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\textsubscript{2}S) is the third gasotransmitter in mammals. It is synthesized from L-cysteine by cystathionine \(\beta\)-synthase, cystathionine y-lyase or by sequential action of alanine aminotransferase and 3-mercaptoppyruvate sulfur transferase. Although initially it was suggested that in the vascular wall H\textsubscript{2}S is synthesized only by smooth muscle cells and relaxes them by activating ATP-sensitive potassium channels, more recent studies indicate that H\textsubscript{2}S is synthesized in endothelial cells as well. The physiological functions of H\textsubscript{2}S are mediated by different molecular targets, such as different ion channels and signaling proteins. Endogenous H\textsubscript{2}S 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\textsubscript{2}S production, and novel H\textsubscript{2}S-delivery compounds have been invented to aid therapeutic intervention of diseases related to abnormal H\textsubscript{2}S metabolism. The primary purpose of this review was to provide an overview of the role of H\textsubscript{2}S 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 20\textsuperscript{th} 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\textsubscript{2}S), 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\textsubscript{2}S field” in biology and medicine is now growing rapidly [8]. H\textsubscript{2}S 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\textsubscript{3}), methane (CH\textsubscript{4}) and hydrogen (H\textsubscript{2}) are suggested to exist as well [8,9]. Epidemiological studies report that a diet rich in organosulfur compounds 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\textsubscript{2}S 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\textsubscript{2}S concentrations ranging from 1 to 500 mM [15,16].

Hydrogen sulfide (H\textsubscript{2}S), 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\textsubscript{2}S in the cardiovascular system varies between 10 nmol/l and 300 μmol/l [1]. Interest in the cytoprotective actions of H\textsubscript{2}S has grown since the discovery that it can induce a hypometabolic state characterized by decreased O\textsubscript{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\textsubscript{2}S with common ion channels used to produce its biological effect within human being.

Chemical properties, synthesis and metabolism of H\textsubscript{2}S

H\textsubscript{2}S 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\textsubscript{2}S is also toxic at high concentrations and shares with them...
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. Desulfhhydration of L-cysteine by cystathionine \( \beta \)-synthase (CBS, EC 4.2.1.22),

b. Desulfhhydration of L-cysteine by cystathionine \( \gamma \)-lyase (CSE, EC 4.4.1.1), and

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

The first two enzymes, CBS and CSE, determine amount \( H_2S \) production than 3-mercaptoppyruvate sulfur transferase and are pyridoxal 5'-phosphate (vitamin B6)-dependent enzymes. Additionally, they are found in the cytosol and act sequentially in the transulfuration pathway to convert L-homocysteine to L-cysteine with 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 \( \beta \)-elimination of cysteine to pyruvate, \( H_2S \) and \( NH_4^+ \), \( \gamma \)-elimination of homocysteine to 2-ketobutyrate, \( H_2S \) and \( NH_4^+ \) and \( \beta \)- or \( \gamma \)-replacement reaction between two cysteine or two homocysteine molecules, with lantionine or homolantionine, 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, cardiomycocytes 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 CAT3-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 \( H_2S \) synthetizing enzymes are present [39]. In specific intracellular spaces (micropores), 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 mmol/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 mM to low \( \mu \)M 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 oxidoreductase (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 persulfide (RSSH) [8,44]. This second 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^2-), 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_4^{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 toCcO, 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 after expiration is negligible, lung is also involved in oxidation 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 heme. 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$_2$S.

CBS catalyzes the first step in H$_2$S 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$_2$S 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$_2$S, albeit at lower levels than CBS and CGL, in mitochondria.
H₂S 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 H₂S 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 H₂S 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 H₂S [55]. In theory, H₂S can also reduce higher thiol oxidation states such as S-nitrosothiols and sulfenic acids [56]. H₂S can also be methylated by the cytosolic enzyme thiol-S methyltransferase to form methane thiol [17]. As with virtually all molecules, H₂S 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 H₂S is O₂. In the presence of molecular O₂ and redox active metals, H₂S will spontaneously oxidize [59]. In an oxygenated biological medium, metalloproteins catalyze H₂S oxidation. This makes O₂ tension a critical methodological consideration when conducting biologically relevant experiments.
Method of Detection of Endogenous H$_2$S Production

Through determination of physiological level of endogenous H$_2$S

The determination of the physiological concentrations of H$_2$S in circulation and in specific tissues is pivotal for determining the impact of H$_2$S on a given physiological function; correlating H$_2$S levels with the specific pathophysiological changes; examining physiological roles of H$_2$S under in vitro conditions at organ, tissue, and cellular levels; and guiding pharmacological and therapeutic administrations of H$_2$S donors not always the measurement of H$_2$S gives the consistent values [27]. The physiological range of H$_2$S 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$_2$S. A study revealed no change in serum H$_2$S concentration among three age groups of humans spanning 50-80 years (34-36 µM) [63].

Rat serum contains 46 µM H$_2$S [38] and it is 34 µM in mouse serum [64]. In New Zealand rabbits, a quantitative assay detects a plasma H$_2$S level around 16.5 µM [65]. Plasma H$_2$S at micromolar ranges has also been reported in many other vertebrates [66]. Endogenous levels of H$_2$S in rat brain homogenates are 50-160 µM [6,67-69]. Similar H$_2$S levels were reported in the liver, kidney and pancreas [68-70]. H$_2$S production was clearly measured in the cardiovascular system [7,38]. Not always the measurement of H$_2$S 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$_2$S levels in lamprey, trout, mouse, rat, pig, and cow blood samples using a special house-made polarographic H$_2$S sensor that can detect 14 nM H$_2$S [39]. One explanation for these low values of H$_2$S is the rapid decay of H$_2$S concentration from micromolar concentration to undetectable level within 30 min in vitro. Whether H$_2$S 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$_2$S 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$_2$S. In two other studies using polarographic sensors, free H$_2$S 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$_2$S 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$_2$S detection would also help validate the actual blood levels of H$_2$S. Finally, in contrast to the sulfur ion selective electrode which detects total sulfur in the blood including its acid labile, bound or free H$_2$S forms, the polarographic sensor is sensitive only to freely dissolved H$_2$S gas [27]. It is possible that a significant amount of H$_2$S 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$_2$S gas.

Whereas whether H$_2$S is a circulating gasotransmitter for both its generation and transportation is still being debated, the paracrine or autocrine effects of H$_2$S may nevertheless be more critical for regulating the functions of the cells, tissues, and organs where H$_2$S is produced in the proximity [27]. Using gas chromatography technique, Furne et al. [25] found very low tissue production of H$_2$S 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$_2$S levels at 35-80 µM. Even using a polarographic sensor, others have
detected significant tissue production of H₂S from the brain and liver [27]. The detection of the volatile gas transmitter 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 H₂S is very narrow [27]. The toxic level of H₂S reported by Warenczyca 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 NaH₂S (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 H₂S with stable COPD [63]. This percentage change translates to a H₂S concentration difference of <20 µM. This narrowness of the transition zone between physiological/biological and toxicological levels of H₂S can also be found in pharmacological studies where the dose-response relationship of H₂S is relatively steep before a given function change occurred and can quickly cause the opposite effect when H₂S concentration further increased [38]. As such, an ideal measurement method for detecting H₂S in mammalians should be sensitive, specific, accurate, noninvasive, on real-time and require a small quantity of samples. Many of the current H₂S measurement techniques, such as spectrophotometry, chromatography, and ion-selective electrode, were originally invented to meet the industrial demand for monitoring H₂S 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 H₂S scavenging molecules, interference of hemoglobin 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 H₂S can be traced back to Fischer's study in 1883 for its principle [74] and to the work by Fogo and Popowski 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 H₂S reacts with ferric chloride (FeCl₃) 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 H₂S concentration and the intensity of the transmitted monochromatic light can then be determined. The minimum detectable concentration of H₂S is determined by the sensitivity of the spectrophotometer to the optical density changes. Photoacoustic spectroscopy of H₂S 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 H₂S level [66]. For animal tissue samples or cells, the methylene blue method has been used often but usually is for detecting the H₂S generation capacity of the samples. In other words, the activity of H₂S-generating enzymes in term of H₂S production rate is assayed, rather than the absolute H₂S 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 H₅S from samples. Step 2 is to transform H₅S to methylene blue. The generated H₂S 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 H₂S 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 H₂S already existent, rather than to be generated, since H₂S generating enzymes are not in the fluid. Therefore, step 1 as described above to maximally activate H₂S-generating enzymes is no longer needed [27]. The fluid sample can be agitated by adding acid to release H₂S into the gas phase, which then interacts with zinc acetate and NDPA to form methylene blue [76]. Alternatively, the acid release of H₂S gas and trapping processes are omitted by directly adding NPDA and trichloracetic acid (TCA) to the plasma to directly form methylene blue [79]. For H₂S in air samples, the methylene blue method can be modified to use an alkaline solution of cadmium hydroxide to absorb H₂S [80].

### Through usage of nanotube-based sensors

Electrochemical detection is the most commonly used technology incorporated in compact and portable H₂S gas monitors [81]. The principle behind it is the conductivity changes of thin films upon exposure to H₂S 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 NH₃ 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-Fe₂O₃ nanotubes has been developed, which can specifically detect H₂S 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 H₂S to occur. It is also not suitable for measuring H₂S in liquid [87]. Other sensors based on SnO₂ nanowire [88], In₂O₃ nanowire [89] and ZnO nanowires [90] with increased sensitivity have been reported. The challenges with these one-dimensional structures are the difficulties in making the nanostructures and in obtaining large quantities as well as their application under in vivo physiological conditions. CuO-SnO₂ and ZnSb₂O₄ have been shown to detect H₂S at concentrations below 1 ppm at 300°C [91]. Using single-wall carbon nanotubes (SWNT) [92] as an H₂S sensor as well as an H₂S carrier has attracted a great attention in recent years. This is because of the adsorption of H₂S 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 H₂S. The activated carbon facilitates H₂S reaction with oxygen at low temperatures, leading to the production of sulfur and water [93]. SWNT-based H₂S biosensor will also potentially reduce the sample volume to nanoscale. The initial attempt of using multi-wall carbon nanotubes to measure H₂S in solution was made by Wu et al. [94]. After carbon nanotubes are immersed in a H₂S solution, on the contact interface between carbon nanotubes and H₂S 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$_2$S by the van der Waals force. The interaction of H$_2$S (hydroxylide 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$_2$S in the solution. In this preliminary study, 10 µM H$_2$S in water was successfully measured [94]. To take one step further toward the biological application of the carbon nanotube-based H$_2$S biosensor, Wu et al. [96] applied this carbon nanotube fluorescence technique to measure H$_2$S level in serum and reported that the binding of H$_2$S to nanotubes was not affected by the presence of proteins in rat serum. After removing endogenous H$_2$S in the serum with hemoglobin, exogenous H$_2$S added to the serum was successfully detected with a linear relationship between H$_2$S concentrations (20, 50 and 100 µM) and fluorescence intensities. The mechanism for using carbon nanotubes to detect H$_2$S, 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$_2$S 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$_2$S 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$_2$S 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$_2$S at room temperature with a detection limit of 3 ppb. The application of these nanostructures for detecting H$_2$S 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$_2$S 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$_2$S 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$^-$ and as such, free H$_2$S 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$^0$ dissociated from H$_2$S and released from proteins. Furthermore, using the electrodes still requires bulky samples and is an off-line measurement [27].

Through usage of polarographic H$_2$S sensors

A novel polarographic H$_2$S sensor (PHSS) was developed in 2005 as a voltammetry, which is a method of determining the chemical makeup of an H$_2$S 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$_2$S. Real-time measurement of the levels of H$_2$S 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$_2$S production in biological samples. It was reported that the miniature PHSS detected H$_2$S 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$_2$S level with commercially available PHSS is more often than not a daunting challenge and a frustrating experience [27]. Because the polargraphic sensor only measures H$_2$S gas, sulfide (H$S^-$ and S$^0$) 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$_2$S 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 α-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$_2$S in air by ion chromatography has the working range of 20-500 µM for a 20 l air sample [101]. Gas chromatography-mass spectrometry has been used to detect H$_2$S in animal tissues based on the amount of trapped S$^-$ after acidification of H$_2$S [25,61]. Reverse-phase (RP)-HPLC for the determination of H$_2$S-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$_2$S, tissue contents of H$_2$S were all significantly increased [73]. Shen et al. [102] reported in 2011 a novel and sensitive method to detect physiological levels of free H$_2$S in cell lysates, tissue homogenates, and body fluids.

This method is built on the rapid reaction of monobromobimane with H$_2$S under basic conditions at room temperature to produce sulfide-dibimane (SDB). SDB is stable, in which it favors over the unstable H$_2$S 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 RP-HPLC analysis reaches the H$_2$S 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$_2$S was achieved. CSE HT mice have lower plasma level of H$_2$S than that of wild-type mice, but higher level than that of CSE KO mice [102]. Sensitive and selective detection of H$_2$S has been one of the hot spots as well as one of the bottlenecks in H$_2$S 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 H₂S in biological samples are the biggest challenges for accurate and rapid measurement of H₂S levels. At this moment, the spectrophotometry-based method is still of the choice to determine tissue or cell production of H₂S, whereas sulfur ion-specific electrodes and polarographic H₂S sensors hold potential for real time measurement of H₂S net levels in blood or other body fluids. For analyzing H₂S in air samples, such as exhaled air from lungs, chromatography analysis of H₂S would be more suitable. Furthermore, the fluorescence-based quantitative or semi-quantitative methods would be useful for detecting H₂S production in specific cellular organelles.

**Biological Roles of H₂S 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, H₂S 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 H₂S include, but are not limited to, differences in the solubility of H₂S in aqueous vs. lipid phases, proximity of the target to H₂S detoxifying enzymes, heme redox state, and inter- and intra-cellular differences in O₂ tension [17]. H₂S plays a role in many physiological processes. However, high O₂ can reverse many of the beneficial roles of H₂S seen at lower O₂ concentrations, resulting in, for example, vasoconstriction rather than vasodilation. Additionally, under hypoxic and normoxic conditions, H₂S promotes angiogenesis. However, at higher concentrations of both O₂ and H₂S, an inhibition of cellular proliferation is seen. H₂S 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 H₂S necessary to induce a hypometabolic effect, can, if pushed further, result in cardiac and respiratory toxicity.

**H₂S 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 H₂S and K_{ATP} channel remains unclear. The whole-cell patch-clamp technique and mutagenesis approach were used to examine the effects of H₂S on different K_{ATP} channel subunits, rvKir6.1 and rvSUR1, heterologous expressed in HEK-293 cells.

H₂S 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 H₂S effects on this recombinant channels. CLT, but not NEM, inhibited basal rvKir6.1/rvSUR1 currents, and both abolished the stimulatory effects of H₂S 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, H₂S lost its stimulatory effects on rvKir6.1/rvSUR1 currents [103]. By targeting K_{ATP} channels, H₂S 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 H₂S-induced vasorelaxation is mainly caused by opening ATP-sensitive potassium channels (K_{ATP}) on the vascular smooth muscle.
cells [38,104,105]. In isolated piglet cerebral arteriole SMCs, a recent study showed that H\textsubscript{S} activated K\textsubscript{ATP} channels at physiological steady state voltage (-50 mV), which was antagonized by glibenclamide [106]. Electrophysiological study provides direct evidence that exogenous H\textsubscript{S} increases macroscopic or unitary K\textsubscript{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\textsubscript{ATP} channels in high-dose H\textsubscript{S}-induced vasorelaxation in isolated rat aortas [38]. Consistent with the role of K\textsubscript{ATP} channels in mediating the effects of H\textsubscript{S}, reduced endogenous synthesis of H\textsubscript{S} decreased K\textsubscript{ATP} channel activity [1]. Moreover, exogenous H\textsubscript{S} administration activated K\textsubscript{ATP} channels and hyperpolarized the membrane of vascular smooth muscle cells isolated from rat mesenteric arteries [107]. H\textsubscript{S}-induced hyperpolarization of SMC membrane is also abolished by glibenclamide. The opening of K\textsubscript{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\textsubscript{ATP} channel blockers glibenclamide and 5-hydroxydecanate [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\textsubscript{S} produces a cardioprotective effect against I/R injury during reperfusion, at least in part by opening K\textsubscript{ATP} channels [109]. The patch-clamp data provide additional electrophysiological evidence that convincingly shows the effect of H\textsubscript{S} on K\textsubscript{ATP} channels. Exposure of single cardiac myocytes to NaHS increased single-channel activity of K\textsubscript{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\textsubscript{S} and its donors cause the negative inotropic and chronotropic action through activating sarcK\textsubscript{ATP} and mitoK\textsubscript{ATP} channels. The cardioprotective effect of H\textsubscript{S} involves not only by the opening of K\textsubscript{ATP} channels, but also through activation of cardiac ERK and/or Akt pathways in addition to preserving mitochondrial structure and function [111,112]. H\textsubscript{S}-induced neuroprotection and suppression of glutamate toxicity was also partially mediated by the activation of K\textsubscript{ATP} channels. Glibenclamide and glipizide dose-dependentely suppress H\textsubscript{S}-induced protection of HT22 cells from oxidative stress. Neuroprotection was increased by the simultaneous application of H\textsubscript{S} and pinacidil or the combined application of cysteine and pinacidil or the combined application of cysteine and pinacidil. While all these results support the involvement of plasma membrane K\textsubscript{ATP} channels in the effects of H\textsubscript{S}, opening (with diazoxide) or blocking (with 5-hydroxydecanate, 5-HT) of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels did not modulate protection by H\textsubscript{S} [113,114]. Distruiti et al. [115] have demonstrated that the systemic administration of different H\textsubscript{S} donors inhibits visceral nociception by opening K\textsubscript{ATP} channels. The activation of K\textsubscript{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\textsubscript{1}, such as morphine and dipyromine, exert their effects by opening K\textsubscript{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\textsubscript{S} on direct hypernociception induced by PGE\textsubscript{1} is dependent on K\textsubscript{ATP} channels in the periphery. Supporting this hypothesis, glibenclamide prevented the anti-nociceptive effect of exogenous H\textsubscript{S} 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\textsubscript{ATP} channel opener also directly blocks hypernociception induced by PGE\textsubscript{1}, which further supports the findings.

Electrophysiologically, it has been shown that K\textsubscript{ATP} channel activation reduces the enhanced excitability of rat nociceptive sensory neurons induced by PGE\textsubscript{1} [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\textsubscript{S} donor limited leukocyte recruitment and trafficking and decreased synovial blood flow. These anti-inflammatory effects of H\textsubscript{S} were mediated via the K\textsubscript{ATP} channel because responses could be blocked by glibenclamide treatment. Intra-articular administration of NaHS had no effect on joint pain sensation or secondary alloydynia in the rat, although this observation needs to be corroborated in other animal species. Thus it is conceivable that H\textsubscript{S} may function as an endogenous regulator of joint function and that its action is distinctly anti-inflammatory [119]. However, exogenously administered H\textsubscript{S} acts on sensitive neurons and has examined the opening of K\textsubscript{ATP} channels and subsequent antinociception [117]. The effects of H\textsubscript{S} on K\textsubscript{ATP} channels also exert influence on pain cognizance. Research has clarified that parental administration of either NaHS or an H\textsubscript{S}-releasing derivative of mesalalmine 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 suggest that H\textsubscript{S} functions as a negative regulator of visceral nociception by activating K\textsubscript{ATP} channels and attenuating pain. NaHS-induced antinociceptive effects are not dependent on the activity of capsacin-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\textsubscript{ATP} channels in the regulation of pancreatic insulin secretion, multiple studies have examined the effect of H\textsubscript{S} on cells. K\textsubscript{ATP} currents were limited after lowering endogenous H\textsubscript{S} 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\textsubscript{S} by oxereexpression of the CSE gene significantly aggrandized whole cell K\textsubscript{ATP} currents in INS-1E cells. Exogenous H\textsubscript{S} markedly increased the open probability of single K\textsubscript{ATP} channels by twofold in inside-out patches, but single-channel conductance and ATP sensitivity of K\textsubscript{ATP} channels were not changed by H\textsubscript{S} [121].

H\textsubscript{S} and Ca\textsuperscript{2+}-sensitive K (K\textsubscript{Ca}) channels

Other than K\textsubscript{ATP}, small, intermediate, and large conductance calcium-dependent potassium channels (SK\textsubscript{ca}, BK\textsubscript{ca} and BK\textsubscript{V}) have also been demonstrated as possible mediators of H\textsubscript{S} vasodilator effects in resistance vessels [122,123]. It has been observed that H\textsubscript{S}-induced vasorelaxation of rat aortic ring was not affected by ibotetoxin or charybdotoxin. This observation suggests that big-conductance Ca\textsuperscript{2+}-sensitive K (BK\textsubscript{ca}) channels might not be responsible for the H\textsubscript{S}-induced vasorelaxation in conduit vessels [124]. Both H\textsubscript{S} and NaHS evoked concentration-dependent relaxation of in vitro perfused...
rat mesenteric artery beds (MAB) [125]. The vascular effects of H$_2$S 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$_2$S in the endothelium intact rat aorta. These data suggest that small to medium conductance K$_{Ca}$ channel (SK$_n$ and IK$_{Ca}$) in MAB and aorta is activated by H$_2$S. Therefore, H$_2$S might fulfill the role of EDHF [115]. The stimulation of SK$_n$ and IK$_{Ca}$ channels by H$_2$S 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$_2$S on BK$_{Ca}$-channel. The same conclusion of H$_2$S-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$_2$S diluted and hyperpolarized rat arteries and that these effects of H$_2$S were blocked by iberiotoxin and paxillin. Thus the stimulation of iberiotoxin sensitive BK$_{Ca}$ channels by H$_2$S is suggested [131].

**H$_2$S 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 indanyl oxyacetic acid (IAA-9) suppress protection by H$_2$S, 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$_2$S against oxidative stress. The recent findings that a decrease in transmembrane Ca$^{2+}$ 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$_2$S on Cl channels in the CNS has been studied. In the research, H$_2$S was seen to activate CFTR Cl channels in HT22 neuronal cell lines which led to neuroprotection during oxtosy. This was demonstrated through dose-dependent suppression of neuroprotection due to H$_2$S using specific CFTR blockers, NPPB and IAA-94, and confirmed using CFTR activator levamisole [113]. Together with the recent observation of H$_2$S activating CI/HCO$_3$ transporters in smooth muscle cells [133], the results suggest possible regulation of Cl fluxes by H$_2$S 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$_2$S in modulating excitability [130].

**H$_2$S and calcium (Ca$^{2+}$) channels**

It is well recognized that voltage-activated Ca$^{2+}$ channels (VDCC) regulate intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]$) 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$^{2+}$ stores, [Ca$^{2+}$], 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 triphosphate receptor (IP$_{3}$R) channels and sarcodoenaplasmic reticular Ca$^{2+}$ ATPases (SERCA) [134].

**H$_2$S and 1-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$_2$S 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 Cav1.2 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 Cav free sulfhydryl groups (143). The H$_2$S 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$^{2+}$ transients in single cardiomyocytes are also reduced by 100 M NaHS [141,142]. Consistently, H$_2$S 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 MµNaHS) [145].

Interestingly, according to a recent study, H$_2$S can reverse the negative inotropic effect induced by NO by causing an increase in the peak amplitude of the electrically stimulated Cav transients [140]. These apparently discrepant data may be reconciled when considering that, under such conditions; the modulation of the Cav1.2 toolkit responsible for the positive inotropic effect is not accomplished by H$_2$S, but by a new thiol-sensitive endogenous modulator deriving from the interaction between the two gasotransmitters [140]. Interestingly, in this report, H$_2$S was provided by NaHS at low micromolar doses (10 µM). The negative effect of H$_2$S on Cav2 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 Cav$^{2+}$ channels [130]. On the other hand the effects of NaHS on neurons, that can express both Cav1.2 and Cav1.3 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\(_{\text{L}1.2}\) or Ca\(_{\text{L}1.3}\)) of L-type channels in these cells. Taken together, these evidences suggest that L-type Ca\(^{2+}\) channels are inhibited by H\(_{2}\)S in the myocardium, whereas they are enhanced by the same H\(_{2}\)S 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\(_{\text{L}1.2}\) in ventricular cardiomyocytes vs. Ca\(_{\text{L}1.3}\) 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\(_{2}\)S. NaHS increases Ca\(_{\text{L}}\) 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\(_{2}\)S-triggered Ca\(_{\text{L}}\) 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\(_{\text{L}}\) 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\(_{\text{L}}\) channels are encoded by the three members of the Ca\(_{\text{L}}\) 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 intracerebrally 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\(_{\text{ATP}}\) channel blocker glibenclamide [130]. Therefore, H\(_{2}\)S 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 intraperantl (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 antiseine oligodeoxycuniolcetides (ODNs) selectively targeting rat Ca\(_{\text{L}3.2}\) [151-153]. The finding that DL propargylglycine (PPG) and -cyaonoalanine, two CSE inhibitors, abish 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 proinflammatory stimulus in the pancreatic duct, albeit at higher concentrations than those reported above (500 nM/rat) [154]. Finally, the neuropathic alldodynia/hyperalgesia induced in rats by damaging the left L5 spinal nerve [155] or by systemic injection of paclitaxel [156], an anticanter drug, was strongly attenuated by either mibefradil or CSE inhibitors, or by antisense ODNs against rat Ca\(_{\text{L}3.2}\). In addition, Ca\(_{\text{L}3.2}\) 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\(_{\text{L}3.2}\) 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\(_{\text{L}3.2}\)-dependent, but not Ca\(_{\text{L}3.1}\)- and Ca\(_{\text{L}3.3.}\)- currents [158]. A mechanistic link between H\(_{2}\)S and the onset of the Ca\(_{\text{L}}\) waves might be provided by the protein-kinase A (PKA)/cAMP pathway. Accordingly, H-89, a rather selective PKA blocker, hinders NaHS-evoked Ca\(_{\text{L}}\) 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\(_{2}\)S and T-type voltage-activated Ca\(^{2+}\) channels (T-type VDCC)

In addition to K\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) T-type VDCCs antinociceptive effects are dependent on the activity of capsaicin-sensitive pathways [120]. Similar to capsaicin, NaHS, administered intracerebrally, 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\(_{\text{ATP}}\) channel blocker glibenclamide modified the intracerebral NaHS-evoked visceral nociception. Furthermore, researchers found that intraperitoneal NaHS facilitated intracerebral 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\(_{2}\)S 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\(_{2}\)S appears to function as a novel nociceptive messenger through sensitization of T-type VDCC in the peripheral tissues, particularly during

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inflammation [164]. In patch-clamp studies using undifferentiated NG108 –15 cells, NaHS enhanced T-type VDCC currents, which may prove that H$_2$S 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$_{3.2}$ isofrom of T channels was activated by H$_2$S, demonstrated by the abolishment of H$_2$S induced-hyperalgesia using a general T-type channel blocker mibebradil, and similar results were produced using ZnCl$_2$ (Ca$_{3.2}$ specific inhibitor) and also with intrathecal administration of Ca$_{3.2}$-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$_2$S induced neurite outgrowth, which was found to be related to the activation of Ca$_{3.2}$ isoform T-type channels demonstrated with the abolishment of neurite outgrowth using general T-type channel inhibitor mibebradil, intracellular Ca$^{2+}$ chelator BAPTA-AM, and Ca$_{3.2}$ isoform specific blocker ZnCl$_2$ [157]. Interestingly, they also discovered that H$_2$S 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$_{3.2}$ isoform, by H$_2$S appears to regulate rhythmic neuronal activity, pain sensation, and differentiation of neurons and boosting of synaptic communication, similar to putative processes regulated by H$_2$S-related L-type channel activation.

**H$_2$S 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, (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, 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$_2$S and capsaicin receptor (TRPV$_1$)**

H$_2$S and its donors activate TRPV$_1$, ion channels in GI tract, airway, pancreas, and urinary bladder, which cause colonic mucosal CI 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 CI 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$_2$S 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$_2$S-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 tetratpentylammonium, which also block K$^+$ channels (K$_{CA}_1$ and K$_{ATP}$) [118]. As glibenclamide may inhibit CFTR, this result could also be interpreted as the direct activation of CFTR by H$_2$S to increase Cl$^-$ secretion. Similar to capsaicin, H$_2$S 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$_2$S 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-α and 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$_2$S 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$_2$S per se may induce neurogenic inflammation, even in the absence of other, often harmful, elements. Further research is still required to solve whether H$_2$S 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$_2$S 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$_2$S 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$_2$S stimulates capsaicin-sensitive primary afferent nerve terminals with the consequent release of tachykinsins, which subsequently produces contractile responses of the detrusor muscle. Furthermore, ruthenium red, a nonspecific blocker of TRPV$_1$ channels, blocked the H$_2$S-induced contractile response [168], but TRPV1-selective antagonist capsazepine and SB366791 failed to do so. It has also been theorized that H$_2$S may stimulate the TRPV$_1$, receptor by a different way from those known activators.

**H$_2$S and ankyrin (TRPA$_1$)**

TRPA, 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, was initially
characterized as a noxious cold receptor [176] and later its role in mechanosensation has been suggested [177,178]. In the rat bladder, TRPA, is expressed in unmethylated sensory nerve fibers with similar pattern to that of TRPV. Interestingly, TRPA, is also present in the urothelium, detected at both transcriptional and protein levels. The stimulation of TRPA, channels induced detrusor overactivity. TRPA, appears to be consistently colocalized with TRPV, in the bladder afferents, which suggests a role of TRPA, 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\(^{2+}\)], in Chinese hamster ovary cells expressing mouse or human TRPA, but not in untransfected cells. This indirect evidence for the activation of TRPA, by H\(_2\)S needs to be validated with more direct electrophysiological recording.

Should this role be confirmed, H\(_2\)S may function as a TRPA, activator potentially involved in inflammatory bladder disease and in lower urinary tract infection. Furthermore, bacterial metabolite H\(_2\)S induced potential shifts in smooth muscle cells. In the heart, H\(_2\)S and its donors cause the negative inotropic and chronotropic action through activating sarCa\(_{11}\), and mitoK\(_{ATP}\) channels and inhibiting L-type Ca\(^{2+}\)-channel activity and exert cardioprotection during I/R injury. H\(_2\)S-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\(_2\)S is via enhancing K\(_{ATP}\) channel and suppressing L-type Ca\(^{2+}\) channel activities. By elevating [Ca\(^{2+}\)], H\(_2\)S may mediate glutamate-induced neurotoxicity and neuronal cell death, but conflicting reports describe the protective effect of H\(_2\)S on neuron cells from oxidative glutamate toxicity by activating K\(_{ATP}\) and Cl- channels. H\(_2\)S-induced hyperalgesia in the colon seems to depend on the sensitization of T-type Ca\(^{2+}\) channels. On the other hand, H\(_2\)S has a pronociceptive role through evoking the excitation of capsaicin-sensitive TRPV\(_1\) containing sensory neurons. H\(_2\)S and its donors also activate TRPV, and TRPA, 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\(_2\)S has been confirmed in cardiovascular, endothelium, and nervous systems, which constitute a major molecular mechanism for many cellular effects of H\(_2\)S. 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\(_2\)S merits further investigation [27]. The effects of H\(_2\)S on voltage-dependent L-type Ca\(^{2+}\) channels or BK\(_{Ca}\) channels are inconclusive.

**Biological Roles of H\(_2\)S Vascular System**

**H\(_2\)S and myogenic tone**

Initial studies suggested that in the vascular wall H\(_2\)S is produced only by smooth muscle cells (SMCs) [38]. However, now it is clear that H\(_2\)S are produced by endothelial cells, peritoneal, 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\(_2\)S as the “third gaseous signaling molecule” was vasodilation. Intravenously administered H\(_2\)S or its donors decrease blood pressure in experimental animals and deficiency of endogenous H\(_2\)S has been implicated as a pathogenic factor in arterial hypertension [185]. In 2001, Zhao et al. showed that H\(_2\)S decreased blood pressure in rats in vivo and caused vascular smooth muscle cell (VSMC) relaxation in vitro [38]. H\(_2\)S-mediated vasodilation has also been shown in the smooth muscle of the ileum and the vas deferens [186]. H\(_2\)S 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\(_2\)S liberates NO from S-nitrosothiols [187]. Others show that endothelial denudation and nitric oxide synthase (NOS) inhibitors shift the concentration-response curve for H\(_2\)S [104]. However, H\(_2\)S increases eNOS phosphorylation and subsequent NO production in an Akt-dependent manner [188]. Teague et al. [186] reported a summation effect between H\(_2\)S and NO on the sublimation of the twitch responses of the ileum to electrical activation. The enhancing effect of H\(_2\)S ONO-induced vasorelaxation is still controversial. Zhao et al. [38] observed that pretreatment of aortic ring preparations with H\(_2\)S inhibited the vasorelaxant effect of the NO-producing agent SNAP [38]. However, Ali et al. [189] have shown that H\(_2\)S induced vasocomstriction and increased the mean arterial pressure in rats likely by scavenging endothelial NO. It is likely that the interaction of NO and H\(_2\)S may alter the vasorelaxant properties of these two gasotransmitters. Also, the common molecular target for NO and H\(_2\)S may become desensitized after firstly encountering one of them. The production of H\(_2\)S in the presence of NO is a different story. H\(_2\)S production by CSE in vascular tissues is increased by SNAP, 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\(_2\)S) resulted in an increase of endogenous H\(_2\)S 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\(_2\)S 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\(_2\)S as a vasorelaxing factor released from perivascular adipose tissue and acting via the stimulation of special K\(_v\) type channels – KCNQ channels [191]. Additionally, small, intermediate, and large conductance calcium-dependent potassium channels (SK\(_{Ca}\), IK\(_a\) and BK\(_{Ca}\)) have also been demonstrated as possible mediators of H\(_2\)S vasodilator effects in resistance vessels [122,123]. An H\(_2\)S-evoked increase in cyclic guanosine monophosphate (cGMP) levels could also be involved in H\(_2\)S-induced vasorelaxation of smooth muscle cells. Bucci et al. confirmed that H\(_2\)S 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\(_{Ca}\) channels to H\(_2\)S-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 vasoreactivity of vascular smooth muscle cells. In another statement, acidification has a vasorelaxant effect, whereas the alkalization of the intracellular environment causes vasoconstriction in most of the vascular bed. According to data published by Lee et al, H\(_2\)S could modify the pH equilibrium in cells by activating the Cl/HCO\(_3\) exchanger and thereby induce acidification [133]. However, the vasoreactive response of vessels to H\(_2\)S 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$_S$ applied [1,104]. H$_S$ relaxes small mesenteric arteries much more potent than aortic tissues [125]. Although rat aortic and mesenteric artery tissues produce similar levels of H$_S$, H$_S$ 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$_S$ speaks for the importance of H$_S$ in regulating peripheral resistance. The mechanisms for differential vasorelaxant effects of H$_S$ are not clear yet, but several possibilities exist. One explanation is the tissue-type specific distribution of the molecular targets of H$_S$. 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$_S$ and to intracellular calcium level may vary between conduit and resistant arteries [125].

Also, different types of blood vessels face different shear 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$_S$ should also be considered. It has been reported that H$_S$ 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$_S$ has the tendency to induce vasoconstriction [72]. This could result from the product of H$_S$ 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$_S$. Another note worth taking is that the release of NO from S-nitrosothiolamine by H$_S$ is oxygen dependent [72]. H$_S$ functions as a vasodilator in cerebral circulation. Topical application of H$_S$ 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$_S$. 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 as a vasodilator in cerebral circulation. Topical application of H$_S$ 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$_S$. 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$_S$ concentration in cerebrospinal fluid was increased about four fold after L-cysteine treatment, measured by GC-MS, which was again blocked by PPG. Whether this vasodilatory effect of H$_S$ 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$_S$. While H$_S$ relaxed phenylephrine- or norepinephrine-precontracted aortic tissues, high concentration of KCl (60 mM)-induced vascular contraction was essentially not affected by H$_S$. In effect, though H$_S$ 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$_S$. Sodium hydrosulphide (NaHS) at concentrations over 100 μmol/L evoked the relaxation of precontracted isolated rat arteries [7,38,189].

Higher concentrations of H$_S$ (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$_S$-gassed solution has much stronger vasorelaxant effects than NaHS solution does. The involvement of various signal transduction pathways in the vasodilator effects of H$_S$ has been examined. NO and CO relax smooth muscle by activating guanylyl cyclase to increase the production of cGMP. H$_S$ does not affect the production of cGMP, which leads to the inference that there is a different mechanism for the effect of H$_S$. Earlier studies also demonstrated that the vasorelaxant effects of H$_S$ on rat vascular tissues are uniquely 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$_S$, indicating that superoxide anion and hydrogen peroxide did not contribute to H$_S$-induced acute vasorelaxation. Although ODQ blocked the vasorelaxation induced by SNP, it had no effect on the vasorelaxant effect of H$_S$ on rat aortic tissues. Therefore, under this experimental condition, the vasorelaxant effect of H$_S$ was not mediated by the cGMP pathway [38]. K$_{ATP}$ channel is the major molecular target of H$_S$ for its vasorelaxant effect and smooth muscle hyperpolarization [38,195]. In the ileum, glibenclamide did not interfere with the relaxation induced by H$_S$ [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$_S$ were shown to be cysteine 6 and 26 of the extracellular portion of the α-subunit of the K$_{ATP}$ channel complex [103]. These vicinal thiol forms a disulphide bond, which H$_S$ reduces, increasing channel conductance and induce hyperpolarization in a tissue-dependent manner. On the other hand, some observations revealed an opposite effect of H$_S$ on smooth muscle cells of the arterial wall. Lower concentrations of H$_S$ (Na$_S$: 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$_S$-induced vasoconstriction. One possible mechanisms of H$_S$-induced vasoconstriction is decreased levels of cyclical adenosin monophosphate (cAMP) in smooth muscle cells. Li et al showed on the rat cerebral artery that H$_S$ evoked a decrease of cAMP levels, an effect that was associated with the promotion of an interaction between actin and myosin [199].

The H$_S$-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$_S$ 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$_S$-induced vasodilatation was due to the inhibition of the cAMP/adenyl cyclase pathway. It was also shown that the administration of low concentrations of H$_S$ (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 vasorelaxation evoked by H$_S$ 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$_S$ 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$_S$, 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$_S$ has marked effects on the circulation, by acting as a hypoxic vasconstrictor or vasodilator in the pulmonary and systemic circulation, respectively [202,203]. H$_S$ 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$_2$S 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$_2$S and vascular endothelial cell proliferation

The same physiological stimuli do not necessarily elicit the same functional responses from different types of cells. While H$_2$S inhibits vascular SMC proliferation, the neurotransmitter 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$_2$S 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$_2$S donors on ECs could not be detected if the concentrations of H$_2$S donors were higher than physiologically relevant levels. The signaling pathways underlying the stimulatory effect of H$_2$S 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$_2$S have all been suggested in ECs [166]. Increased intracellular calcium concentration ([Ca$^{2+}$]) in cultured human saphenous vein endothelial cells by NaHS treatment has also been reported [210]. This increase in [Ca$^{2+}$], was mostly due to calcium release from ryandoline 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$_2$S on [Ca$^{2+}$], levels in ECs to H$_2$S-stimulated EC proliferation [210]. H$_2$S 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$_2$S 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 aortal 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$_2$S in protecting ECs from hyperglycemic damage was further demonstrated in CSE KO mice. The isolated thoracic aortal 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$_2$S 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$_2$S is an important endogenous modulator of cell proliferation and apoptosis [211]. Serum deprivation up-regulated CSE expression and H$_2$S 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$_2$S 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 wild-type mice [214]. Clearly, endogenous CSE/H$_2$S 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$_2$S affects the effect of exogenous H$_2$S on cell apoptosis. Yang et al. [213] found that NaHS induced apoptosis of human aortic SMCs at concentrations 200 M. After inhibition of endogenous H$_2$S 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 wild-type (WT) mice [214]. CSE/H$_2$S 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$_2$S 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$_2$S 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$_2$S 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$_2$S was significantly attenuated. Therefore, the activation of ERK and its downstream factor caspase-3 likely mediates H$_2$S-induced cell apoptosis [211]. It is worth pointing out that in many other cell types or tissues, ERK activation serves as a proliferative/apoptotic signal. It has been reported that the proliferation of cultured rat aortal 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$_2$S-treated intestinal epithelial cells (IEC-18) [218]. The pro-apoptotic effect of H$_2$S 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-1,2,3-thione-5-yl)phenyl ester] is a novel molecule comprising an H$_2$S-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 GI phase were not affected by S-diclofenac but asynchronous SMCs manifested with an increase in apoptotic cell death. S-diclofenac stabilized p35 and induced p21, p53,Alf1 and Bax. But the anti-apoptotic factor Bcl-2 was not affected [219]. In CSE-overexpressed cell or exogenous H2S-treated cells, there are also an increased expression of p21/Gip/WAK-1 and a down-regulation of cyclin D1 [217].

The anti-proliferative and/or pro-apoptotic effect of H2S 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].

H2S and angiogenesis

H2S 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 H2S cytotoxicity [207]. In cell culture experiments, low micromolar concentrations of H2S 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 H2S [208]. Additionally, aortic tissue isolated from transgenic mice lacking CSE, the primary H2S-producing enzyme in the endothelium, exhibit marked decreases in angiogenesis [208]. The mechanism of H2S-induced angiogenesis operates through several pathways, including activation of ATP-sensitive potassium (KATP) channels [38]. Papapetropoulos et al. showed that treatment of endothelial cells with the KATP channel inhibitor glibenclamide reduced cell migration, which was accompanied by decreased H2S-induced p38 and heat shock protein 27 (Hsp27) phosphorylation [208]. Additionally, H2S can stimulate angiogenesis through phosphatidylinositol 3-kinase (PI3K) and Akt activation [166]. H2S can also activate hypoxia inducible factor-1a (HIF-1a) and thus increase expression of vascular endothelial growth factor (VEGF) [221]. Conversely, VEGF-stimulated angiogenesis is suppressed in CSE knockout mice [166]. Endogenous H2S production is known to be upregulated during wound healing [222]. Topically applied H2S 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 H2S treatment [224]. These studies indicate that endogenous H2S is crucial in physiological angiogenesis and that those capabilities can be employed in disease treatment.

Conclusion

H2S 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 KATP and KCNQ channels, respectively. The mechanism of vascular effect of H2S is controversial with opposite data sometimes provided by different studies. These discrepancies result most likely from using different animal species, different H2S donors and their concentrations, as well as experimental conditions, such as buffer composition or oxygen levels. Generally H2S has marked effects on the circulation, by acting as a hypoxic vasoconstrictor or vasodilator in the pulmonary and systemic circulation, respectively. Although H2S 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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