

# Hydrogen sulfide: a new EDRF

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**The first endothelium-derived relaxing factor (EDRF) ever identified is a gasotransmitter, nitric oxide (NO). Recent studies have provided several lines of evidence to support the premise that hydrogen sulfide (H<sub>2</sub>S), another gasotransmitter, is a new EDRF. H<sub>2</sub>S production is catalyzed in mammalian cells by cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE). The expression of CSE proteins and the activity of CBS have been observed in vascular endothelial cells. A measurable amount of H<sub>2</sub>S is produced from endothelium upon muscarinic cholinergic stimulation. The endothelium-dependent vasorelaxation induced by H<sub>2</sub>S shares many common mechanistic traits with those of endothelium-derived hyperpolarizing factor (EDHF). Deficiency in CSE expression increases blood pressure in CSE knockout mice and significantly diminishes endothelium-dependent relaxation of resistance arteries. More extensive and mechanistic studies in the future will help to determine whether H<sub>2</sub>S is a new EDRF or the very EDHF.**

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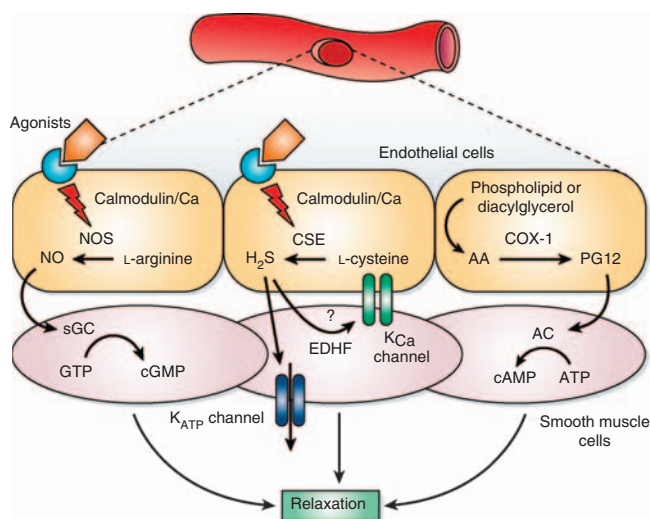
Dilation of blood vessels can be regulated by a group of labile humoral substances released from the endothelium, namely endothelium-derived relaxing factors (EDRFs). The paradigm-shaking discovery of nitric oxide (NO) as an EDRF decades ago<sup>1</sup> marked the beginning of a new era in cardiovascular research. Upon activation by different stimuli, such as acetylcholine and bradykinin, endothelial cells have their intracellular calcium level increased, which subsequently activates the endothelial NO synthase (eNOS). This leads to the production of NO and citrulline from the substrates L-arginine and molecular O<sub>2</sub>. NO is released from endothelial cells and stimulates guanylyl cyclase in vascular smooth muscle cells (SMCs). Consequently, cGMP levels in vascular SMCs are elevated and the cells relax (Figure 1).

The initial excitement of NO discovery has transformed into many new explorations and triggered wave after wave of aftershocks. It is generally agreed upon that there are more than one EDRF, which are produced by the endothelium and cause blood vessel dilation. Prostacyclin (PGI<sub>2</sub>) is produced from the endothelium through cyclooxygenase-1, and binds to specific receptors in SMCs and activates adenylate cyclase. Thus, increased cAMP levels in SMCs relax the cells (Figure 1). In most cases, NO-mediated endothelium-dependent vasorelaxation occurs in large conduit arteries whereas the endothelium-dependent relaxation of peripheral resistance arteries does not depend on NO production. After eliminating the endothelial production of NO and PGI<sub>2</sub> by knocking out the expression of eNOS and cyclooxygenase-1, there is still residual endothelium-dependent relaxation.<sup>2</sup> Even considering only non-prostanoid EDRF, NO may not be alone. The long-standing mystery surrounding the endothelium-derived hyperpolarizing factor (EDHF) has not been solved, and the roles of other gasotransmitters<sup>3</sup> in endothelium-dependent vasorelaxation have been questioned in recent years.

The chemical profile of hydrogen sulfide (H<sub>2</sub>S) is similar to that of NO. Both are small molecules of gas, generated in mammalian cells by enzymatic catalyzation, and freely permeable to lipid bilayer. H<sub>2</sub>S has been traditionally viewed as a toxic gas, and is infamous for its of 'rotten egg' smell. Its toxicological effect is mainly manifested as acute intoxication and loss of central respiratory drive. Only in the last decade, biological and physiological importance of this gas molecule has been exposed under limelight. In mammalian cells, H<sub>2</sub>S is produced from L-cysteine, catalyzed by one of two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE).<sup>3</sup> In addition to H<sub>2</sub>S, L-cysteine

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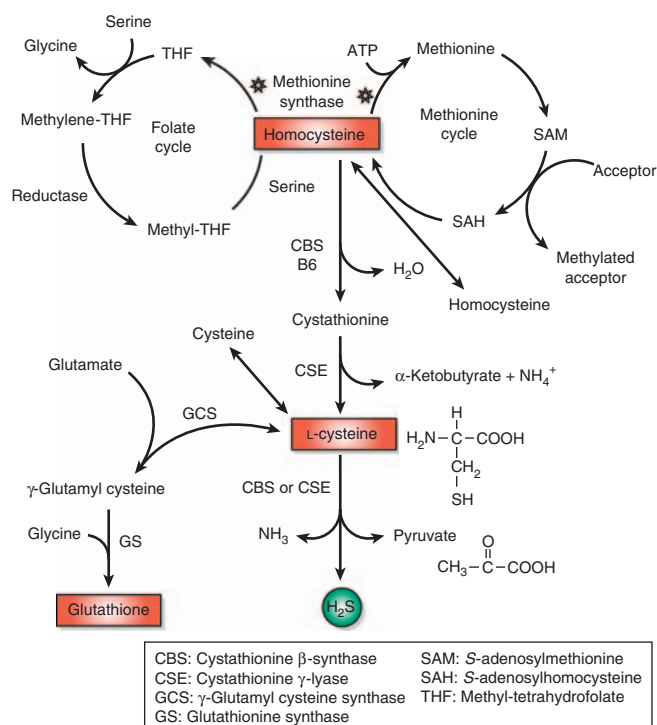
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**Figure 1 | Molecular basis for endothelium-dependent vasorelaxation.** With different substrates, NO, H<sub>2</sub>S, and PGI<sub>2</sub> are produced from the endothelium by NOS, CSE, and COX-1, respectively. They are released from the endothelium and act on the adjacent smooth muscle cells to induce relaxation. NO and H<sub>2</sub>S are also produced in smooth muscle cells. For brevity of description, smooth muscle production of these substances is not included in this diagram. NOS, NO synthase; CSE, cystathionine  $\gamma$ -lyase; COX-1, cyclooxygenase-1; AA, arachidonic acid; EDHF, endothelium-derived hyperpolarizing factor.

catabolism also generates ammonium and pyruvate (Figure 2). The expressions of CBS and CSE are tissue-type specific. CBS is reported to be the predominant H<sub>2</sub>S-generating enzyme in the brain and nervous system and CSE is the predominant H<sub>2</sub>S-generating enzyme in pancreatic  $\beta$ -islets, different vascular tissues, and the heart.<sup>3</sup> Physiological range of H<sub>2</sub>S in circulation is 10–100  $\mu$ M in fish, rats, and human. Endogenous level of H<sub>2</sub>S in brain tissue is approximately 50–160  $\mu$ M.

Our knowledge on the cardiovascular effects of H<sub>2</sub>S has amounted in recent years, and the momentum in searching the physiological and pathophysiological roles of H<sub>2</sub>S has been built up. H<sub>2</sub>S induces relaxation of different vascular tissues (for example, rat aorta and mesenteric arteries), and protects the heart from ischemia/reperfusion damage. H<sub>2</sub>S can induce vasorelaxation by directly opening K<sub>ATP</sub> channels in vascular SMCs. K<sub>ATP</sub> channel blocker, glibenclamide, inhibited H<sub>2</sub>S-induced dilation of aorta,<sup>4</sup> mesenteric artery beds,<sup>5</sup> or hepatic microcirculation<sup>6</sup> and abolished H<sub>2</sub>S-evoked blood pressure decrease *in vivo*.<sup>4</sup> Using the whole-cell and single-channel patch-clamp technique, direct evidence was obtained that exogenous H<sub>2</sub>S activated K<sub>ATP</sub> channels and hyperpolarized cell membrane of rat aorta and mesenteric artery SMCs.<sup>4</sup> Glibenclamide also abolished H<sub>2</sub>S-facilitated carotid sinus baroreflex.<sup>7</sup> Inhibition of endogenous H<sub>2</sub>S production with D,L-propargylglycine, a membrane permeable and irreversible inhibitor of CSE, reduced whole-cell K<sub>ATP</sub> currents.<sup>4</sup> In myocardium, a mitochondrial K<sub>ATP</sub> channel blocker (5-hydroxydecanoate) or a plasma membrane K<sub>ATP</sub> channel blocker (glibenclamide) abolished H<sub>2</sub>S-offered cardioprotection against myocardial infarction damage.<sup>8</sup>



**Figure 2 | Endogenous synthesis of hydrogen sulfide in mammalian cells.**

With a broader concept to encompass the origination from endothelium and downstream target of SMCs, we have more than one EDRF. Certainly, EDHF can be categorized into the EDRF class and the idea of H<sub>2</sub>S being a new EDRF is both intriguing and appealing.

## LINES OF EVIDENCE SUPPORT THE IDENTIFICATION OF H<sub>2</sub>S AS AN EDRF

### Endothelium production of H<sub>2</sub>S

Elucidation of the source of H<sub>2</sub>S production in the blood vessel wall and the physiological role of this gas in regulating cardiovascular function may pave the way for new therapeutic approach, either pharmacologically or genetically, to treat and prevent many different cardiovascular diseases, including hypertension and heart damage. In an earlier study, CSE mRNA expression was detected in smooth muscles of rat aorta wall, using an *in situ* hybridization method.<sup>4</sup> In cultured rat aortic endothelial cells, CSE mRNA was not detected using reverse transcription-PCR.<sup>4</sup> CSE protein expression was not conducted at that time due to the lack of CSE-specific antibody.<sup>4</sup> In the liver, CSE was found in hepatocytes and hepatic stellate cells, but not in sinusoidal endothelial cells.<sup>6</sup> It appeared that the vascular endothelium *per se* was not equipped with H<sub>2</sub>S-generating enzymes. This notion has been recently challenged and it no longer holds true. CSE protein has been detected in cultured bovine aortic endothelial cells and human umbilical vein endothelial cells. Yang *et al.*<sup>9</sup> provided immunohistological evidence for a predominant localization of CSE protein in the endothelial layer of vascular tissues of wild-type mice. This newly defined

endothelial localization of CSE can be largely ascribed to the availability of newly developed selective and specific anti-CSE antibody. Direct evidence for the endothelium production of H<sub>2</sub>S was also obtained. Cultured vascular endothelial cells produce measurable H<sub>2</sub>S and silencing CSE in these cells significantly decreases H<sub>2</sub>S production.<sup>9</sup>

#### Physiological stimulus for endothelial H<sub>2</sub>S production

Similar to NO, acute production of H<sub>2</sub>S from vascular endothelial cells is triggered by muscarinic cholinergic activation. Methacholine, a cholinergic receptor agonist, increased H<sub>2</sub>S levels in cultured endothelial cells by more than twofold. Atropine, a cholinergic antagonist, abolished methacholine effect. In the presence of the calcium ionophore A23187, endothelial production of H<sub>2</sub>S is significantly increased. Chelating of intracellular-free calcium with BAPTA abolished the effect of A23187 on H<sub>2</sub>S production as well as lowering the basal level of H<sub>2</sub>S in endothelial cells. Furthermore, co-immunoprecipitation study demonstrated the binding of calmodulin to recombinant CSE *in vitro*. This binding is contingent on the presence of calcium, and abolished by calmodulin antagonist W7. These results demonstrated that a calcium-calmodulin system is the prerequisite for muscarinic activation of CSE in endothelial cells. Once CSE is knocked down from endothelial cells, the effects of methacholine and A23187 on H<sub>2</sub>S production were abolished, indicating CSE activation is the key link in calcium-calmodulin-dependent H<sub>2</sub>S production.<sup>9</sup>

#### Endothelium-dependent vasorelaxation induced by H<sub>2</sub>S

The vasorelaxant effect of H<sub>2</sub>S is also endothelium-dependent. The removal of endothelium attenuated the relaxation of rat aortic tissues induced by H<sub>2</sub>S and shifted H<sub>2</sub>S concentration-response curve to the right with EC<sub>50</sub> changed from 136 to 273 μM.<sup>10</sup> Although this observation did not address whether H<sub>2</sub>S is endothelium derived or not, it shows that the presence of an intact endothelium does affect H<sub>2</sub>S-induced vasorelaxation. The endothelium dependence of H<sub>2</sub>S effect was more pronounced in isolated and perfused rat mesenteric artery bed.<sup>5</sup> The removal of the functional endothelium significantly reduced H<sub>2</sub>S-induced relaxation of rat mesenteric artery bed by about sevenfold with EC<sub>50</sub> of H<sub>2</sub>S changed from 25 to 161 μM ( $P < 0.05$ ). This tissue type-selective endothelium-dependent effect of H<sub>2</sub>S is similar to that of EDHF. The EC<sub>50</sub> of H<sub>2</sub>S in inducing vasorelaxation is quite close to the reported endogenous level of H<sub>2</sub>S in plasma, which suggests that under physiological *in vivo* conditions the vascular tone of resistance arteries is likely regulated by endogenous H<sub>2</sub>S. Long-term effect of H<sub>2</sub>S on the endothelium is observed as the increased proliferation of vascular endothelial cells, although H<sub>2</sub>S inhibits proliferation of many types of cells, such as vascular SMCs.<sup>11</sup> NaHS (10–20 μM), an H<sub>2</sub>S donor, increased cell growth, migration, scratched wound healing, and tube-like structure formation in cultured human umbilical vein endothelial cells. These effects of NaHS were mediated by PI3K activation and Akt

phosphorylation. Furthermore, NaHS treatment (10 and 50 μmol/kg/day, intraperitoneally) of mice significantly promoted neovascularization *in vivo*.<sup>12</sup> Deficiency in the expression of CSE significantly lowers the H<sub>2</sub>S level in cardiovascular tissues, especially in endothelial cells. Methacholine-induced endothelium-dependent relaxation of resistance mesenteric arteries was significantly diminished in CSE-deficient mice.<sup>9</sup>

#### Stability and removal of H<sub>2</sub>S

The half-life of NO in blood is counted in seconds and it can be scavenged by oxyhemoglobin. H<sub>2</sub>S seems to be more stable in protein-free solution.<sup>13</sup> However, H<sub>2</sub>S can also be scavenged by methemoglobin or disulfide-containing molecules such as oxidized glutathione.<sup>3</sup> Therefore, the half-life of free H<sub>2</sub>S in blood may also be short.

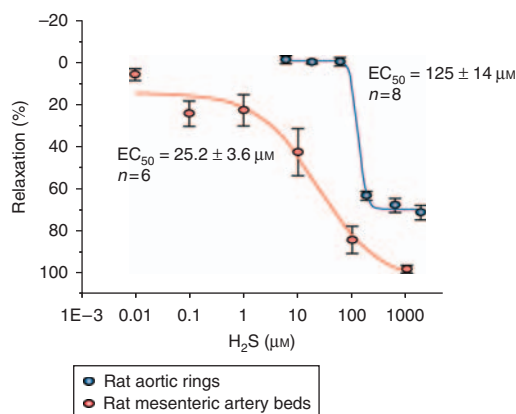
#### THE EDHF RESEMBLANCE OF H<sub>2</sub>S

The unique property of EDHF, different from other putative EDRFs, is its specific action of hyperpolarizing vascular SMCs so as to close voltage-dependent calcium channels. The effect of EDHF is mainly mediated by small-conductance K<sub>Ca</sub> channels and aided by intermediate-conductance K<sub>Ca</sub> channels, which can be blocked by the co-application of apamin and charybdotoxin. It has been debated, and the case has not yet been settled, whether EDHF is a diffusible EDRF or a simple electrotonic phenomenon that relays membrane potential change in endothelium to the underneath SMCs through low-electrical resistance myoendothelial gap junctions. Among diffusible chemical candidates for EDHF are K<sup>+</sup> ions, endothelium-derived C-type natriuretic peptide, H<sub>2</sub>O<sub>2</sub>, and P450 metabolites/epoxyeicosatrienoic acids. H<sub>2</sub>S possesses many common features of EDHF.

- (1) Some studies have shown that the vasorelaxing effect of H<sub>2</sub>S appears to be more potent on small-resistance mesenteric arteries than on large aortic vascular tissues (Figure 3). It is known that the contribution of EDHF to endothelium-dependent vasorelaxation is much greater in smaller arteries, including mesenteric artery and coronary artery.
- (2) Zhao *et al.*<sup>4</sup> has shown that the co-application of apamin (50 nM) and charybdotoxin (50 nM) significantly weakened H<sub>2</sub>S-induced relaxation of endothelium-intact rat aortic tissues. The removal of endothelium similarly reduced H<sub>2</sub>S effect as apamin/charybdotoxin did. The same inhibitory effect of apamin/charybdotoxin on H<sub>2</sub>S-induced relaxation was also observed in rat mesenteric arteries.<sup>5</sup> On the other hand, whether treatment of the whole animal with apamin/charybdotoxin can affect H<sub>2</sub>S-induced hypotension has not been reported. These results suggest that either H<sub>2</sub>S itself is an EDHF or that H<sub>2</sub>S releases EDHF from the endothelium.

#### INTERACTION OF H<sub>2</sub>S AND NO

As both H<sub>2</sub>S and NO are bioactive gas molecules, interaction between these two gasotransmitters has long been



**Figure 3 | Comparison of the vasorelaxant potencies of H<sub>2</sub>S on endothelium-intact aorta and mesenteric arteries from rats.** Previously published data (Figure 2a from Zhao *et al.*<sup>4</sup>, and Figure 2a from Cheng *et al.*<sup>5</sup>) are pooled to synthesize and reproduce this summary figure.

suspected. Pre-treating rat aortic tissues with 60 μM H<sub>2</sub>S inhibited the vasorelaxant effect of sodium nitroprusside, an NO donor. Incubation of NaHS with a range of NO donors or NO gas *in vitro* was reported to form a nitrosothiol molecule. In this way, H<sub>2</sub>S acts similar to an NO scavenger.<sup>14</sup> The formation of this nitrosothiol molecule inhibited the vasorelaxant effect of NO both *in vitro* and *in vivo*. Low concentrations of NaHS or H<sub>2</sub>S gas in solution reversed the relaxant effects of acetylcholine and histamine on rat aortae. NaHS also inhibited the conversion of [<sup>3</sup>H]-arginine into [<sup>3</sup>H]-citrulline by recombinant eNOS.<sup>15</sup> In another study on isolated rat aortas and cultured human umbilical vein endothelial cells, NaHS incubation reduced eNOS activity, eNOS transcript abundance, and L-arginine transport. H<sub>2</sub>S treatment reduced protein expression of eNOS, but not of nNOS and iNOS. The downregulation of the vascular L-arginine/eNOS/NO pathway was observed after intraperitoneal injection of NaHS (14 μmol/kg) into rats.<sup>16</sup> In contrast, H<sub>2</sub>S by itself had no effect on NO production from the rat vascular SMCs, but it augmented interleukin-β-induced NO production and this effect was associated with increased expression of iNOS.<sup>17</sup> Whether H<sub>2</sub>S and NO at low concentrations have a synergistic effect on vascular relaxation has been controversial.

To be equipped with both NO and H<sub>2</sub>S as EDRFs will enable blood vessels with heterogeneity to relax with selective and defined stimuli. Multiple EDRFs may help to add layers of control or backup mechanisms should one EDRF fail under pathological conditions. Moreover, the interaction between these two EDRFs will either compensate the loss or deficit, or buffer the overproduction or overaction, of one of the two.

### PERSPECTIVES AND CHALLENGES

Accumulating data on the cardiovascular effects of endogenous H<sub>2</sub>S has stimulated mounting speculation that H<sub>2</sub>S might be another EDRF or the very EDHF. Whereas the score for

the home team is up and all fans are celebrating, it is still too early to announce the winner before hitting a home run. Many challenges should be appropriately addressed regarding the viewpoint of H<sub>2</sub>S being a new EDRF.

The premise that H<sub>2</sub>S is an EDRF for small resistance arteries that NO is an EDRF for large arteries, NO-mediated endothelium-dependent relaxation of large arteries should be investigated in CSE knockout mice so that the relative contribution of NO and H<sub>2</sub>S to endothelium-dependent vasorelaxation can be deciphered.

As mentioned earlier, endogenous H<sub>2</sub>S can be generated by another enzyme, CBS. Wang *et al.*<sup>18</sup> reported the activity of CBS as reflected by the production of cystathionine in cultured human umbilical venous endothelial cells. However, no attempt was taken then to detect CBS mRNA or protein in these cells, which had been cultured for 14 days with the addition of 100 μM L-homocysteine. This observation hints that CBS may function as an inducible H<sub>2</sub>S-generating enzyme in the vascular endothelial cells, which merits further investigation. The upregulation of CBS may occur when homocysteine level or other links of *trans*-sulfuration pathway are altered. As such, not only the sulfur metabolism is affected, endothelium-dependent production of H<sub>2</sub>S and vasorelaxation would also be regulated under different physiological and pathophysiological conditions.

It is of note that EDHF plays a critical role in regulating endothelium-dependent vasorelaxation in female mice, whereas NO and PGI<sub>2</sub> are the predominant EDRFs in male mice.<sup>2</sup> Whether heterogeneity of gender affects the role of H<sub>2</sub>S as an EDRF should be examined.

The qualification of H<sub>2</sub>S as an EDHF needs electrophysiological recording of membrane potential changes.

H<sub>2</sub>O<sub>2</sub> has been suggested as the EDHF.<sup>19</sup> In cultured rat aortic A-10 cells, low level of NaHS decreased the production of H<sub>2</sub>O<sub>2</sub>, ONOO<sup>-</sup>, and O<sub>2</sub><sup>-</sup> in the presence of homocysteine, and improved cell viability.<sup>20</sup> Whether the endothelium-dependent vasorelaxant effect of H<sub>2</sub>S is mediated by altered H<sub>2</sub>O<sub>2</sub> production or altered redox status in endothelial cells remains unclear.

Relative contributions of K<sub>ATP</sub> channels and small/intermediate K<sub>Ca</sub> channels to the vascular effect of H<sub>2</sub>S need to be further clarified in different vascular tissues in order to determine whether H<sub>2</sub>S assumes the role of EDRF or EDHF depending on vascular tissue types.

In conclusion, H<sub>2</sub>S is an accredited candidate for EDRF and shares common features with the putative EDHF. It is produced both in vascular endothelial cells and SMCs. Upon muscarinic cholinergic stimulation, endothelium releases H<sub>2</sub>S, which has two different but complementary vascular actions. The activation of K<sub>ATP</sub> channels in vascular SMCs and of charybdotoxin/apamin sensitive K<sub>Ca</sub> channels by H<sub>2</sub>S would compound to hyperpolarize SMCs, leading to vasorelaxation (Figure 1). Being an EDRF, H<sub>2</sub>S plays an important role in regulating endothelium-dependent vasoactive activities. Once endothelium-derived H<sub>2</sub>S is diminished, selective resistance blood vessels will over-constrict and blood

pressure increases.<sup>9</sup> The H<sub>2</sub>S deficiency will also likely affect the pathogenesis progress of vascular complications of diabetes, coronary vascular diseases, atherosclerosis, and cardiac ischemic damage, to name a few. More extensive and mechanistic studies in the future are expected to further stimulate and intensify the debate on the candidacy of H<sub>2</sub>S as a new EDRF/EDHF. No matter what the verdict of this debate, the importance of H<sub>2</sub>S in regulating cardiovascular function will be certainly better appreciated.

#### DISCLOSURE

The author declared no competing interests.

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