Hydrogen sulfide, an enhancer of vascular nitric oxide signaling: mechanisms and implications

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Szabo C. Hydrogen sulfide, an enhancer of vascular nitric oxide signaling: mechanisms and implications. *Am J Physiol Cell Physiol* 312: C3–C15, 2017. First published October 26, 2016; doi:10.1152/ajpcell.00282.2016.—Nitric oxide (NO) vascular signaling has long been considered an independent, self-sufficient pathway. However, recent data indicate that the novel gaseous mediator, hydrogen sulfide (H2S), serves as an essential enhancer of vascular NO signaling. The current article overviews the multiple levels at which this enhancement takes place. The first level of interaction relates to the formation of biologically active hybrid S/N species and the H2S-induced stimulation of NO release from its various stable “pools” (e.g., nitrite). The next interactions occur on the level of endothelial calcium mobilization and PI3K/Akt signaling, increasing the specific activity of endothelial NO synthase (eNOS). The next level of interaction occurs on eNOS itself; H2S directly interacts with the enzyme: sulfhydration of critical cysteines stabilizes it in its physiological, dimeric state, thereby optimizing eNOS-derived NO production and minimizing superoxide formation. Yet another level of interaction, further downstream, occurs at the level of soluble guanylate cyclase (sGC): H2S stabilizes sGC in its NO-responsive, physiological, reduced form. Further downstream, H2S inhibits the vascular cGMP phosphodiesterase (PDE5), thereby prolonging the biological half-life of cGMP. Finally, H2S-derived polysulfides directly activate cGMP-dependent protein kinase (PKG). Taken together, H2S emerges an essential endogenous enhancer of vascular NO signaling, contributing to vasorelaxation and angiogenesis. The functional importance of the H2S/NO cooperative interactions is highlighted by the fact that H2S loses many of its beneficial cardiovascular effects when eNOS is inactive.

angiogenesis; cGMP; hydrogen sulfide; nitric oxide; vascular

The Vascular eNOS/sGC/cGMP/PKG Pathway May Not Be Completely Self-Sufficient

Vascular NO production (overviewed in 12, 39, 43, 53, 74, 89, 90, 107) is predominantly due to endothelial NO synthase (eNOS), a calcium-dependent enzyme constitutively expressed in vascular endothelial cells. Various vasorelaxant and angiogenic hormones and factors, as well as shear stress, lead to calcium mobilization in the endothelial cells, which activates eNOS in a calmodulin-dependent manner. In the presence of various co-factors (e.g., NADPH and BH4), eNOS converts its physiological substrate L-arginine to NO and L-citrulline. In addition to calcium, eNOS is also regulated by phosphorylation/dephosphorylation at several critical regulatory amino acid residues. NO, produced by eNOS, either reaches its targets within the endothelial cell itself, or diffuses to the underlying vascular smooth muscle cells. In turn, NO binds to the heme group of its target enzyme, soluble guanylate cyclase (sGC), and activates it. The sGC-mediated production of cGMP, via stimulation of downstream enzymes (cGMP-dependent protein kinases, PKGs) is primarily responsible for the biological effects of eNOS, such as vascular relaxation and angiogenesis. Vascular cGMP levels are physiologically degraded by phosphodiesterase 5 (PDE5) (19, 26, 105). The vascular eNOS/sGC/cGMP/PKG pathway, one of the most intensively studied signaling pathways in biology, is generally considered a stand-alone, self-sufficient pathway that does not rely on external biochemical enhancers.

Several decades after the discovery of the essential role of the NO/sGC/cGMP pathway in the control of the cardiovascular system, the regulatory roles of another gaseous mediator, hydrogen sulfide (H2S), started to emerge (overviewed in 55, 57, 60, 61, 66, 85, 108, 109, 125–129, 139, 143, 145). In brief,

1 The organization and physiological role of this pathway is now textbook material; the only reason that the core pathway is outlined in the section below is to provide a common starting point and reference to the subsequent sections where the interactions with H2S will be introduced.

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H₂S is produced in the vascular system by three distinct enzymes, cystathionine-gamma-lyase (CSE), cystathionine-beta-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). The substrates of CBS and CSE are l-cysteine and homocysteine; the substrate of 3-MST is 3-mercaptopyruvate which is produced from l-cysteine. H₂S exerts its biological effects via a variety of mechanisms including posttranscriptional modification of critical cysteines in various enzymes via a novel process entitled S-sulfhydration. Similar to NO, H₂S causes vasorelaxation (143, 144), participates in the physiological maintenance of blood pressure (149), and serves an endogenous stimulator of angiogenesis (15, 106, 124). In addition, similar to NO, which converts into various stable or semi-stable “pools” (e.g., nitrite) and can be regenerated from it under certain conditions (59, 76, 77), H₂S converts into thiosulfate, which can regenerate biologically active H₂S (79, 117, 141).

Not only do NO and H₂S exhibit biological and functional similarities in the cardiovascular system, but several lines of data, most of which has emerged over the last 5 years, indicate that the two pathways, in fact, cooperate with each other. In the vascular system, H₂S, in many respects, is now viewed as an enhancer of the NO/cGMP/sGC/PKG pathway, without which eNOS cannot function to its fullest physiological extent. The biosynthesis, biological effects, metabolism, and physiological and pathophysiological roles of H₂S in a variety of diseases are subject to separate review articles (55, 57, 60, 61, 66, 85, 108, 109, 125, 127, 128, 139, 143, 145). The sole focus of the current review is to summarize the mechanisms by which H₂S acts as an enhancer of the vascular eNOS/sGC/cGMP/PKG system.

**H₂S Stimulates NO Release from Its Stable or Semi-Stable Pools**

Starting with the work of Moore, Whiteman, and coworkers (1, 147, 150) the notion began to emerge that the vascular effects of NO and H₂S may be interdependent, and may be, at least in part, related to the formation of a combined NO/H₂S species, i.e., a nitrosothiol (147). These findings, together with earlier observations of Kimura and coworkers who demonstrated that H₂S enhances the vascular relaxant effect of NO (47), suggested that H₂S may act as an enhancer of vascular eNOS/sGC/cGMP/PKG signaling, an effect, which, perhaps, is most relevant in the microvasculature (143, 144).

There are multiple levels of direct chemical interactions between NO (and its various metabolites) and H₂S (and its various metabolites) such that the field of NO/H₂S “cross-talk” has emerged as a separate area of biochemistry, which investigates (among others) the reaction products of H₂S with nitrosothiols, peroxynitrite, and nitrite (8, 17, 22–25, 37, 63, 73, 134, 146, 147). The biological responses induced by HNO (nitroxy1), one of the products of the H₂S/NO interactions, has recently been reviewed by Nagpure and Bian (91). There are several open areas in this rapidly growing field, which are overviewed in several recent articles (24, 41, 63, 67, 73, 78, 91).

Although the goal of this article is not to outline the complex chemistry of H₂S-related biological species, it must be mentioned that in biological systems, at or near physiological pH, in the presence of various biologically relevant and redox active molecules (e.g., thiols, transition metals, proteins), even when starting out from “pure” H₂S gas (e.g., by “bubbling” H₂S into the culture medium of cells), a complex mixture of sulfur species will be created. Because the dissociation constants of H₂S are pK₅1 = 6.8 and pK₅₂ >12, at pH 7.4, only about a quarter of H₂S gas will remain as dissolved gas: H₂S will be predominantly present in the form of hydrosulfide anion (HS⁻). At physiological pH, only minute amounts of H₂S will convert into S₂⁻ (73, 92). In addition to these (primary) H₂S-related species, in biological systems, due to a complex sequence of (bio)chemical interactions that are presently only partially understood, H₂S will also lead to the formation of a complex mixtures of organic persulfides and polysulfides (52, 100). While H₂S and HS⁻ are generally believed to have similar (although probably not identical) biological character, the chemical reactions elicited by polysulfides are drastically different (6, 52, 61, 100, 135). Downstream from H₂S, the biological liberation of sulfur (thyl) radical, the highly unstable intermediate HS molecule, the reactive S⁻ molecule, as well as the formation of other reactive sulfur species including S₂⁻, is also chemically feasible (24, 92, 94, 103). When this “soup” of H₂S-derived reactive species reacts with NO (or with a “soup” of NO-derived species), a wide variety of bioactive intermediates can form, including nitrosopersulfide (SSNO⁻), and SULFI/NO [ON(ON)SO₃⁻] (24). These molecules are biologically active: therefore, via this interaction, H₂S may create complex NO metabolites that modify (perhaps enhance) the biological action of NO (8, 23) (Fig. 1, arrow 1). The exact (patho)physiological role of the various S/N hybrid species remains to be further elucidated.

For the purpose of the current review, the next relevant reaction is that of H₂S and various semistable pools of NO (e.g., nitrite) leading to the generation of NO (11) (Fig. 1, arrow 2). As overviewed elsewhere (59, 76, 77), nitrite was initially believed an inactive, stable metabolite of NO, but it is now recognized as an important constituent of a biologically active, physiological “NO pool”; in fact, via administration of nitrite, therapeutically relevant, NO-mediated biological responses can be achieved (such as vascular relaxation, cardioprotection, and angiogenesis). The fact that H₂S can stimulate NO release from nitrite, as shown in hypoxic endothelial cells in vitro (11), and furthermore, the fact that the reaction of H₂S with peroxynitrite can produce the NO donor sulfinyl nitrite (37) may suggest that in the presence of H₂S, nitrite may become more biologically active and/or perhaps therapeutically more efficacious. Moreover, peroxynitrite, a reactive NO-derived species (7, 123), may be rendered less noxious by H₂S. In cultured endothelial cells the H₂S-induced release of NO from nitrite is, at least in part, catalyzed by xanthine oxidase (11). In addition to nitrite and peroxynitrite, H₂S can also induce NO release from nitrosothiols and metal nitrosyl complexes (99). It is worth mentioning that not only H₂S can stimulate NO production, but also nitrite can stimulate H₂S production: in a mouse model of chronic heart failure, treatment of the animals with sodium nitrite was found to induce the upregulation of the H₂S-producing enzymes CSE and CBS in the heart; consequently, nitrite treatment was found to elevate circulating H₂S levels (31).
**H2S Stimulates eNOS Activity via Stimulation of Calcium Mobilization**

Since eNOS is a calcium-dependent enzyme, intracellular calcium mobilization, in response to endothelium-dependent vasodilators and angiogenic hormones, is a rapid, potent activator of eNOS. Several lines of studies indicate that this process can be stimulated in the presence of H2S. First, in rat aorta endothelial cells, Moccia and colleagues demonstrated that H2S stimulates Ca2+ entry in a tetraethylammonium- and glybenclamide-inhibitable manner, suggesting the involvement of reverse-mode of KATP channels (84). H2S may also recruit the Na+/Ca2+ exchanger in a reverse mode (84). Subsequent studies showed that H2S increased intracellular Ca2+ levels in cultured endothelial cells, and this response was inhibitable by dantrolene or xestospongin C (58). Finally, in the HUVEC-derived cell line Ea.hy926, Potenza and colleagues demonstrated H2S elicited a time- and concentration-dependent intracellular Ca2+ response, which was attributed to inositol-1,4,5-trisphosphate-dependent Ca2+ release (111). The findings of Potenza and colleagues (showing that the endothelial calcium signal is identical in the presence or absence of extracellular calcium) are in disagreement with the findings of Moccia (suggesting that endothelial cell calcium mobilization in response to H2S may require the influx of extracellular calcium). Whether the source of calcium is extra- or intracellular, the findings discussed above identify endothelial calcium mobilization as the first, most upstream level at which H2S may enhance the activity of the eNOS/sGC/cGMP/PKG system (84) (Fig. 1, arrow 3). It must be pointed out, nevertheless, that the above-referenced studies investigate calcium mobilization (in response to H2S), and present functional outcome variables (e.g., endothelial cell migration), but do not directly (e.g., by...
measuring L-arginine to L-citrulline conversion) show in the endothelial cells that the increased Ca\(^{2+}\) signal, is, indeed, directly responsible for the activation of eNOS; further studies, directly testing the link of the H\(_2\)S-induced Ca\(^{2+}\) signal with eNOS activity, remain to be conducted.

### H\(_2\)S Stimulates eNOS Activity via AKT-Mediated Phosphorylation

A key level of the regulation of eNOS activity occurs at the level of phosphorylation/dephosphorylation of its critical regulatory amino acids. The best established regulatory amino acid residue is Ser1177 (the phosphorylation of which stimulates eNOS activity). Ser1177 phosphorylation, which increases the specific activity of eNOS, are facilitated by Akt activation in endothelial cells (29, 42, 82). Upstream, the activation of Akt in endothelial cells, is regulated by intracellular PIP3 levels, which are under the control of the PI3 kinase (PI3K) system (92).

The H\(_2\)S-induced cytoprotection and cell migration involves the PI3K system which stimulates its downstream effectors such as Akt and Rac-1 (15, 152). In various cultured endothelial cell systems, several lines of studies demonstrate that H\(_2\)S stimulates Akt by promoting its phosphorylation at its activating site (Ser493) (15, 21). H\(_2\)S also induces Akt activation in other cell types (50, 78, 132). Whether the H\(_2\)S-mediated increase in Akt is due to a direct effect of H\(_2\)S on PI3K remains to be determined. Part of the stimulatory effect on Akt activity is likely to be related to H\(_2\)S-mediated inhibition of Phosphatase and Tensin homolog (PTEN), an essential counterregulatory enzyme of the PI3K pathway, via sulfhydration of Cys124 (78, 98) (Fig. 1, arrow 4). The activity of PTP1B (another counterregulatory enzyme in the PI3K pathway) is also inhibited by H\(_2\)S, via sulfhydration at Cys215 (71) (Fig. 1, arrow 5).

Whatever the upstream processes are (direct stimulation of PI3K and/or indirect effects via PTEN or PTP1B or via other mechanisms), elevated PIP3 levels by H\(_2\)S would be expected to induce Akt activation, which, in turn, would be expected to produce the characteristic changes in the phosphorylation of regulatory amino acids on eNOS. Indeed, this is the case, as demonstrated by multiple studies in response to administration of various H\(_2\)S donors in vitro and in vivo (2, 3, 6, 11, 18, 21, 49, 56, 62, 75, 80, 102, 112, 113). For instance, in endothelial cells incubated with H\(_2\)S donors, the activation of Akt (evidenced by phosphorylation at Ser473) was accompanied by the phosphorylation of Ser1177 on eNOS (21). In endothelial cells subjected to shear stress, H\(_2\)S was found to stimulate eNOS phosphorylation at Ser1177 (49). Similarly, H\(_2\)S was shown in multiple studies (in the heart as well as in the coronary arterioles) to stimulate Ser1177 phosphorylation of eNOS in vivo (62, 103, 113). In endothelial cells subjected to shear stress, Ser1177 phosphorylation was diminished after siRNA-mediated silencing of either CSE, CBS, or 3-MST genes (49); likewise, in CSE\(^{-/-}\) mice the degree of basal eNOS phosphorylation at Ser1177 is markedly lower than the corresponding basal Ser1177 phosphorylation in wild-type mice (62). These findings indicate that basal, physiological Ser1177 phosphorylation of eNOS is maintained by endogenous H\(_2\)S through the basal physiological actions of various H\(_2\)S-producing enzymes. The functional relevance of the H\(_2\)S-mediated eNOS phosphorylation is highlighted by the results Lefer and co-workers who demonstrated that the protective effect of H\(_2\)S was markedly lower in eNOS phosphomutant S1179A mice, than in the corresponding wild-type control mice (62).

There may be additional levels at which H\(_2\)S stimulates the generation of PIP3, even further upstream from PI3K and PTEN. One example relates to VEGF (a proangiogenic and vasorelaxant hormone). The endothelial cell receptor of VEGF (VEGFR2) is a strong activator of PI3K through VEGFR autophosphorylation (150). Tao and coworkers demonstrated that H\(_2\)S can reduce the intramolecular Cys1024-Cys1045 disulfide bond in VEGFR2, which, in turn, increases the PI3K stimulating activity of this receptor, culminating in an increased angiogenic response (133).

### H\(_2\)S Stimulates eNOS Activity via Direct Sulfhydration

eNOS homodimers represent the physiological state of the enzyme, where it produces NO in the most efficient manner, as well as the least amount of superoxide. On the other hand, monomeric eNOS is prone to superoxide generation (33, 114, 131). Wang and colleagues have demonstrated that H\(_2\)S sulfhydrates Cys443 of eNOS, which, in turn, stimulates the catalytic activity of eNOS, thereby keeping the enzyme in the dimeric state and suppressing superoxide generation by eNOS and maximizing physiological NO generation (2) (Fig. 1, arrow 6).

### H\(_2\)S Stimulates eNOS mRNA Synthesis

H\(_2\)S was found to increase the expression of eNOS (Fig. 1, arrow 7) in some systems, for instance in EAhy926 cells in vitro (18). In contrast, in other experimental settings only a slight and nonsignificant trend towards increased eNOS protein expression was noted in response to H\(_2\)S exposure (3). In some experimental systems, H\(_2\)S can also induce an upregulation of eNOS mRNA in vivo. For instance, in a murine model of thrombus formation induced by phototoxic light/dye-injury, H\(_2\)S administration caused the upregulation of eNOS in venules of the ear of hairless SKH1-hr mice (69). Moreover, in kidney reperfusion model in the rat, H\(_2\)S administration maintained eNOS expression levels during ischemia (while eNOS was downregulated in the ischemic control group that did not receive H\(_2\)S) (51). eNOS mRNA expression also increased after H\(_2\)S treatment in the corpus cavernosum (81). While H\(_2\)S donation can stimulate eNOS mRNA expression, basal physiological production of H\(_2\)S does not appear to be an absolute requirement for eNOS expression: eNOS expression levels were comparable in wild-type mice and CSE\(^{-/-}\) mice (62).

### H\(_2\)S Maintains sGC in an NO-Activatable State

Cells contain two distinct sGC pools: the reduced, ferrous heme group of the enzyme (Fe\(^{2+}\)) is the physiological form, which is responsive to NO in the classical NO/sGC/cGMP/PGK pathway. However, the heme group of sGC can also be oxidized (e.g., in various pathophysiological states) to yield the ferric (Fe\(^{3+}\)) that is no longer NO-activatable (9, 28, 34, 38, 105). Recent studies show that H\(_2\)S can act as a reducing agent.
for the heme group of sGC, catalyzing a ferric to ferrous heme transition (154). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) is a sGC inhibitor that acts via oxidizing the heme group (46). Treatment of recombinant sGC with ODQ reduced the responsiveness of the enzyme to NO, an effect that was attenuated if in the presence of H2S. On the other hand, H2S attenuated the response to the heme-independent activator BAY58-2667 that targets oxidized sGC. The beneficial effect of H2S on maintaining an NO-activatable state of sGC (Fig. 1, arrow 8) has been confirmed in oxidatively stressed endothelial cells as well as in isolated vascular rings (154). The above-described redox effect of H2S on the heme group of sGC appears to be most relevant under conditions of oxidative stress, because in the absence of oxidative stimuli, H2S does not influence sGC activity, neither under basal conditions, nor in response to NO donors (21, 154).

H2S Reacts with cGMP to Yield 8-SH-cGMP

The next level of cooperation between H2S and the vascular NO/sGC/cGMP/PKG pathway is at the level of cGMP itself. Modified cyclic nucleotides, including 8-nitro-cGMP, play key roles in vascular regulation (95, 96). Recent data show that H2S directly reacts with 8-nitro-cGMP, yielding 8-SH-cGMP. This molecule appears to be more resistant to PDE5 than “normal” cGMP (95, 96) and, in turn stimulates PKG activity (Fig. 1, arrow 9). 8-Nitro-cGMP/8-SH-cGMP imbalance has been implicated in a variety of pathophysiological conditions including cardiac ischemia and remodeling (95, 96, 100).

H2S Inhibits PDE5 Activity

Vascular cGMP phosphodiesterases (PDEs), predominantly PDE5, are essential negative regulators of cGMP levels. Inhibition of PDE5 activity suppresses cGMP degradation and, by maintaining/prolonging intracellular cGMP levels, enhances downstream signaling. In effect, in the presence of PDE inhibitors, the same amount of NO gets a “bigger bang for the buck” in terms of cGMP-dependent downstream vascular responses. PDE5 inhibitors, by boosting cGMP signaling, have successfully been employed not only in the therapy of male erectile dysfunction, but also pulmonary hypertension and other cardiovascular diseases (19, 26, 105). Two independent laboratories have demonstrated that H2S inhibits PDE activity (13, 21), which, in turn, boosts NO-stimulated cGMP levels in vascular endothelial cells and vascular smooth muscle cells (Fig. 1, arrow 10). Although H2S inhibits all known isoforms of both cAMP- and cGMP phosphodiesterases, it is most potent in inhibiting PDE5 (104). The mechanism of the inhibition does not appear to involve PDE sulfhydration (9, 10). The functional consequence of this interaction is that H2S elevates intracellular cGMP in vascular cells and tissues, thereby promoting and enhancing NO-mediated vascular responsiveness. This is also evidenced by H2S-induced increases in the phosphorylation of the PKG target VASP (13, 14, 21). There is also evidence for inhibition of the mitochondrial cAMP PDE isoform (PDEA2) by H2S (88); the functional consequence of this response is the stimulation of mitochondrial electron transport; this effect cooperates with the other mechanisms, including direct electron donation, and activation of ATP synthase (44, 88, 87, 122, 125), by which physiological concentrations of H2S stimulate mitochondrial electron transport and cellular bioenergetics.

Polysulfides Directly Activate PKG

Another layer of interaction between H2S and the NO/sGC/cGMP/PKG pathway occurs at the level of protein kinase G (PKG) activation. PKG is the essential effector of eNOS-mediated vasorelaxant, angiogenic, and cardioprotective responses (40, 115). Greiner and coworkers demonstrated that polysulfides (molecules that are formed from H2S intracellularly via a variety of mechanisms, see above) are capable of catalyzing the formation of an activating interprotein disulfide within PKG Iα, thereby stimulating the activity of PKG (45) (Fig. 1, arrow 11). This has important consequences for the cardiovascular regulatory effect of H2S: while in control mice H2S lowers blood pressure, in genetically engineered mice in which PKG’s Cys42 redox sensor has been rendered redox-insensitive, the blood pressure effect of H2S was attenuated (120). PKG may also have other levels of interaction with the NO or H2S systems, although the information on this subject remains somewhat fragmented. In some cases, evidence of a positive interaction has been presented, for instance PKG was reported to induce CSE expression in the heart (27), whereas in other experimental systems PKG-induced phosphorylation and consequent inhibition of CSE have been observed (4).

Consequences of the NO-H2S Cooperation for the Control of the Cardiovascular System

If the cooperative biochemical interactions between H2S and the NO/sGC/cGMP system outlined above and summarized in Fig. 1 are physiologically or pathophysiologically relevant, then the following functional consequences would be expected: 1) H2S administration would be expected to increase vascular NO production, with a consequent boost of vascular cGMP levels; 2) H2S administration would be expected to potentiate the vasorelaxant and angiogenic effect of NO; 3) if endogenously produced H2S plays a cooperative role with the NO system, then inhibition of vascular H2S biosynthesis would be expected to attenuate the vasorelaxant and angiogenic effect of NO; 4) if the cardiovascular effect of H2S primarily occurs via enhancement of the NO pathway, then in the absence of functional NO/sGC/cGMP/PKG system the vasorelaxant and angiogenic effects of H2S would be expected to be diminished. There is, indeed, direct experimental evidence for all of the above-mentioned scenarios, as outlined below.

1) H2S administration elevates vascular NO and cGMP levels. In various models of cultured endothelial cells, H2S has been found to both increase NO levels (3, 18, 112) and to elevate cGMP levels (9, 21). These effects were also associated with an increase in VASP phosphorylation (21), indicative of the fact that H2S exposure results in the stimulation of the full NO/sGC/cGMP pathway, culminating in an increase in PKG activity. Not only authentic H2S, but also the CBS/CSE substrate l-cysteine, was found to increase endothelial NO levels, and inactivate the CBS/CSE sub-strate l-cysteine, was found to increase endothelial NO levels, in a manner that was reduced by the CSE inhibitor (5) PAG (3), confirming the ability of the endogenous endothelial H2S biosynthesis to contribute to NO production. In a cultured rat corpus cavernosum preparation, various H2S donors were also found to increase NO levels (81). Importantly, the vasorelaxant and angiogenic responses to H2S are attenuated by the PKG-Iα inhibitor DT-2 (14, 21). In addition, l-cysteine- and H2S-induced relaxations are less pronounced in aortae isolated from PKG-I KO mice than the corresponding control relaxations in
aortae isolated from wild-type mice (14). These data, taken together, support the notion that stimulation of NO production and/or enhancement of the cGMP/sGC system, culminating on PKG activation, represent essential components of the vascular signaling induced by H2S. There are examples of H2S donation increasing circulating NO levels as well. For instance, it was demonstrated that the H2S donor diallyl trisulfide increases circulating NO levels in a pressure overload-induced heart failure model in mice (107). Moreover, Bir and colleagues demonstrated that H2S donor therapy increases circulating NO levels in mice (11). Finally, Wang and colleagues found that plasma NO levels are lower in CSE-deficient mice than in wild-type mice (2), concluding that endogenous H2S production contributes to the physiological maintenance of circulating NO levels.

2) H2S administration potentiates the vascular effect of NO. Evidence for a potentiating effect of H2S on vascular relaxations induced by exogenous NO was first provided by Kimura and co-workers in 1997: H2S donation was found to enhance the relaxant effect of various NO donors (Na-nitroprusside and morpholinosydnonimine) in rat portal vein and thoracic aortic ring preparations, in an endothelium-independent fashion (47). We have demonstrated in 2012 that the acetylcholine-induced, endothelium-dependent relaxations, as well as the relaxant responses to the NO donor DEA/NO, are enhanced (concentration-response curves shifted to the left) in the presence of exogenously administered H2S, and this functional synergy was accompanied with a synergistic enhancement of vascular cGMP levels (21). With respect to angiogenesis, vascular overexpression of CSE was found to enhance the stimulating effect of both DEA/NO and VEGF in an aortic ring sprouting assay (21). Moreover, a recent study demonstrated that ZYZ-803 (a novel synthetic H2S/NO hybrid molecule) induces endothelial cell proliferation, migration, and tube-like structure formation and angiogenesis in rat aortic rings and Matrigel plug assay: all of these actions occur at greater potency than the effect of either H2S or a NO donor on its own (48). ZYZ-803 also exerts vasorelaxant effects via stimulation of the cGMP pathway (148).

3) Inhibition of vascular H2S biosynthesis attenuates the vascular effect of NO. Although the eNOS/sGC/cGMP/PKG system is generally believed to be an independent signaling pathway, surprisingly, it relies, at least in part, on endogenous H2S production. This component is relatively smaller in the context of vascular relaxation, but it is surprisingly marked in the context of angiogenesis. Wang and Snyder have observed that vasorelaxant effect of metacholine (which, similar to acetylcholine, relaxes blood vessels via activation of cholinergic receptors and stimulation of the eNOS/sGC/cGMP/PKG system) is lower in blood vessels of CSE<sup>−/−</sup> mice than the corresponding response in wild-type mice (149). In addition, the vasorelaxant effect of the NO donor SNAP is less pronounced in vascular rings of CSE<sup>−/−</sup> mice than the vasorelaxation observed in rings of wild-type control mice (149). Confirming and extending these observations, we have demonstrated that acetylcholine, as well as DE/NO-induced vascular relaxation dose-responses, are shifted to the right in thoracic aortic rings after vascular CSE silencing; this was also associated with a reduction in vascular cGMP levels (21). As far as interactions in angiogenesis: DEA/NO-induced in vitro angiogenic activity (as well as the ability of VEGF to induce angiogenesis) was markedly attenuated after silencing of CSE in endothelial cells; once again, after CSE silencing, the NO donors failed to induce elevations in endothelial cGMP levels (21). H2S-induced angiogenesis (which served as the control group in this experiment) remained unaffected after CSE silencing. In line with these findings, Wang and coworkers have demonstrated that the angiogenic effect of the eNOS substrate l-arginine is markedly less pronounced in aortic rings of CSE<sup>−/−</sup> mice compared with the responses in wild-type mice (3).

4) Inhibition of eNOS attenuates the vascular effect of H2S. Moore and colleagues observed in 2006 that the hypotensive effect of intravenous H2S infusion in rats is suppressed by treatment with the NOS inhibitor l-NAME (1), and similar findings were recently reported in human volunteers: the local vasodilatory response to administration of H2S was reduced by l-NAME (73). The in vitro vascular relaxant effect of H2S is also reduced by pretreatment with the NOS inhibitor l-NAME, and it is shifted to the right in vascular rings from eNOS-deficient mice (21), indicating that part (but not all) of the vascular relaxations induced by H2S require the eNOS/sGC/cGMP/PKG pathway. Since H2S exerts vascular relaxant effects via a number of additional mechanisms, including K<sub>ATP</sub> channel opening, as well as, at higher concentrations, by direct metabolic effects (64, 66), it is not surprising that NOS inhibition only abrogates part of its vascular relaxant responses. However, it appears that the NO system is obligatory for H2S-induced angiogenesis: H2S mediated in vitro angiogenic activity (as well as the ability of H2S to induce an elevation of endothelial cGMP levels) was completely abrogated by pretreatment of the endothelial cell cultures with the NOS inhibitor l-NAME (21); likewise, the stimulatory effect of H2S donors in the Matrigel plug assay in vivo was absent in eNOS<sup>−/−</sup> mice (21). In agreement with these findings, Wang and colleagues demonstrated that l-NAME, as well as vascular eNOS silencing, abrogates H2S-induced endothelial cell proliferation, tube formation, and aortic ring sprouting responses in vitro, whereas eNOS overexpression facilitates the angiogenic effect of H2S (3).

5) H2S-induced cardiovascular and therapeutic actions are abrogated in eNOS-deficient systems. Examples for this are shown in Table 1. Given the fact that (as discussed above) H2S-induced angiogenic responses (Matrigel plug responses) are suppressed in eNOS-deficient mice (21), it should not come as a surprise that the stimulating effect of H2S on wound healing (an effect, which, at least in part, relies on the stimulation of angiogenesis) is also suppressed after pretreatment of the animals with the NOS inhibitor l-NAME (21). In isolated perfused hearts, the protective effect of H2S against isoproterenol-induced cardiac injury is attenuated by l-NAME pretreatment (118). In addition, the well-established (32) ability of H2S pretreatment to reduce infarct size in a murine model of ischemia-reperfusion injury is absent in eNOS deficient mice (62). Similarly, chronic treatment with SG-1001 (a novel H2S

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3 Please note that l-NAME, similar to most pharmacological agents, can have independent pharmacological actions unrelated to NOS inhibition. The interrelationships between NO and H2S that are based solely on pharmacological evidence are, therefore, less robust than those interrelationships that are both supported by pharmacological (NOS inhibitors) as well as genetic (siRNA-mediated silencing and/or knockout mice) proof.
The H2S treatment, administered shortly prior to the start of the CPR procedure, prolongs survival in wild-type animals, but is without significant therapeutic effect in eNOS-deficient mice (83). Finally, the H2S-induced preconditioning responses on leukocyte rolling, but not on leukocyte adhesion, are reduced in eNOS−/− mice, compared with wild-type mice (151). There are, however, examples when eNOS deficiency does not abrogate the beneficial effect of H2S. For instance, in a model of angiogenesis induced by permanent femoral artery ligation, the beneficial effects of H2S are maintained even on eNOS-deficient background (11). 

**Implications**

There are several practical/translational implications of the above outlined multiple layers of intricate interplay between the vascular NO and H2S systems. The first one relates to the fact that the eNOS/sGC/cGMP/PKG system becomes impaired in a variety of pathophysiological conditions (from hypertension to atherosclerosis and diabetic complications). As reviewed elsewhere in more detail (121, 129, 138, 142, 143, 145), in many forms of vascular disease (diabetic vascular complications, preeclampsia, vascular aging, atherosclerosis) H2S levels are impaired; the absence of endogenous H2S production (e.g., CSE deficient systems) exacerbates the onset and severity of the vascular dysfunction, while H2S donation improves vascular function. Under conditions of vascular disease, given the partial or absolute requirement of the NO system for H2S to be fully effective, one would expect that the therapeutic benefit provided by H2S donation would be diminished. Under such conditions, one would also expect that simultaneous substitution of both NO and H2S may be more beneficial than the effect of H2S alone. H2S levels are dynamically and delicately regulated by production and consumption (121, 129, 138), and some of these combinations may simultaneously enhance the therapeutic benefit provided by H2S donation would be diminished.

H2S has been demonstrated to protect against the development of endothelial dysfunction in various pathophysiological conditions including ischemia-reperfusion, diabetes, and preeclampsia (32, 102, 124, 143). Under conditions of vascular oxidative stress, the biological profile of both NO and H2S will not be fully efficacious, unless not only NO, but also H2S is also substituted. Multiple mechanisms contribute to the loss of vascular H2S levels in diabetic animals: part of it relates to mitochondrial dysfunction, excessive ROS production, and consequent excessive H2S consumption (121); another part relates to the oxidative inactivation of the H2S-producing enzyme 3-MST (20). Under such conditions, appropriately selected antioxidant therapies may be useful to restore biologically active H2S (as well as NO) levels.

Recognizing the simultaneous need for the restoration of vascular NO and H2S homeostasis, several groups started to design and test various “hybrid” molecules. H2S-donating groups have been placed on a large number of existing drugs (reviewed in 119, 142), and some of these combinations may simultaneously enhance NO and H2S signaling. For instance, ACS6, a H2S-donating derivative of sildenafil (116), boosts both NO-induced cGMP signaling and H2S signaling (via PDE5 inhibition and direct chemical H2S donation, respectively). The angiogenic and vascular effects of the combined NO/H2S donor ZYZ-803 (48, 148) have already been discussed above.

**Table 1. Examples for abrogation of H2S-induced cardiovascular responses in eNOS-deficient systems**

<table>
<thead>
<tr>
<th>Experimental System</th>
<th>Mode of eNOS Inhibition</th>
<th>Biological Response to H2S That Is Impaired in the Absence of Functional eNOS</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure measurement in anesthetized rats</td>
<td>L-NAME</td>
<td>Hypotension</td>
<td>1</td>
</tr>
<tr>
<td>Cultured BEnd3 endothelial cell angiogenesis</td>
<td>L-NAME</td>
<td>Migration, proliferation, tube formation</td>
<td>21</td>
</tr>
<tr>
<td>Cultured BEnd3 endothelial cells</td>
<td>L-NAME</td>
<td>cGMP formation</td>
<td>21</td>
</tr>
<tr>
<td>Cultured BEnd3 endothelial cell angiogenesis</td>
<td>eNOS silencing</td>
<td>Migration, proliferation, tube formation</td>
<td>3</td>
</tr>
<tr>
<td>Aortic ring sprouting assay</td>
<td>eNOS−/−</td>
<td>Endothelial cell migration</td>
<td>21</td>
</tr>
<tr>
<td>Isolated murine aortic rings</td>
<td>L-NAME</td>
<td>Vasorelaxation</td>
<td>21</td>
</tr>
<tr>
<td>Isolated murine aortic rings</td>
<td>eNOS−/−</td>
<td>Vasorelaxation</td>
<td>21</td>
</tr>
<tr>
<td>Normothermic cardiac arrest, followed by resuscitation by cardioprotective defibrillation and respiration in mice</td>
<td>eNOS−/−</td>
<td>Prolongation of survival</td>
<td>81</td>
</tr>
<tr>
<td>Matrigel plug assay in mice</td>
<td>eNOS−/−</td>
<td>Angiogenesis</td>
<td>21</td>
</tr>
<tr>
<td>Burn induced wound healing in rats</td>
<td>L-NAME</td>
<td>Wound closure</td>
<td>21</td>
</tr>
<tr>
<td>Isoproterenol-induced cardiac injury in mice</td>
<td>L-NAME</td>
<td>Cardiac lactate dehydrogenase and creatine kinase release, cardiac TBARS, glutathione, SOD and catalase activity, TNF expression, histopathological alterations</td>
<td>118</td>
</tr>
<tr>
<td>Myocardial infarction induced by transient LAD occlusion in mice</td>
<td>eNOS−/−</td>
<td>Infarct size</td>
<td>62</td>
</tr>
<tr>
<td>Myocardial infarction induced by transient LAD occlusion in mice</td>
<td>L-NAME</td>
<td>Infarct size</td>
<td>10</td>
</tr>
<tr>
<td>Chronic heart failure induced by aortic constriction in mice</td>
<td>eNOS−/−</td>
<td>Cardiac hypertrophy, BNP plasma levels, myocardial contractility</td>
<td>68</td>
</tr>
<tr>
<td>Intestinal ischemia-reperfusion in mice, intravital microscopy</td>
<td>L-NAME</td>
<td>Leukocyte rolling</td>
<td>151</td>
</tr>
</tbody>
</table>

BNP, B-type natriuretic peptide; LAD, left anterior descending coronary artery; LDH, lactate dehydrogenase; L-NAME: Nω-nitro-L-arginine methyl ester; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substrates; TNF, tumor-necrosis factor alpha.

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a variety of other potentially cytotoxic or detrimental species, and H2S can also convert into a variety of reactive molecules. Under such conditions, the antioxidant character of H2S may become more important. To some extent, such antioxidant effects may be related to direct interactions with ROS species, even though the corresponding reaction rate constants, as determined by stopped flow studies, are widely variable, some of them being rather low (17, 25, 93). Probably more important is the fact that H2S can also exert antioxidant effects through various indirect mechanisms, including the stimulation of the antioxidant vascular “master switch” Nrf2 and stimulation of intracellular glutathione generation; these effects may also contribute to the phenomenon of H2S-induced preconditioning (4, 16, 54, 112). Importantly, the absence of vascular H2S biosynthesis, on its own, induces an elevation of oxidative stress (61). When considering the somewhat controversial and fragmented body of evidence, the role of H2S/NO interactions, on the background of vascular oxidative stress, may be summarized in the following working hypothesis (Fig. 2). 1) Vascular oxidative stress increases the consumption of NO, and converts it into more deleterious species (e.g., peroxynitrite). 2) Oxidative stress may also consume cofactors of eNOS (e.g., tetrahydrobiopterin, NADPH), which impairs eNOS activity and leads to further oxidant generation from eNOS. 3) Vascular oxidant generation may also oxidize sGC, increasing the pool of sGC that is no longer activatable by NO. 4) These processes may form self-amplifying cycles of injury, increasing oxidant generation from various sources including mitochondrial electron transport chain. 5) The vascular oxidative stress response diminishes cellular H2S levels, either by directly interacting with them, or by inactivating some of the physiological enzymatic sources of H2S (e.g., 3-MST). 6) When ambient H2S levels are diminished, vascular oxidative stress further increases (62, 121). Moreover, the stimulatory effect of H2S on the eNOS/sGC/cGMP/PKG pathway are diminished, because aging, in turn, causes further impairment in the biological effectiveness of the vascular NO system. 7) All of the above processes may be even further exacerbated in aging blood vessels, which appears to suppress H2S levels, and H2S-dependent bioenergetic responses in various tissues (125, 154) (although this has not yet been sufficiently investigated in vascular tissues). Aged blood vessels also have lower levels of endogenous antioxidants and have a diminished ability to mobilize antioxidant responses (e.g., aging blood vessels have a markedly diminished ability to mount an Nrf2-mediated antioxidant response) (136, 137). 8) Antioxidant therapy and/or replacement therapy with H2S donors, including mitochondrially targeted H2S donors, which have been shown to protect endothelial cells from oxidative injury (130), may be useful to restore the functionality of the eNOS/sGC/cGMP/PKG pathway. It must be reiterated that the above chain of processes represents a working hypothesis, which remains to be experimentally evaluated in the future.

**Some Open Questions**

There are numerous areas, some more theoretical, some more practical from either the standpoint of physiology or...
pathophysiology, that remain severely underdeveloped and open for further investigation. We briefly summarize some of these areas, and hope that the current review will stimulate thought and future experimentation in these areas. The first theoretical area focuses around the following question: what (evolutionary) purpose does it serve to “double down” on NO signaling with H2S-associated enhancers and facilitators? A related question relates to the relative importance of H2S in regulating various proteins that are traditionally thought to be regulated by ROS-sensitive mechanisms. Several authors speculate on the evolutionary roots of NO, H2S, and ROS, and attempt to explain the necessity of these interactions (e.g., 35, 99), but the topic, in the specific context of vascular regulation, remains to be further explored. Another topic relates to the currently unknown levels (and possible regional differences) of H2S in various parts of the vasculature, as well as the potential regional differences in the expression and activity of the various H2S-producing enzymes. The studies investigating the role of endogenous H2S production in various vascular function have, so far, typically only focused on one particular enzyme, such as, in some cases, the coronary arterial bed, where 3-MST has been proposed as the main H2S-dependent regulatory system (71). However, systematic studies comparing the relative importance of the three H2S-producing enzymes in regulating various vascular responses in various vascular regions remain to be conducted. This subject remains to be studied, both as a stand-alone matter as well as in relation to eNOS signaling. Another area that remains to be studied is the role of H2S in the regulation of vascular permeability (and the potential interplay between NO and H2S in regulating it). Yet another area that remains to be studied much more extensively is the interactions between the H2S pathways and the various constituents of the ROS “world,” including the regulation by H2S of the expression and activity enzymes involved in ROS production and ROS neutralization; the responses of the antioxidant systems to various levels of H2S exposure, and the pathophysiological interplay between the H2S and ROS systems. Once again, this subject remains to be studied, both as a stand-alone matter as well as in relation to eNOS signaling. A working hypothesis related to the interactions of NO and H2S in the context of vascular overproduction is presented in Fig. 2 in the context of vascular dysfunction; however, the various steps of this working hypothesis remain to be further studied.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

C.S. interpreted results of experiments; C.S. prepared figures; C.S. drafted manuscript; C.S. edited and revised manuscript; C.S. approved final version of manuscript.

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H2S regulation of nitric oxide metabolism.


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