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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_2S), another gasotransmitter, is a new EDRF. H_2S 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_2S is produced from endothelium upon muscarinic cholinergic stimulation. The endothelium-dependent vasorelaxation induced by H_2S 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_2S 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[1] 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_2. 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 (PGI2) 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 PGI2 by knocking out the expression of eNOS and cyclooxygenase-1, there is still residual endothelium-dependent relaxation.[2] 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[3] in endothelium-dependent vasorelaxation have been questioned in recent years.

The chemical profile of hydrogen sulfide (H_2S) 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_2S 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_2S 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).[3] In addition to H_2S, L-cysteine

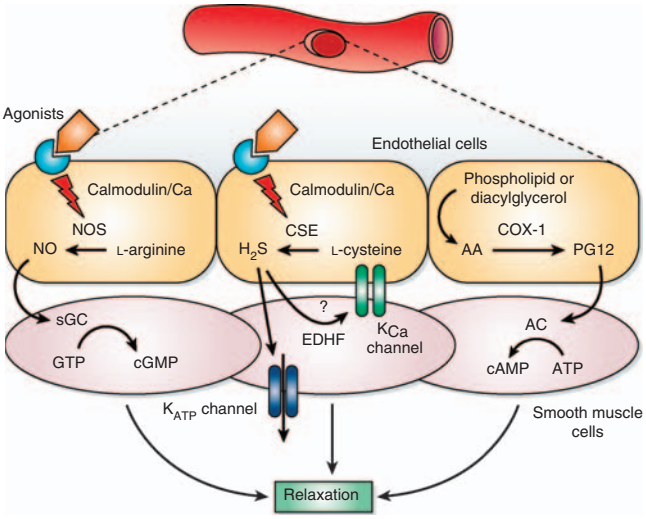

Figure 1 | Molecular basis for endothelium-dependent vasorelaxation. With different substrates, NO, H_2S, and PGI2 are produced from the endothelium by NOS, CSE, and COX-1, respectively. They are released from the endothelium and act on the adjacent smooth muscle cells to induce relaxation. NO and H_2S 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 γ-lyase; COX-1, cyclooxygenase-1; AA, arachidonic acid; EDHF, endothelium-derived hyperpolarizing factor.

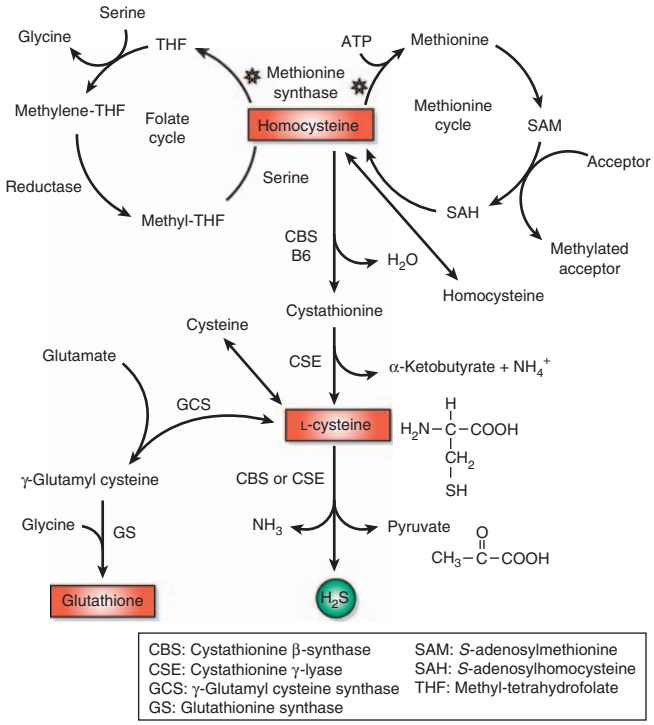

Figure 2 | Endogenous synthesis of hydrogen sulfide in mammalian cells.

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_2S-generating enzyme in the brain and nervous system and CSE is the predominant H_2S-generating enzyme in pancreatic β-islets, different vascular tissues, and the heart.[3] Physiological range of H_2S in circulation is 10–100 μM in fish, rats, and human. Endogenous level of H_2S in brain tissue is approximately 50–160 μM.

Our knowledge on the cardiovascular effects of H_2S has amounted in recent years, and the momentum in searching the physiological and pathophysiological roles of H_2S has been built up. H_2S induces relaxation of different vascular tissues (for example, rat aorta and mesenteric arteries), and protects the heart from ischemia/reperfusion damage. H_2S can induce vasorelaxation by directly opening K_{ATP} channels in vascular SMCs. K_{ATP} channel blocker, glibenclamide, inhibited H_2S-induced dilation of aorta,[4] mesenteric artery beds,[5] or hepatic microcirculation[6] and abolished H_2S-evoked blood pressure decrease *in vivo*.[4] Using the whole-cell and single-channel patch-clamp technique, direct evidence was obtained that exogenous H_2S activated K_{ATP} channels and hyperpolarized cell membrane of rat aorta and mesenteric artery SMCs.[4] Glibenclamide also abolished H_2S-facilitated carotid sinus baroreflex.[7] Inhibition of endogenous H_2S production with D,L-propargylglycine, a membrane permeable and irreversible inhibitor of CSE, reduced whole-cell K_{ATP} currents.[4] In myocardium, a mitochondrial K_{ATP} channel blocker (5-hydroxydecanoate) or a plasma membrane K_{ATP} channel blocker (glibenclamide) abolished H_2S-offered cardioprotection against myocardiac infarction damage.[8]

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_2S being a new EDRF is both intriguing and appealing.

LINES OF EVIDENCE SUPPORT THE IDENTIFICATION OF H_2S AS AN EDRF

Endothelium production of H_2S

Elucidation of the source of H_2S 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.[4] In cultured rat aortic endothelial cells, CSE mRNA was not detected using reverse transcription-PCR.[4] CSE protein expression was not conducted at that time due to the lack of CSE-specific antibody.[4] In the liver, CSE was found in hepatocytes and hepatic stellate cells, but not in sinusoidal endothelial cells.[6] It appeared that the vascular endothelium *per se* was not equipped with H_2S-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.*[9] 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_2S was also obtained. Cultured vascular endothelial cells produce measurable H_2S and silencing CSE in these cells significantly decreases H_2S production.[9]

Physiological stimulus for endothelial H_2S production

Similar to NO, acute production of H_2S from vascular endothelial cells is triggered by muscarinic cholinergic activation. Methacholine, a cholinergic receptor agonist, increased H_2S 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_2S is significantly increased. Chelating of intracellular-free calcium with BAPTA abolished the effect of A23187 on H_2S production as well as lowering the basal level of H_2S 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_2S production were abolished, indicating CSE activation is the key link in calcium–calmodulin-dependent H_2S production.[9]

Endothelium-dependent vasorelaxation induced by H_2S

The vasorelaxant effect of H_2S is also endothelium-dependent. The removal of endothelium attenuated the relaxation of rat aortic tissues induced by H_2S and shifted H_2S concentration–response curve to the right with EC_{50} changed from 136 to 273 μM.[10] Although this observation did not address whether H_2S is endothelium derived or not, it shows that the presence of an intact endothelium does affect H_2S-induced vasorelaxation. The endothelium dependence of H_2S effect was more pronounced in isolated and perfused rat mesenteric artery bed.[5] The removal of the functional endothelium significantly reduced H_2S-induced relaxation of rat mesenteric artery bed by about sevenfold with EC_{50} of H_2S changed from 25 to 161 μM ($P<0.05$). This tissue type-selective endothelium-dependent effect of H_2S is similar to that of EDHF. The EC_{50} of H_2S in inducing vasorelaxation is quite close to the reported endogenous level of H_2S in plasma, which suggests that under physiological *in vivo* conditions the vascular tone of resistance arteries is likely regulated by endogenous H_2S. Long-term effect of H_2S on the endothelium is observed as the increased proliferation of vascular endothelial cells, although H_2S inhibits proliferation of many types of cells, such as vascular SMCs.[11] NaHS (10–20 μM), an H_2S 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*.[12] Deficiency in the expression of CSE significantly lowers the H_2S level in cardiovascular tissues, especially in endothelial cells. Methacholine-induced endothelium-dependent relaxation of resistance mesenteric arteries was significantly diminished in CSE-deficient mice.[9]

Stability and removal of H_2S

The half-life of NO in blood is counted in seconds and it can be scavenged by oxyhemoglobin. H_2S seems to be more stable in protein-free solution.[13] However, H_2S can also be scavenged by methemoglobin or disulfide-containing molecules such as oxidized glutathione.[3] Therefore, the half-life of free H_2S in blood may also be short.

THE EDHF RESEMBLANCE OF H_2S

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_{Ca} channels and aided by intermediate-conductance K_{Ca} 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^+ ions, endothelium-derived C-type natriuretic peptide, H_2O_2, and P450 metabolites/epoxyeicosatrienoic acids. H_2S possesses many common features of EDHF.

(1) Some studies have shown that the vasorelaxing effect of H_2S 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.*[4] has shown that the co-application of apamin (50 nM) and charybdotoxin (50 nM) significantly weakened H_2S-induced relaxation of endothelium-intact rat aortic tissues. The removal of endothelium similarly reduced H_2S effect as apamin/charybdotoxin did. The same inhibitory effect of apamin/charybdotoxin on H_2S-induced relaxation was also observed in rat mesenteric arteries.[5] On the other hand, whether treatment of the whole animal with apamin/charybdotoxin can affect H_2S-induced hypotension has not been reported. These results suggest that either H_2S itself is an EDHF or that H_2S releases EDHF from the endothelium.

INTERACTION OF H_2S AND NO

As both H_2S and NO are bioactive gas molecules, interaction between these two gasotransmitters has long been

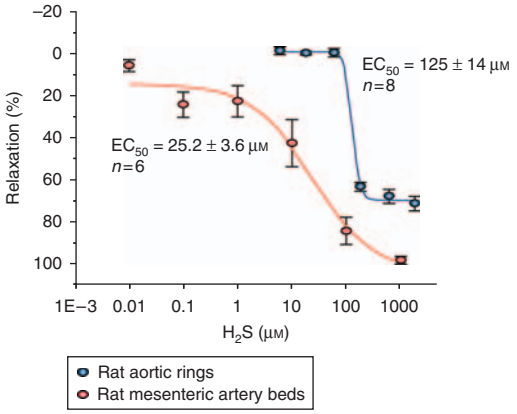

Figure 3 | Comparison of the vasorelaxant potencies of H_2S on endothelium-intact aorta and mesenteric arteries from rats. Previously published data (Figure 2a from Zhao *et al.*[4], and Figure 2a from Cheng *et al.*[5]) are pooled to synthesize and reproduce this summary figure.

suspected. Pre-treating rat aortic tissues with 60 μM H_2S 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_2S acts similar to an NO scavenger.[14] The formation of this nitrosothiol molecule inhibited the vasorelaxant effect of NO both *in vitro* and *in vivo*. Low concentrations of NaHS or H_2S gas in solution reversed the relaxant effects of acetylcholine and histamine on rat aortae. NaHS also inhibited the conversion of [^{3}H]-arginine into [^{3}H]-citrulline by recombinant eNOS.[15] 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_2S 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.[16] In contrast, H_2S 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.[17] Whether H_2S and NO at low concentrations have a synergistic effect on vascular relaxation has been controversial.

To be equipped with both NO and H_2S 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_2S has stimulated mounting speculation that H_2S 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_2S being a new EDRF.

The premise that H_2S 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_2S to endothelium-dependent vasorelaxation can be deciphered.

As mentioned earlier, endogenous H_2S can be generated by another enzyme, CBS. Wang *et al.*[18] 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_2S-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_2S 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 PGI2 are the predominant EDRFs in male mice.[2] Whether heterogeneity of gender affects the role of H_2S as an EDRF should be examined.

The qualification of H_2S as an EDHF needs electrophysiological recording of membrane potential changes.

H_2O_2 has been suggested as the EDHF.[19] In cultured rat aortic A-10 cells, low level of NaHS decreased the production of H_2O_2, $ONOO^-$, and O_2^- in the presence of homocysteine, and improved cell viability.[20] Whether the endothelium-dependent vasorelaxant effect of H_2S is mediated by altered H_2O_2 production or altered redox status in endothelial cells remains unclear.

Relative contributions of K_{ATP} channels and small/intermediate K_{Ca} channels to the vascular effect of H_2S need to be further clarified in different vascular tissues in order to determine whether H_2S assumes the role of EDRF or EDHF depending on vascular tissue types.

In conclusion, H_2S 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_2S, which has two different but complementary vascular actions. The activation of K_{ATP} channels in vascular SMCs and of charybdotoxin/apamin sensitive K_{Ca} channels by H_2S would compound to hyperpolarize SMCs, leading to vasorelaxation (Figure 1). Being an EDRF, H_2S plays an important role in regulating endothelium-dependent vasoactive activities. Once endothelium-derived H_2S is diminished, selective resistance blood vessels will over-constrict and blood

pressure increases.[9] The H_2S 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_2S as a new EDRF/EDHF. No matter what the verdict of this debate, the importance of H_2S in regulating cardiovascular function will be certainly better appreciated.

DISCLOSURE

The author declared no competing interests.

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