^{3+} and Gd^{3+}) and in a concentration range (100–500 M) similar to that affecting VOCs [130,147]. It appears that H_2S-triggered Ca_i waves are due to influx through Ca^{2+} channels on plasma membrane and, to a lesser extent, to the release from intracellular Ca^{2+} stores [130,147].

In contrast, a recent report showed that NaHS-induced Ca_i increase in isolated rat colonic crypts was not dependent on extracellular Ca^{2+}, but was affected by blockade of either ryanodine receptors (RyRs) or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [148]. T-type Ca^{2+} channels are encoded by the three members of the Ca_V3 subfamily and display different biophysical and pharmacological features as compared to L-type Ca^{2+} channels: activation at lower membrane potentials, faster inactivation, slower deactivation, smaller permeability to Ba^{2+}, insensitivity to dihydropyridines and block by $ZnCl_2$ [149]. T-type Ca^{2+} currents are involved in a great number of physiological processes, such as neuronal firing, hormone secretion, smooth muscle contraction, myoblast fusion, and fertilization [149]. Moreover, they play critical roles in mediating either somatic or visceral nociceptive information. Similarly to capsaicin, NaHS, injected intracolonically at 0.5-5 nM per mouse, triggers visceral nociceptive responses *in vivo*, which are completely abolished by mibefradil, an unspecific T-type channel blocker, and insensitive to verapamil and to the K_{ATP} channel blocker glibenclamide [130]. Therefore, H_2S may function as a novel nociceptive messenger through the activation of peripheral T-type Ca^{2+} channels, particularly during inflammatory processes. However, since mibefradil is not selective for T-type channels, this conclusion should be confirmed by future investigations [150]. Furthermore, both intraplantar (1 nM/paw) and intratechal (0.01-0.1 nM/animal) administration of NaHS caused a prompt hyperalgesia in rats, an effect that was abolished by mibefradil, $ZnCl_2$ or antisense oligodeoxynucleotides (ODNs) selectively targeting rat $Ca_V3.2$ [151-153]. The finding that DL propargylglycine (PPG) and -cyanoalanine, two CSE inhibitors, abolish the l-cysteine-induced hyperalgesia and attenuate the lipopolysaccharide-induced hyperalgesia, an effect reversed by NaHS, supports these observations [151,152]. Moreover, mibefradil suppressed the phosphorylation of ERK induced by the infusion of NaHS, a pronociceptive stimulus in the pancreatic duct, albeit at higher concentrations than those reported above (500 nM/rat) [154]. Finally, the neuropathic allodynia/hyperalgesia induced in rats by damaging the right L5 spinal nerve [155] or by systemic injection of paclitaxel [156], an anticancer drug, was strongly attenuated by either mibefradil or CSE inhibitors, or by antisense ODNs against rat $Ca_V3.2$. In addition, $Ca_V3.2$ was significantly up-regulated in the ipsilateral L4, L5 and L6 dorsal root ganglia of rats subjected to spinal nerve injury, but not treated with paclitaxel [155].

A redox modulation of $Ca_V3.2$ has been proposed, since NaHS increases the amplitude of T-type Ca^{2+} currents in a neuroblastoma cell line without affecting their kinetics. This effect was reversed by the oxidizing agent, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and mimicked by the reducing compound, dithiothreitol (DTT) [152]. It should be pointed out that the elevation in the density of T-type Ca^{2+} currents was observed at 0.5-1.5 mM NaHS. The enhancement of T-type Ca^{2+} current by the exogenous application of H2S, in turn, induces neuronal differentiation, as revealed by neurite outgrowth and functional expression of high voltage-activated Ca^{2+} currents, including L-, P/Q- and N type channels [157]. Once again, these effects arose when NaHS was administrated at 1.5-13.5 mM. Interestingly, earlier reports demonstrated that L-cysteine selectively potentiates recombinant $Ca_V3.2$-dependent, but not $Ca_V3.1$- and $Ca_V3.3$-, currents [158]. A mechanistic link between H_2S and the onset of the Ca_i waves might be provided by the protein-kinase A (PKA)/cAMP pathway. Accordingly, H-89, a rather selective PKA blocker, hinders NaHS-evoked Ca_i signals in both neurons and microglial cells [159,160]. Moreover, PKA-dependent phosphorylation may increase the Ca^{2+} permeability of T-type channels, NMDA receptors and RyRs [159].

H_2S and t-type voltage-activated Ca^{2+} channels (T-type VDCC)

In addition to K_{ATP} channels, T-type VDCC also has critical roles to play in the processing of either somatic [149] or visceral [161] nociceptive information and in control of pain [152]. However, unlike K_{ATP}, T-type VDCC's antinociceptive effects are dependent on the activity of capsaicin-sensitive pathways [120]. Similar to capsaicin, NaHS, administered intracolonically, triggered visceral nociceptive behavior that was accompanied by referred abdominal hyperalgesia/allodynia [162]. These responses are completely abolished by pre-administered intraperitoneally mibefradil [162]. In contrast, mibefradil at the same dose failed to attenuate the intracolonic capsaicin-induced visceral nociception. Neither L-type VDCC blocker verapamil nor K_{ATP} channel blocker glibenclamide modified the intracolonic NaHS-evoked visceral nociception. Furthermore, researchers found that intraperitoneal NaHS facilitated intracolonic capsaicin-evoked visceral nociception, which was also abolished by intraperitoneal pretreatment with mibefradil. Similarly, intraplantar administration of NaHS induced prompt mechanical hyperalgesia in rat hindpaw, which is blocked by mibefradil but not by glibenclamide [152]. Therefore, H_2S likely functions as a novel nociceptive messenger through the activation of T-type VDCC during inflammation. Furthermore, PPG or BCA (CSE inhibitors) abolished the L-cysteine-induced hyperalgesia and attenuated the lipopolysaccharide-induced hyperalgesia, an effect being reversed by NaHS [152]. Like the reducing agent dithiothreitol, NaHS increased T-type VDCC currents without alteration of their kinetics in undifferentiated NG108 –15 cells, an effect being abolished by an oxidizing agent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Suppression of T-type VDCC by DTNB at a high concentration was reversed by NaHS and dithiothreitol at subeffective concentrations. T-type VDCC is also involved in pancreatic nociception in rodents [163]. Either NaHS or capsaicin induced the expression of Fos protein in the superficial layers of the T8 and T9 spinal dorsal horn of rats or mice [27]. The induction of Fos by NaHS but not capsaicin was abolished by mibefradil. In conscious mice, repeated doses of cerulein produced pancreatitis, accompanied by abdominal allodynia/hyperalgesia. Pretreatment with PPG prevented the allodynia/hyperalgesia, but not the pancreatitis. A single dose of mibefradil reversed the established pancreatitis-related allodynia/hyperalgesia. Taken together, H_2S appears to function as a novel nociceptive messenger through sensitization of T-type VDCC in the peripheral tissues, particularly during

inflammation [164]. In patch-clamp studies using undifferentiated NG108 –15 cells, NaHS enhanced T-type VDCC currents, which may prove that H_2S activates these channels [152]. These authors also reported that intraplantar [151,152] and intrathecal [151] injections of NaHS promptly induced hyperalgesia in rats through T-type VDCC activation. Further investigation suggested that the $Ca_v3.2$ isoform of T channels was activated by H_2S, demonstrated by the abolishment of H_2S induced-hyperalgesia using a general T-type channel blocker mibefradil, and similar results were produced using $ZnCl_2$ ($Ca_v3.2$ specific inhibitor) and also with intrathecal administration of $Ca_v3.2$-specific antisense nucleotides to the rat [151]. Using high (4.5-13.5 mM) concentrations of NaHS on undifferentiated NG108 –15 cells, the same group was able to demonstrate that H_2S induced neurite outgrowth, which was found to be related to the activation of $Ca_v3.2$ isoform T-type channels demonstrated with the abolishment of neurite outgrowth using general T-type channel inhibitor mibefradil, intracellular Ca^{2+} chelator BAPTA-AM, and $Ca_v3.2$ isoform specific blocker $ZnCl_2$ [157]. Interestingly, they also discovered that H_2S induced high-voltage-activated Ca^{2+} currents that were composites of L-type, N-type, and P/Q-type channel activation [157]. Therefore, by compiling the evidence by various authors, T-type channel activation, in particular the $Ca_v3.2$ isoform, by H_2S appears to regulate rhythmic neuronal activity, pain sensation, and differentiation of neurons and boosting of synaptic communication, similar to putative processes regulated by H_2S-related L-type channel activation.

H_2S and transient receptor potential (trp) ion channels

The mammalian TRP superfamily consists of 28 different proteins that may be subdivided into six main subfamilies. They are TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) [165]. Several members that make up this protein superfamily have been found to be nonselective cation channels, of which many are located on primary sensory neurons and involved in somatosensory procedures, such as the transduction of chemical, thermal, and mechanical stimuli. $TRPV_1$ (also called capsaicin receptor) is a nonselective cation channel with high permeability of Ca^{2+} and activated by capsaicin and other vanilloid compounds [165]. However, $TRPA_1$ is activated by a variety of plant-derived and environmental irritants all of which interact with cysteine residues in the ion channel proteins and is present on capsaicin-sensitive primary sensory neurons, which upon activation elicit pain, protective reflexes, and local release of neurotransmitters in the periphery [166].

H_2S and capsaicin receptor ($TRPV_1$)

H_2S and its donors activate $TRPV_1$ ion channels in GI tract, airway, pancreas, and urinary bladder, which cause colonic mucosal Cl^- secretion, gut motility, airway constriction, acute pancreatitis, detrusor muscle contraction, and bladder contractility through a neurogenic inflammation mechanism [167-170]. Serosal application of NaHS and L-cysteine stimulates luminal Cl^- secretion by guinea pig and human colonic tissues [171]. This effect is blocked by TTX, desensitization of afferent nerves with capsaicin, or by the TRPV1 antagonist capsazepine. As such, the stimulatory effects of H_2S on TTX-sensitive Na^+ channels as well as $TRPV_1$ channels are theorized [130]. Interestingly, the secretory effect of NaHS is not observed in a human colonic epithelial cell line (T84 cells) [171].

It appears that H_2S-stimulated mucosal secretion cannot be realized in the absence of either TTX-sensitive Na^+ channels and/or $TRPV_1$ channels from sensory nerve endings. In addition, NaHS-induced Cl^- secretion in rat distal colon is inhibited by serosally applied glibenclamide and tetrapentylammonium, which also block K^+ channels (K_{ATP} and K_{Ca}) [148]. As glibenclamide may inhibit CFTR, this result could also be interpreted as the direct activation of CFTR by H_2S to increase Cl^- secretion. Similar to capsaicin, H_2S donors induce CGRP and substance P release from the sensory nerves in the guinea pig airways and cause *in vivo* bronchoconstriction and microvascular leakage in a capsazepine-sensitive manner. This adds to the irritant action of H_2S in the respiratory system [170]. It has been found that NaHS induces a dose-dependent contraction of isolated bronchial and tracheal rings *in vitro* and this effect is denigrated by the desensitization of sensory nerves with high concentration of capsaicin, by $TRPV_1$ antagonists (capsazepine), as well as by a mixture of neurokinin NK1 (a substance P receptor) and NK2 receptor (CGRP receptor) antagonists. Interestingly, intraperitoneal injection of NaHS to healthy mice induced substantial inflammatory reaction in the lung, as evidenced by increased concentration of substance P, pro-inflammatory cytokines, TNF-αand IL-1β and lung MPO activity [172]. These effects were abolished by a specific NK1 receptor antagonist, but not by NK2 receptor antagonists. In addition, the inflammatory effect of H_2S was abolished by capsazepine and was not observed in mice lacking substance P and neurokinin-A due to the knockout of their common precursor gene, preprotachykinin-A [172]. These data indicated that H_2S per se may induce neurogenic inflammation, even in the absence of other, often harmful, elements. Further research is still required to solve whether H_2S acts as an endogenous ligand of $TRPV_1$ or not [130]. Activation of $TRPV_1$ has been reported to mediate neurogenic inflammation in cerulein-evoked pancreatitis [173]. Intravenous injection of the $TRPV_1$ agonist capsaicin activated a dose-dependent increase in Evan blue aggregation in the rat pancreas. This effect was halted by the pretreatment with the $TRPV_1$ antagonist capsazepine or the neurokinin-1 receptor antagonist CP96, 345. Capsazepine also limited cerulein-induced Evans blue, MPO and histological severity of inflammation in the pancreas, but no effect was seen on serum amylase [173]. Consequently, enhanced plasma H_2S levels have recently been demonstrated in cerulein-induced pancreatitis [167], and administration of PPG reduces the morphological changes in acute pancreatitis, which consists mainly of edema, inflammation and acinar cell injury/necrosis. In contrast to its vasorelaxant effect, NaHS actually created concentration-dependent contractile responses in the detrusor muscle of the rat urinary bladder [174]. This response generated rapid and persistent tachyphylaxis similar to the responses of capsaicin. However, this cannot be seen as a direct effect of H_2S on the muscle because it was destroyed by the combination of NK1 and NK2 receptor-selective antagonists as well as by high-capsaicin pretreatment, which could desensitize capsaicin-sensitive primary afferent neurons. The response to NaHS is mostly resistant to TTX, as is the effect of capsaicin in this organ. The results may be able to provide pharmacological proof that H_2S stimulates capsaicin-sensitive primary afferent nerve terminals with the consequent release of tachykinins, which subsequently produces contractile responses of the detrusor muscle. Furthermore, ruthenium red, a nonspecific blocker of $TRPV_1$ channels, blocked the H_2S-induced contractile response [168], but TRPV1-selective antagonist capsazepine and SB366791 failed to do so. It has also been theorized that H_2S may stimulate the $TRPV_1$ receptor by a different way from those known activators.

H_2S and ankyrin ($TRPA_1$)

$TRPA_1$ is activated by a variety of plant-derived and environmental irritants, such as allyl isothiocyanate (AI), cinnamaldehyde (CA), allicin and acrolein, all of which interact with cysteine residues in the ion channel proteins [175]. Interestingly, acrolein and similar aldehydes are formed endogenously during inflammation. $TRPA_1$ was initially

characterized as a noxious cold receptor [176] and lately its role in mechanosensation has been suggested [177,178]. In the rat bladder, $TRPA_1$ is expressed in unmyelinated sensory nerve fibers with similar pattern to that of $TRPV_1$. Interestingly, $TRPA_1$ is also present in the urothelium, detected at both transcriptional and protein levels. The stimulation of $TRPA_1$ channels induced detrusor overactivity. $TRPA_1$ appears to be consistently colocalized with $TRPV_1$ in the bladder afferents, which suggests a role of $TRPA_1$ in bladder chemosensation and mechanotransduction [169]. Following pretreatment with protamine sulfate, NaHS increased maximal bladder pressure and reduced voided and infused volumes. NaHS evoked a time- and concentration-dependent increase in $[Ca^{2+}]_i$ in Chinese hamster ovary cells expressing mouse or human $TRPA_1$, but not in untransfected cells. This indirect evidence for the activation of $TRPA_1$ by H_2S needs to be validated with more direct electrophysiologial recording.

Should this role be confirmed, H_2S may function as a $TRPA_1$ activator potentially involved in inflammatory bladder disease and in lower urinary tract infection. Furthermore, bacterial metabolite H_2S induced by potential pathogens such as *Escherichia coli* [179] might activate $TRPA_1$ in lower urinary tract infections. In generally, H_2S is the first identified gaseous opener of K_{ATP} channels in vascular SMCs and regulates vascular tone by relaxing smooth muscle cells. In the heart, H_2S and its donors cause the negative inotropic and chronotropic action through activating sarcK_{ATP} and mitoK_{ATP} channels and inhibiting L-type Ca^{2+} channel activity and exert cardioprotection during I/R injury. H_2S-induced reduction of blood pressure can be related to the activation of peripheral K_{ATP} channels in resistant vessel SMCs. The regulation of insulin secretion from pancreatic β-cells by H_2S is via enhancing K_{ATP} channel and suppressing L-type Ca^{2+} channel activities. By elevating $[Ca^{2+}]_i$, H_2S may mediate glutamate-induced neurotoxity and neuronal cell death, but conflicting reports describe the protective effect of H_2S on neuron cells from oxidative glutamate toxicity by activating K_{ATP} and Cl- channels. H_2S-induced hyperalgesia in the colon seems to depend on the sensitization of T-type Ca^{2+} channels. On the other hand, H_2S has a pronociceptive role through evoking the excitation of capsaicin-sensitive $TRPV_1$-containing sensory neurons. H_2S and its donors also activate $TRPV_1$ and $TRPA_1$ channels in nonvascular smooth muscle such as urinary bladder, airways and GI tract, regulating smooth muscle contractility. The opening of K_{ATP} channels by H_2S has been confirmed in cardiovascular, endocrine, and nervous systems, which constitute a major molecular mechanism for many cellular effects of H_2S. However, the molecular interaction of this gasotransmitter with K_{ATP} channel complex has not been clear and the relative contribution of cysteine sulfhydration in K_{ATP} channel proteins by H_2S merits further investigation [27]. The effects of H_2S on voltage-dependent L-type Ca^{2+} channels or BK_{Ca} channels are inconclusive.

Biological Roles of H_2S Vascular System

H_2s and myogenic tone

Initial studies suggested that in the vascular wall H_2S is produced only by smooth muscle cells (SMCs) [38]. However, now it is clear that H_2S are produced by endothelial cells, perirenal, epididymal and perivascular white adipose tissue, as well as in brown adipose tissue [8,180-184].

One of the first physiological roles that prompted investigators to regard H_2S as the "third gaseous signaling molecule" was vasodilation. Intravenously administered H_2S or its donors decrease blood pressure in experimental animals and deficiency of endogenous H_2S has been implicated as a pathogenic factor in arterial hypertension [185]. In 2001, Zhao et al. showed that H_2S decreased blood pressure in rats *in vivo* and caused vascular smooth muscle cell (VSMC) relaxation *in vitro* [38]. H_2S-mediated vasodilation has also been shown in the smooth muscle of the ileum and the vas deferens [186]. H_2S produced in vascular smooth muscle and endothelial cells dilates blood vessels in part by activating ATP-sensitive potassium channels (K_{ATP}) in smooth muscle cells and inducing cell hyperpolarization [185] and in part by stimulating endothelium-derived NO production [104]. Studies suggest that H_2S liberates $NO^{\cdot}$ from S-nitrosothiols [187]. Others show that endothelial denudation and nitric oxide synthase (NOS) inhibitors shift the concentration-response curve for H_2S [104]. However, H_2S increases eNOS phosphorylation and subsequent $NO^{\cdot}$ production in an Akt-dependent manner [188]. Teague et al. [186] reported a summation effect between H_2S and NO on the sublimation of the twitch responses of the ileum to electrical activation. The enhancing effect of H_2S on NO-induced vasorelaxation is still controversial. Zhao et al. [38] observed that pretreatment of aortic ring preparations with H_2S inhibited the vasorelaxant effect of the NO-producing agent SNP [38]. However, Ali et al. [189] have shown that H_2S induced vasoconstriction and increased the mean arterial pressure in rats likely by scavenging endothelial NO. It is likely that the interaction of NO and H_2S may alter the vasorelaxant properties of these two gasotransmitters. Also, the common molecular target for NO and H_2S may become desensitized after firstly encountering one of them. The production of H_2S in the presence of NO is a different story. H_2S production by CSE in vascular tissues is increased by SNP, while the expression of CSE is up-regulated by another NO-producing agent, SNAP [38]. CSE contains 12 cysteine residues that are potential targets for S-nitrosylation. S-nitrosylation of CSE has the potential to increase the enzymatic activities [72]. Perfusion of the mesenteric system with 1 mmol/L cysteine (precursor of H_2S) resulted in an increase of endogenous H_2S production and a dilation of the mesenteric circulation [125]. Cheang et al. showed that K_{ATP} channels were not involved in mediating effects of H_2S in rat coronary arteries [190]. These authors suggested voltage-dependent potassium (K_V) channels as possible mediators of NaHS-evoked vasorelaxation.

Schleifenbaum et al proposed H_2S as a vasorelaxing factor released from perivascular adipose tissue and acting via the stimulation of special K_v type channels – KNCQ channels [191]. Additionally, small, intermediate, and large conductance calcium-dependent potassium channels (SK_{Ca}, IK_{Ca} and BK_{Ca}) have also been demonstrated as possible mediators of H_2S vasodilator effects in resistance vessels [122,123]. An H_2S-evoked increase in cyclic guanosine monophosphate (cGMP) levels could also be involved in H_2S-induced vasorelaxation of smooth muscle cells. Bucci et al. confirmed that H_2S results in vasorelaxation by non-selectively inhibiting endogenous phosphodiesterase (PDE) [192]. This effect would increase tissue levels of cyclic nucleotides, such as cGMP. Recently, conflicting reports have emerged showing that the contribution of the K_{ATP} channels to H_2S-induced vasodilation is minimal and that vasodilation is due to metabolic inhibition (i.e., decrease in ATP), intracellular pH changes, and modulation of Cl^-/HCO_3^- channels [193]. A change in the intracellular acid-base balance is one of the factors that influence the vasoactivity of vascular smooth muscle cells. In another statement, acidification has a vasorelaxant effect, whereas the alkalinization of the intracellular environment causes vasoconstriction in most of the vascular bed. According to data published by Lee et al, H_2S could modify the pH equilibrium in cells by activating the Cl^-/HCO_3^- exchanger and thereby induce acidification [133]. However, the vasoactive response of vessels to H_2S differs in dependence on several factors, for example, the type of vessel (conduit arteries, resistance arteries) endothelium, the substance

used for precontraction and the concentration of H_2S applied [1,104]. H_2S relaxes small mesenteric arteries much more potent than aortic tissues [125]. Although rat aortic and mesenteric artery tissues produce similar levels of H_2S, H_2S is nearly six fold more potently in relaxing rat mesenteric artery beds than relaxing rat aortic tissues. The higher sensitivity of mesenteric arteries to H_2S speaks for the importance of H_2S in regulating peripheral resistance. The mechanisms for differential vasorelaxant effects of H_2S are not clear yet, but several possibilities exist. One explanation is the tissue-type specific distribution of the molecular targets of H_2S. For example, the expression of K_{ATP} channels possibly differs in various vascular tissues with different isoforms. The second explanation is that sensitivities of contractile proteins to H_2S and to intracellular calcium level may vary between conduit and resistant arteries [125].

Also, different types of blood vessels face different sheer stress levels, possess different cellular components (smooth muscle cells, endothelial cells and connective tissues, etc.) and have different stiffness. Finally, oxygen-dependent sensitivity of blood vessels to H_2S should also be considered. It has been reported that H_2S induced vasorelaxation at physiological O_2 levels and this vasorelaxation occurred much faster at below physiological O_2 levels. With higher than physiological O_2 levels (200 mM), H_2S has the tendency to induce vasoconstriction [72]. This could result from the product of H_2S oxidation, which may mediate vasoconstriction. Blood in small peripheral vessels has lower oxygen partial pressure, and these small vessels consume oxygen at higher rate due to the high content of smooth muscle cells and low collagen. The situation is just opposite in large conduit arteries [72]. The difference in tissue oxygen level may explain different vascular effects of H_2S. Another note worth taking is that the release of NO from S-nitrosoglutathione by H_2S is oxygen dependent [72]. H_2S functions as a vasodilator in cerebral circulation. Topical application of H_2S to the newborn pigs induces dilation of pial arterioles [194]. Leffler et al. [194] further showed that L-cysteine per se dilated pial arterioles. Additionally, others have shown that transgenic mice deficient in CBS are chronically hypertensive [37]. Three lines of evidence were given to demonstrate the effect of L-cysteine was the outcome of CSE-generated H_2S. First, PPG at 10 mM blocked the vasorelaxant effect of L-cysteine, but AOA at 1 mM failed to do the same. Second, CSE proteins were detected in cerebral microvessels. While CBS proteins were detected in brain parenchyma, it was not detectable in cerebral microvessels. Third, H_2S concentration in cerebrospinal fluid was increased about fourfold after L-cysteine treatment, measured by GC-MS, which was again blocked by PPG. Whether this vasodilatory effect of H_2S is unique to newborn animal or ubiquitous to cerebral circulation at other stages of development is not known. The stimulus used to precontract vascular tissues also significantly affects the effect of H_2S. While H_2S relaxed phenylephrine- or norepinephrine-precontracted aortic tissues, high concentration of KCl (60 mM)-induced vascular contraction was essentially not affected by H_2S. In effect, though H_2S inhibits KCl (20 mM)-induced contractions of aortic tissues, it does not change the contraction of ileum induced by the same concentration of KCl [186]. Therefore, different vascular tissues manifest different sensitivities to H_2S. Sodium hydrosulphide (NaHS) at concentrations over 100 μmol/L evoked the relaxation of precontracted isolated rat arteries [7,38,189].

Higher concentrations of H_2S (sodium disulphide (NaHS): 2.8 and 14 μmol/kg; 0.1-1 mmol/L) evoked decrease of blood pressure or vasorelaxation in some types of isolated vessels [38,104]. At the same concentration level, H_2S-gassed solution has much stronger vasorelaxant effects than NaHS solution does. The involvement of various signal transduction pathways in the vasodilator effects of H_2S has been examined. NO and CO relax smooth muscle by activating guanylyl cyclase to increase the production of cGMP. H_2S does not affect the production of cGMP, which leads to the inference that there is a different mechanism for the effect of H_2S. Earlier studies also demonstrated that the vasorelaxant effects of H_2S on rat vascular tissues are unlikely mediated by prostaglandin, protein kinase C or cAMP pathways [38,104,125]. Superoxide dismutase and catalase in the bath solution also did not alter the vasorelaxant effect of H_2S, indicating that superoxide anion and hydrogen peroxide did not contribute to H_2S-induced acute vasorelaxation. Although ODQ blocked the vasorelaxation induced by SNP, it had no effect on the vasorelaxant effect of H_2S on rat aortic tissues. Therefore, under this experimental condition, the vasorelaxant effect of H_2S was not mediated by the cGMP pathway [38]. K_{ATP} channel is the major molecular target of H_2S for its vasorelaxant effect and smooth muscle hyperpolarization [38,195]. In the ileum, glibenclamide did not interfere with the relaxation induced by H_2S [186]. This finding may be seen as there are participation of several additional signaling pathways and mechanisms [48]. Furthermore, the specific molecular targets of H_2S were shown to be cysteine 6 and 26 of the extracellular portion of the rvSUR2B subunit of the K_{ATP} channel complex [103]. These vicinal thiols form a disulfide bond, which H_2S reduces, increasing channel conductance and induce hyperpolarization in a tissue-dependent manner. On the other hand, some observations revealed an opposite effect of H_2S on smooth muscle cells of the arterial wall. Lower concentrations of H_2S (Na_2S: 3 μmol/kg; 10-100 μmol/L) resulted in blood pressure increase and vasoconstriction of the same vessels [105,196-198]. Published data indicate numerous possible mechanisms of H_2S-induced vasoconstriction. One possible mechanisms of H_2S-induced vasoconstriction is decreased levels of cyclic adenosine monophosphate (cAMP) in smooth muscle cells. Li et al showed on the rat cerebral artery that H_2S evoked a decrease of cAMP levels, an effect that was associated with the promotion of an interaction between actin and myosin [199].

The H_2S-mediated decrease in cAMP concentrations stimulated the activation of myosin light chain kinase, an enzyme that mediates the interaction between actin and myosin [197]. Li et al also proved that H_2S did not directly influence cAMP levels but significantly reduced forskolin-stimulated adenylyl cyclase activity in human brain vascular smooth muscle cells [199]. This result demonstrated that H_2S-induced vasoconstriction was due to the inhibition of the cAMP/adenylyl cyclase pathway. It was also shown that the administration of low concentrations of H_2S (5-100 μmol/l) inhibited forskolin-induced cAMP accumulation in aortic smooth muscle. Moreover, NaHS was observed to inhibit vasorelaxing effects via β-adrenergic vasodilators and to induce vasoconstricting effects via adenylate cyclase and cAMP inhibition [200]. Ping et al. found that prostanoids could be involved in NaHS-induced vasoconstriction because the vasoconstriction evoked by H_2S was markedly attenuated in the presence of a cyclooxygenase inhibitor (indomethacin, 10 μmol/l) [201]. It was concluded by the same authors that the contractile effect of H_2S was mediated by an influx of extracellular Ca^{2+} because the effect was totally inhibited in a Ca^{2+}- free solution and following incubation with the Ca^{2+} influx blocker nifedipine [1]. H_2S, in contrast to NO, which has a clear vasorelaxant action, has both vasorelaxing and vasoconstricting effects on the arterial system [8]. In another terms, H_2S has marked effects on the circulation, by acting as a hypoxic vasoconstrictor or vasodilator in the pulmonary and systemic circulation, respectively [202,203]. H_2S is a potent vasodilator in the systemic circulation and it produce reduces cardiac output and prolongs body energy stores by redistributing blood flow to the most demanding organs [38]. Conversely, in the lung

circulation, H_2S may contribute to hypoxic vasoconstriction [204,205]; thereby helping to maintain a high arterial O_2 saturation at the low ventilation rates [202,206].

H_2S and vascular endothelial cell proliferation

The same physiological stimuli do not necessarily elicit the same functional responses from different types of cells. While H_2S inhibits vascular SMC proliferation, the gasotransmitter stimulates the proliferation and migration of vascular endothelial cells either in culture or in the whole blood vessel walls. To this end, the stimulatory effect of H_2S on ECs has been reported with cultured human umbilical vein endothelial cells (HUVECs) [207,208] and bEnd3 microvascular endothelial cells [209].

It should be noticed that the pro-proliferative effect of H_2S donors on ECs could not be detected if the concentrations of H_2S donors were higher than physiologically relevant levels. The signaling pathways underlying the stimulatory effect of H_2S on EC proliferation are complex and inconclusive. The stimulation of PI-3K/Akt pathway, K_{ATP} channels, and MAPK and the inhibition of sGC/cGMP pathway by H_2S have all been suggested in ECs [166]. Increased intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured human saphenous vein endothelial cells by NaHS treatment has also been reported [210]. This increase in $[Ca^{2+}]_i$ was mostly due to calcium release from ryanodine receptor-coupled endoplasmic reticulum and due to capacitative Ca^{2+} entry to a smaller extent. Also note that endothelial cells in intact blood vessels (not in culture) do not express functional ryanodine receptors, so this effect is irrelevant. To date, there is no report to link the effect of H_2S on $[Ca^{2+}]_i$ levels in ECs to H_2S-stimulated EC proliferation [210]. H_2S also protects ECs from the damages of different stressors. Hyperglycemia decreased the viability of ECs by increasing oxidative stress and nuclear DNA injury. This hyperglycemia stress results in impaired endothelium-dependent vasorelaxation. In cultured microvascular ECs, the hyperglycemia-induced EC damage was suppressed by supplementation of exogenous H_2S to the culture media. CSE overexpression increased EC viability by 6% compared with the native ECs, facing the same hyperglycemic culture conditions. On the other hand, knocking down the expression of endogenous CSE with siRNA reduced hyperglycemia-enhanced oxidative stress in ECs [209]. Extending their observations from cultured endothelial cells, Suzuki et al. [209] overexpressed CSE gene in thoracic aortic rings isolated from Sprague-Dawley rats. This *in vitro* transfection preserved the endothelium-dependent vasorelaxant properties of the vascular rings in the presence of hyperglycemia [209]. The important role of CSE/H_2S in protecting ECs from hyperglycemic damage was further demonstrated in CSE KO mice. The isolated thoracic aorta rings from CSE KO mice were manifested with much more severely damaged endothelium-dependent relaxations than that from WT mice when incubated with the same hyperglycemic conditions *in vitro* [209].

H_2S and vascular smooth muscle cell proliferation

The proliferation of vascular SMCs plays a critical role in the maintenance of vascular structure and functions, and its alteration leads to vascular remodeling and various proliferative vascular diseases. However, cellular and molecular mechanisms that regulate SMC proliferation and differentiation are not fully understood. H_2S is an important endogenous modulator of cell proliferation and apoptosis [211]. Serum deprivation up-regulated CSE expression and H_2S production in cultured human aorta SMCs in concert with the induced SMC differentiation marker gene expressions, such as SM-MHC, calponin and SM-actin [212]. Overexpression of CSE in human aortic SMCs inhibited cell growth and induced cell apoptosis [213]. Absence of endogenous H_2S in vascular SMCs, such as those isolated from CSE gene deficient mice (KO mice), led to a significant surge in cell growth rate [214]. The percentage of BrdU-positive cells in cultured SMCs and in the media of the aorta was also significantly greater in CSE KO mice than in age matched and wide-type mice [214]. Clearly, endogenous CSE/H_2S limits the proliferation and growth of SMCs. Furthermore, increased SMC proliferation in CSE KO mice was not secondary to the development of hypertension. The normalization of blood pressure in CSE KO mice by captopril did not reduce aortic SMC proliferation when compared with untreated age-matched CSE KO mice [214]. The endogenous level of H_2S affects the effect of exogenous H_2S on cell apoptosis. Yang et al. [213] found that NaHS induced apoptosis of human aortaic SMCs at concentrations 200 M. After inhibition of endogenous H_2S production by PPG pretreatment or by knocking down endogenous CSE gene with short-interfering RNA approach, the proapoptotic effect of NaHS becomes significant at 50-100 M. Another study reported that exogenously applied NaHS at 100 M inhibited proliferation and induced apoptosis of vascular SMCs from CSE KO mice, but not of SMCs from wide-type (WT) mice [214]. CSE/H_2S pathway is also involved in the development of balloon injury-induced neointima formation of rat carotid arteries. The transcriptional expression levels of CSE, CSE activity and endogenous H_2S production were all decreased in balloon-injured carotid arteries [215]. Treatment of the rats with NaHS significantly weakened balloon injury-induced neointimal hyperplasia and reduced vascular smooth muscle cell proliferation in the lesions *in vivo*. Similar observations were made in the mouse where carotid artery ligation resulted in enhanced neointima formation and down-regulation of CSE expression [212].

The mechanisms underlying the antiproliferative and/or proapoptotic effect of H_2S are multifaceted. One of the focal points of these studies is the involvement of the mitogen-activated protein kinase (MAPK) superfamily, including three parallel cascades which are the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) cascade, the p38-MAPK cascade, and the classical extracellular signal-regulated kinase (ERK)/MAPK cascade. In human aortic SMCs, for example, exogenous H_2S induced apoptosis through activation of MAPK pathway. The phosphorylation of ERK transduces the apoptotic signal to its downstream enzyme cascades and eventually activates caspase-3. After the activities of ERK and caspase-3 were inhibited, the apoptosis of human aortic SMCs induced by H_2S was significantly attenuated. Therefore, the activation of ERK and its downstream factor caspase-3 likely mediates H_2S-induced cell apoptosis [211]. It is worth pointing out that in many other cell types or tissues, ERK activation serves as a proliferative/antiapoptotic signal. It has been reported that the proliferation of cultured rat aortic vascular SMCs was inhibited by NaHS. At the same concentration range (50-500 M), NaHS also inhibited ERK activity [216]. Whether activation of ERK could reverse NaHS induced proliferation inhibition was not conducted by the same researchers [216]. Therefore, it is not sure whether the decreased ERK activity can account for the reported effect of NaHS on rat vascular SMCs. In CSE overexpressed HEK-293 cells, ERK and p38 MAPK activities were significantly increased, but not in Ad-lacZ infected cells or control cells, and the cell growth was inhibited [217]. The activations of ERK and p38 MAPK were also involved in H_2S-treated intestinal epithelial cells (IEC-18) [218]. The pro-apoptotic effect of H_2S may also be related to the cell cycle due to the stimulation of cyclin-dependent kinases. S-diclofenac {2-[(2,6-dichlorophenyl)amino]benzene acetic acid 4-(3H-1,2,dithiol-3-thione-5-yl)phenyl ester} is a novel molecule comprising an H_2S-releasing dithiolthione moiety attached by an ester

linkage to diclofenac [219]. S-diclofenac induces a dose-dependent decrease in the survival of primary and immortalized rat aortic vascular SMCs. The cells in G1 phase were not affected by S-diclofenac but asynchronized SMCs manifested with an increase in apoptotic cell death. S-diclofenac stabilized p53 and induced p21, p53AlP1 and Bax. But the anti-apoptotic factor Bcl-2 was not affected [219]. In CSE-overexpressed cell or exogenous H_2S-treated cells, there are also an increased expression of p21Cip/WAK-1 and a down-regulation of cyclin D1 [217].

The anti-proliferative and/or pro-apoptotic effect of H_2S may be of importance for the prevention of cell proliferation in disorders such as atherosclerosis, vascular graft occlusion and neointimal hyperplasia leading to restenosis after angioplasty [220].

H_2S and angiogenesis

H_2S can cause cell proliferation and migration [166,208]; however, there appears to be a narrow concentration range of the proliferative effect, below which no effect is seen and above which there is anti-proliferation and H_2S cytotoxicity [207]. In cell culture experiments, low micromolar concentrations of H_2S increase endothelial cell number, proto-vessel formation, and cell migration [166]. Chicken chorioallantoic membranes, an *in vivo* model of angiogenesis, display increased branching and lengthening of blood vessels in response to 48 h incubation with H_2S [208]. Additionally, aortic tissue isolated from transgenic mice lacking CSE, the primary H_2S-producing enzyme in the endothelium, exhibit marked decreases in angiogenesis [208]. The mechanism of H_2S-induced angiogenesis operates through several pathways, including activation of ATP-sensitive potassium (K_{ATP}) channels [38]. Papapetropoulos et al. showed that treatment of endothelial cells with the K_{ATP} channel inhibitor glibenclamide reduced cell migration, which was accompanied by decreased H_2S-induced p38 and heat shock protein 27 (Hsp27) phosphorylation [208]. Additionally, H_2S can stimulate angiogenesis through phosphatidylinositol 3-kinase (PI_3K) and Akt activation [166]. H_2S can also activate hypoxia inducible factor-1α (HIF-1α) and thus increase expression of vascular endothelial growth factor (VEGF) [221]. Conversely, VEGF-stimulated angiogenesis is suppressed in CSE knockout mice [166]. Endogenous H_2S production is known to be upregulated during wound healing [222]. Topically applied H_2S accelerates wound closure and healing [208]. Angiogenesis is very important in both acute and chronic ischemia as poorly vascularized tissue will lose function and possibly become necrotic. In models of chronic hind limb ischemia, sodium hydrosulfide (NaHS) increased capillary formation and blood flow [223]. Similar results were found in chronically ischemic hearts with improvements in cardiac function following H_2S treatment [224]. These studies indicate that endogenous H_2S is crucial in physiological angiogenesis and that those capabilities can be employed in disease treatment.

Conclusion

H_2S can directly regulate vascular tone by acting up on specific targets receptors and induces hyperpolarization of adjacent vascular smooth muscle cells in large conduit and small resistance arteries by activating K_{ATP} and KCNQ channels, respectively. The mechanism of vascular effect of H_2S is controversial with opposite data sometimes provided by different studies. These discrepancies result most likely from using different animal species, different H_2S donors and their concentrations, as well as experimental conditions, such as buffer composition or oxygen levels. Generally H_2S has marked effects on the circulation, by acting as a hypoxic vasoconstrictor or vasodilator in the pulmonary and systemic circulation, respectively. Although H_2S is a potent vasodilator of isolated systemic vessels, its effect in living hibernating animals would probably be overwhelmed by a strong adrenergic tone that may constrict peripheral systemic blood vessels.

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