

# Actions of hydrogen sulfide in the vascular wall

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## Abstract

In the vascular wall, hydrogen sulfide ( $H_2S$ ) inhibits inflammation, stimulates angiogenesis, and activates vasodilation. There are multiple sources of  $H_2S$  in the vascular wall, but cystathionine gamma-lyase (CSE) within endothelial cells appears to produce most of the local, vascular  $H_2S$ . Generated  $H_2S$  acts on smooth muscle, endothelial, inflammatory, and adipose cells within the vascular wall and contributes to circulating levels of  $H_2S$  and  $H_2S$  metabolites.  $H_2S$  signaling is generally beneficial with evidence that it inhibits inflammation and protects cells from oxidative stress.  $H_2S$  also stimulates vasodilation and suppresses cytokine generation. Oxidized low-density lipoproteins (oxLDL) and hyperglycemia downregulate the pathway in cultured cells and disease states. Lower plasma and urine levels have been reported in human studies of diabetes, hypertension, and atherosclerosis so that suppression of this system may contribute to vascular disease. Activation of the system or supplementation with exogenous donors of  $H_2S$  appears to protect from vascular and inflammatory diseases. Areas of active research include delineating signal transduction pathways both upstream of CSE and downstream of released  $H_2S$  as well as defining more accurate and user-friendly ways to measure endogenous production.

## Introduction

Hydrogen sulfide's ( $H_2S$ ) role in regulating the cardiovascular system is not completely defined, but  $H_2S$  is rapidly gaining credentials as an important player in cardiovascular health. Recent publications describe pathways of  $H_2S$  signaling and define some endogenous regulators of its production. Additional studies have shown that  $H_2S$  produced throughout the body regulates many functions and this review will focus on recent work describing roles in the cardiovascular system, especially in the vascular wall.

Within the vascular wall, several pathways produce  $H_2S$ . A recent study by Leskova and co-workers in cultured HUVECS observed that these cells take up exogenous thiosulfate for later release as free  $H_2S$  while numerous other studies report  $H_2S$  production in the vascular wall by cystathionine gamma-lyase (CSE) [1-7], cystathionine beta-synthase (CBS) [8-10] and 3-mercaptopyruvate sulfurtransferase (3-MST) [11,12]. Thus, the synthesis of this gasotransmitter is not completely defined and remains an area of active investigation as discussed below.

After synthesis and release,  $H_2S$  has effects in the vascular wall that include inhibition of inflammation [13-17], stimulation of angiogenesis [18-22], increased production of endothelium-derived relaxing factors [23-25], activation of antioxidant pathways [26], and direct stimulation of vascular smooth muscle cell vasodilation [8,27,28]. These pathways will be discussed below along with a discussion of  $H_2S$  production and activity in several cardiovascular diseases.

## Synthesis of hydrogen sulfide

Hydrogen sulfide ( $H_2S$ ) is a very important signaling gas that is acquired through both diet and bacterial flora and also produced endogenously by a number of enzymes. The major enzymes responsible for the endogenous production of  $H_2S$  are CBS, cystathionine  $\gamma$ -lyase CSE, and 3-MST. (Figure 1) These pyridoxal 5'-phosphate-dependent enzymes act as catalysts in the desulfuration of cysteine [29]. CSE mRNA, but not CBS, has been detected in endothelium-denuded aorta,

mesenteric-, tail-, and pulmonary arteries, demonstrating that CSE is found in vascular smooth muscle cells [30]. Similarly, Shibuya et al. showed that CSE and CBS protein is not expressed in rat thoracic aortic endothelial cells. However, recent studies have demonstrated that CSE is in fact expressed in mesenteric endothelial cells [31]. Others have also demonstrated the presence of 3-MST in rat aortic endothelial cells and smooth muscle cells [32]. Thus, these enzymes are present in both smooth muscle and endothelial cells in the vasculature and more than one type of  $H_2S$  synthesis enzyme may be co-expressed in some cells.

CSE proteins are localized in the cytosol under physiological conditions. In response to high calcium levels, CSE has been shown to translocate to the mitochondria where cysteine is metabolized, and  $H_2S$  is produced [33]. However, much is still unknown about the regulation of this enzyme and its ability to move between intracellular compartments and this is an active area of research.

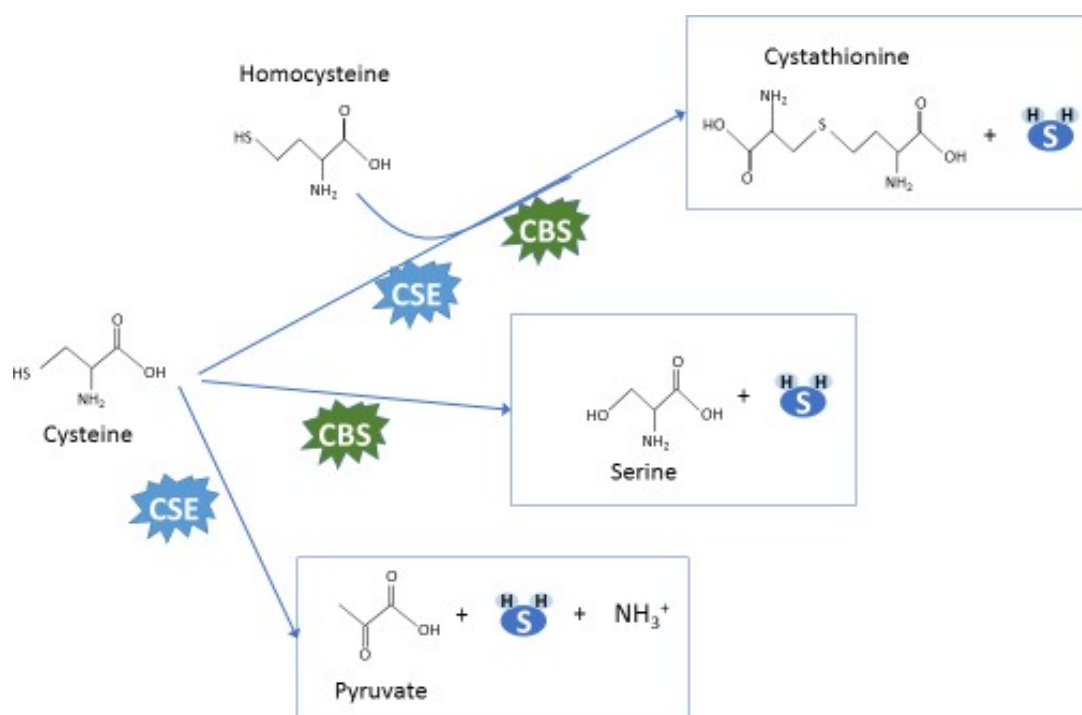
Sulfur exists in the body in several forms that are frequently categorized into one of three pools- free sulfide, acid labile sulfide, and reductant-labile or bound sulfane sulfur. Depending on the local environment (pH, temperature, etc.), sulfur can be liberated from each pool and modified to increase the bioavailability of specific sulfur species including  $H_2S$ . In addition to occurring in the free state,  $H_2S$  reacts to form species belonging to the acid labile or reductant-labile bound pool.

$H_2S$  is water-soluble and dissociates into  $H^+$  and  $HS^-$  with further dissociation to  $S^{2-}$  under alkaline conditions. These ions are referred to as free sulfide. Acid-labile sulfide pools include iron-sulfur cluster-containing proteins. Acid-labile  $H_2S$  is released in

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**Figure 1.** Synthesis of Hydrogen Sulfide in Vascular Endothelial Cells. Hydrogen sulfide (H<sub>2</sub>S) can be synthesized in the vascular wall from cysteine by both cystathionine gamma lyase (CSE) and by cystathionine beta synthase (CBS). CSE is the primary pathway identified in most endogenous endothelial cells.

buffer solutions at pH 5.4 or lower. Within cells, acid-labile H<sub>2</sub>S is present in the mitochondria at pHs between 7 and 8. One possible mechanism of this greater acid-labile H<sub>2</sub>S release in the mitochondria is by detachment via enzymes, but this has not been convincingly established [34]. Oxidation of H<sub>2</sub>S generates disulfide bonds between two sulfur atoms to generate hydrodisulfides, hydropolysulfides, and polysulfides, collectively known as reductant-labile bound sulfane sulfur [34,35]. Reducing agents such as tris(2-carboxyethyl) phosphine hydrochloride (TCEP) have been shown to induce the release of H<sub>2</sub>S from bound sulfur sulfane pools [35]. Ogasawara *et al.* were the first to investigate tissue distribution of bound and acid-labile H<sub>2</sub>S *in vivo*. They demonstrated that the majority of H<sub>2</sub>S in liver, kidney, brain, and spleen of Wistar rats is in the form of sulfane sulfurs, while acid-labile sulfur is the predominant form of H<sub>2</sub>S in the heart. The authors further investigated the subcellular distribution of sulfane sulfurs and acid-labile sulfurs in cells from the kidney and liver and found that acid-labile sulfur is predominantly found in the mitochondria while sulfane sulfurs predominate in the cytosol [36]. In another study, mouse aorta had the highest concentration of free H<sub>2</sub>S, while the heart and kidney contained the highest concentrations of acid-labile sulfides [37].

### Inhibition of inflammation

Inflammatory pathways have been implicated in many cardiovascular diseases and pathways that limit inflammation have been shown to be protective on many levels as discussed in multiple recent reviews [38-41]. A part of the protective effect of H<sub>2</sub>S against the development and progression of cardiovascular disease is via inhibition of inflammation and oxidative stress. A recent study by Li *et al.* [4] observed that CSE KO mice have significantly higher plasma levels of homocysteine and are resistant to the protective effects of estrogen on the development of atherosclerotic lesions. However, administering exogenous H<sub>2</sub>S was not sufficient to restore responsiveness to estrogen in the CSE KO mice suggesting both the level and the location of H<sub>2</sub>S

production are critical in the anti-inflammatory response to estrogen.

H<sub>2</sub>S suppresses macrophage-mediated inflammation by increasing heme oxygenase-1 expression in macrophages to inhibit nuclear factor-kappa-b (NFκB)-dependent cytokine production [42]. A similar effect is observed in endothelial cells exposed to lipopolysaccharide (LPS), where suppression of endogenous H<sub>2</sub>S production leads to augmented cytokine production and cell hyperpermeability [43]. In this study by Bourque and co-workers, LPS decreased expression of CSE in cultured endothelial cells, while overexpression of CSE suppressed LPS activation of NFκB to protect cells from LPS-induced inflammation. Treating macrophages with oxidized low-density lipoproteins (ox-LDL) is another *in vitro* model that has been used to demonstrate that CSE overexpression reduces cytokine generation and the resultant systemic inflammatory response [44]. H<sub>2</sub>S inhibition of cytokine generation is thought to protect the vascular wall from the initiation and growth of atherosclerotic lesions. *In vivo* studies have confirmed that CSE KO in ApoE KO mice increases lesion development and this is ameliorated by administration of exogenous H<sub>2</sub>S donors [45]. Furthermore, blood levels of H<sub>2</sub>S correlate inversely with the severity of atherosclerosis in chronic hemodialysis patients [46] and higher levels of urinary sulfates are associated with a lower risk of renal events in patients with type 2 diabetes [47]. This is corroborated in animal and cell-based studies of hyperglycemia showing that exogenous H<sub>2</sub>S prevents diabetic complications in rats [48-51] and mice [52-56] through improved vascular function, decreased cardiac fibrosis, and improved renal function.

Direct actions of H<sub>2</sub>S to protect endothelial cells from stress signals and enhance cell survival are in part responsible for this effect [9,32, 57-61]. Studies by Xie *et al.* [62] aimed to uncover the mechanism by which H<sub>2</sub>S protects against oxidative damage in endothelial cells. Cell counting kit 8 (CCK8), Annexin V/PI staining and lactate dehydrogenase (LDH) release analysis showed that pretreatment with the slow releasing H<sub>2</sub>S

donor, GYY4137, protected EA.hy926 endothelial cells from  $H_2O_2$ -induced cell injury and death. The endothelial cells treated with  $H_2O_2$  had increased ROS formation and decreases in oxygen consumption and ATP production and pretreatment with GYY4137 prevented these changes. Notably, when expression of the mitochondrial enhancer of ROS scavenging, NAD-dependent histone deacetylase sirtuin-3 (SIRT3) [42, 55] was reduced by siRNA, the preventive effect of  $H_2S$  against  $H_2O_2$ -induced damage was abolished. Finally, luciferase reporter assays revealed that  $H_2S$  increases expression of SIRT3 and superoxide dismutase 2 (SOD2). These findings demonstrate that  $H_2S$  improves mitochondrial function and protects endothelial cells from cytotoxicity to enhance cell survival. Others have also observed that  $H_2S$  protects mitochondrial function from oxidative damage [54, 61]. Thus, both endogenous and exogenous  $H_2S$  are able to prevent inflammation and oxidative damage through multiple pathways and this prevention contributes to protection from disease related morbidity. (Figure 2)

### Vasodilation

Hydrogen sulfide induces vasodilatory responses via endothelium-independent and endothelium-dependent mechanisms. Zhao *et al.* examined the vasoactive effects of  $H_2S$  in rat aortic tissues [63-70]. The report shows a dose-dependent inhibition of  $H_2S$ -induced vasodilation of rat aortic rings by the  $K_{ATP}$  channel inhibitor, glibenclamide. In isolated smooth muscle cells,  $H_2S$  increased  $K_{ATP}$  current amplitudes and hyperpolarized the cells similar to the responses to the  $K_{ATP}$  channel agonist, pinacidil and glibenclamide reversed the  $H_2S$ -induced responses.

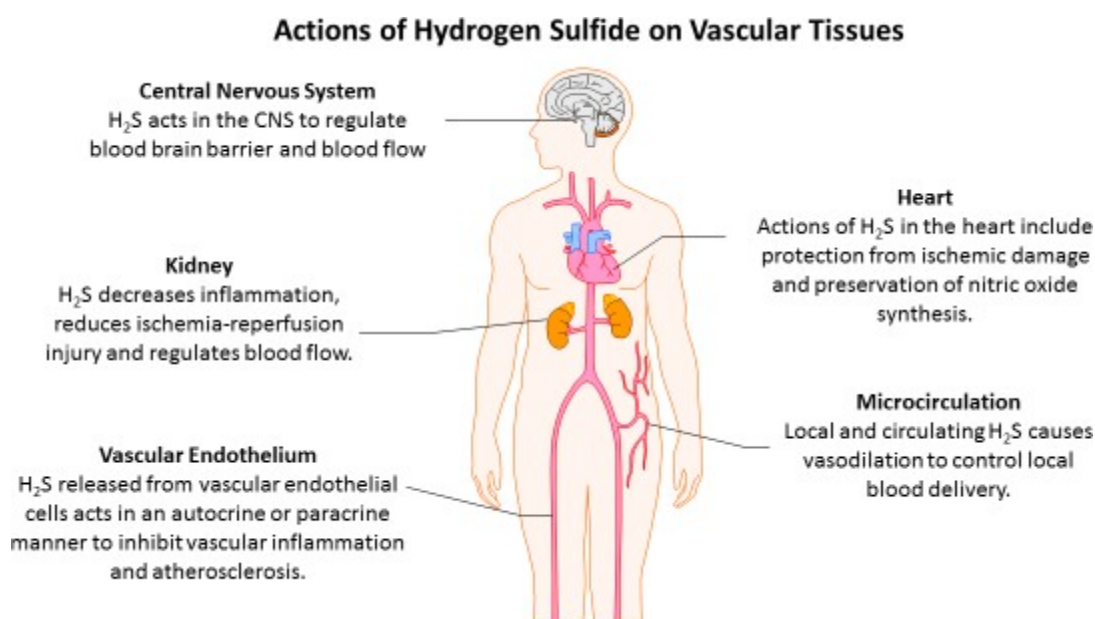
This report demonstrated that  $H_2S$  can activate VSMC  $K_{ATP}$  channels to induce membrane hyperpolarization and vasodilation. In a later study, Zhao *et al.* [69] examined the actions of  $H_2S$  on the endothelium to cause vasorelaxation. In this study, removal of the endothelium with saponin did not affect maximum  $H_2S$ -induced relaxation of rat aortic rings but require higher concentrations of  $H_2S$  to elicit a response suggesting the endothelium is a potential target of  $H_2S$ . Other studies, however, have observed a significant role of the endothelium in  $H_2S$ -induced vasodilation. Our group reported a dramatic loss of sodium

hydrogen sulfide (NaHS)-induced dilation in endothelium-denuded mesenteric arteries [21]. NaHS-induced dilation is also abrogated in arteries after luminal application of the  $BK_{Ca}$  inhibitor, iberiotoxin (IbTX). This study identified EC- $BK_{Ca}$  as targets of  $H_2S$  and support an endothelium-dependent mechanism of  $H_2S$ -induced vasodilation.

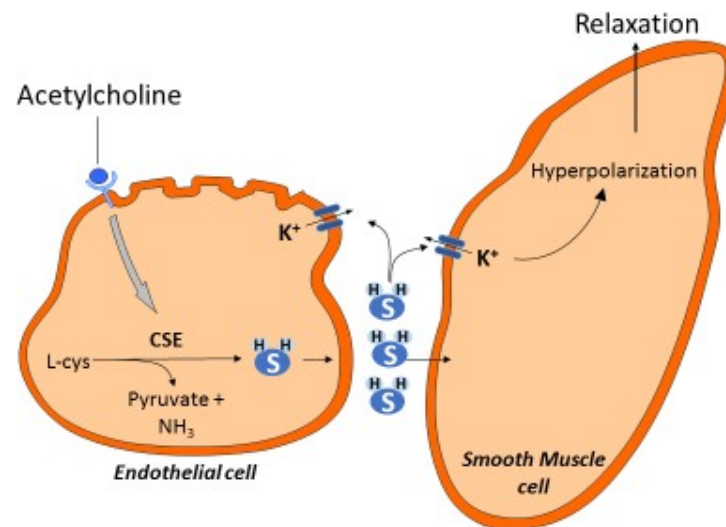
Mustafa *et al.* also reported that  $H_2S$  is an endothelial-derived hyperpolarizing factor (EDHF) [36]. Their studies in arteries and cultured endothelial cells found that acetylcholine hyperpolarized cells from wild-type mice but did not affect cells from mice lacking CSE.

Additionally, arteries from the CSE+/+ mice did not dilate to  $H_2S$  in the presence of apamin and charybdotoxin, inhibitors of small and intermediate conductance calcium-activated potassium channels (SK and IK) respectively. These findings identified  $H_2S$  as a physiological EDHF. (Figure 3)

The role of  $H_2S$  as an EDHF is not limited to the sulphydration of potassium channels. In fact,  $H_2S$  also enhances production and vascular signaling of another well described EDHF, nitric oxide (NO) [22, 5]. Chen *et al.* [8] examined the signaling pathways of  $H_2S$  and NO in human EA.hy926 endothelial cells. They report a significant increase in the NO producing enzyme, endothelial nitric oxide synthase (eNOS) and in NO levels in response to the hydrogen sulfide donor NaHS.  $H_2S$ -induced increases in eNOS expression has also been observed by others [31,35,41]. The enhancement of NO production by  $H_2S$  is not limited to eNOS expression. Multiple studies have shown direct activation of eNOS by  $H_2S$ . Huang *et al.* [19] found increased phosphorylation of the activating serine residue eNOS1177 following NaHS incubation. King *et al.* [23] also that knockout of CSE reduced phosphorylation of eNOS1177 while increasing phosphorylation of an inhibitory site eNOST495. These changes correlated with impaired eNOS function in the CSE KO mice. These findings highlight the dual effect of  $H_2S$  to enhance the activating phosphorylation and reduce the inhibiting phosphorylation state of eNOS. In addition to enhancing the activity of eNOS via phosphorylation,  $H_2S$  can directly stabilize the dimeric state of eNOS to enhance NO production as examined by Altaany *et al.* [3]. In their studies, NaHS increased eNOS dimer/eNOS monomer ratio,



**Figure 2.** Hydrogen sulfide ( $H_2S$ ) acts on the vasculature throughout the body to elicit protective and beneficial actions. In addition, it has direct effects on cardiac muscle [1] and on renal tissues [22] that have been recently reviewed.



**Figure 3.** Hydrogen Sulfide Acts as an Endothelial Dilating Factor. Hydrogen sulfide ( $H_2S$ ) is synthesized in vascular endothelial cells and then diffuses out of the cell to act in a paracrine manner on adjacent vascular smooth muscle cells or in an autocrine manner on endothelial cells. It has been shown to activate potassium channels in both cell types contributing to smooth muscle cell relaxation and subsequent vasodilation.

**Table 1.** Source of  $H_2S$  Effect in different Species Tissues.

Tissue	Species	Source-Effect of $H_2S$	References
Kidney	Mice	CBS- increases amino acid reabsorption, protects from ischemia/reperfusion injury	[57,58]
Kidney	Rat	Donor: improved endothelial dilation, increased eNOS phosphorylation, increases GFR	[59,60]
Adipocytes	Mice	CSE-promotes glucose uptake and lipid storage	[61]
VSMC	Mice	CSE-inhibits $IP_3$ receptor $Ca^{2+}$ release	[62]
Endothelial Cells (EC)	Mice, Human umbilical vein EC, rat aortic EC	CSE-reduces eNOS phosphorylation and activity, contributes to acetylcholine-induced dilation, Donor: sulfhydrates Kir 6.1 channels, upregulates SIRT3 pathway, increases NO production, blocks NFkB activation, decreases atherosclerotic plaque formation	[43,45,63-68]
Mitochondria	Mice	Donor-Induces nrf2 to upregulate antioxidant pathways, increases mitochondrial electron transport (3MST), prevents ROS damage	[69,70]
Liver	Mice	High fat diet increases CSE and CBS expression, elevates homocysteine metabolism, gluconeogenesis	[71,72]
Cerebral circulation	Mice	Donor-Protects from ischemia	[73]
Cerebral circulation	Rats	Donor: Constricts basilar artery	[74]
Heart	Frog	Donor-Decreased stroke volume and phosphorylated eNOS,	[75]
Heart	Rat	Donor-Decreased stroke volume and phosphorylated eNOS, ERK and AKT phosphorylation to protect from ischemia/reperfusion, decreased fibrosis	[75,76]
Pancreas	Rat	CBS-Releases $Ca^{2+}$ from acinar cells	[77]
Macrophage	Mouse	CSE-Increases HO-1 expression, inhibits iNOS activity, increased sulfhydration of KEAP1 to activate Nrf2	[42,52]

demonstrating the ability of  $H_2S$  to stabilize and enhance activity of eNOS. Taken together,  $H_2S$  increases eNOS expression, stability, and activation, ultimately leading to elevate NO production.

## Conclusion

Understanding of how and where  $H_2S$  acts in the vasculature is much greater than it was 10 years ago, including the discovery of new pathways for synthesis, additional vascular targets, and increased understanding of the biochemistry and metabolism in the vascular wall. It is clear that there are many parallels to the nitric oxide system as well as some important differences. That is, both are synthesized

within vascular endothelial cells from an amino acid precursor and act on both autocrine and paracrine targets to protect the vascular wall from oxidative stress and to regulate local blood flow. However, it is not clear if  $H_2S$  synthesis is elevated by acute receptor activation similar to receptor or flow activation of eNOS or if regulation of the enzyme is primarily at the transcriptional level, more analogous to activation of endothelin generation. In addition, plasma levels of  $H_2S$  have not been clearly demonstrated to reflect tissue levels and more information is needed to determine appropriate ways to evaluate  $H_2S$ -producing enzyme activity of the system. This is in part due to the high volatility of  $H_2S$ , which is rapidly depleted into the headspace of solutions and



is also subject to rapid oxidation. Therefore, accurate analysis of both tissue and plasma levels of H<sub>2</sub>S is one of the greatest challenges in better understanding the role of this compound under different disease conditions. Finally, much has been elucidated in the H<sub>2</sub>S signaling pathways, but questions remain on where and how production is stimulated, what are physiologically relevant tissue and plasma concentrations and what are the cellular targets for modification by H<sub>2</sub>S. Future studies are expected to continue to clarify the roles of this important gasotransmitter in health and disease. Source of H<sub>2</sub>S Effect in different Species Tissues is given in Table 1. [71-84]

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