Redox Biology 57 (2022) 102504

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Overview on hydrogen sulfide-mediated suppression of vascular calcification and hemoglobin/heme-mediated vascular damage in atherosclerosis

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ARTICLE INFO

Keywords:
Hemoglobin oxidation
Heme
Vascular calcification
Valvular calcification
Hydrogen sulfide

ABSTRACT

Vulnerable atherosclerotic plaques with hemorrhage considerably contribute to cardiovascular morbidity and mortality. Calcification is the main characteristic of advanced atherosclerotic lesions and calcified aortic valve disease (CAVD). Lyses of red blood cells and hemoglobin (Hb) release occur in human hemorrhagic complicated lesions. During the interaction of cell-free Hb with plaque constituents, Hb is oxidized to ferric and ferryl states accompanied by oxidative changes of the globin moieties and heme release. Accumulation of both ferryl-Hb and metHb has been observed in atherosclerotic plaques. The oxidation hotspots in the globin chain are the cysteine and tyrosine amino acids associated with the generation of Hb dimers, tetramers and polymers. Moreover, fragmentation of Hb occurs leading to the formation of globin-derived peptides. A series of these pro-atherogenic cellular responses can be suppressed by hydrogen sulfide (H_2S). Since H_2S has been explored to exhibit a wide range of physiologic functions to maintain vascular homeostasis, it is not surprising that H_2S may play beneficial effects in the progression of atherosclerosis. In the present review, we summarize the findings about the effects of H_2S on atherosclerosis and CAVD with a special emphasis on the oxidation of Hb/heme in atherosclerotic plaque development and vascular calcification.

1. Introduction

According to the WHO, vulnerable atherosclerotic plaques, especially complicated lesions with hemorrhage, contribute considerably to deaths (31%). Complicated atherosclerotic lesions are characterized by ruptures on the plaque surface and/or hemorrhage into the plaque, followed by severe vascular pathophysiology [1]. Intraplaque hemorrhage also evolves by the rupture of the neovascularized vessel derived from the vasa vasorum [1–3]. Calcification is the main characteristic of advanced atherosclerotic lesions [4]. Calcific aortic valve disease (CAVD), the most common valvular heart disease with high morbidity and mortality, and atherosclerosis share similar characteristics, such as the oxidation of lipoproteins/lipids, inflammation, and calcification [5,6].

In contrast to early opinions that considered hydrogen sulfide (H_2S) as a toxic gas, discoveries of this century have shown that H_2S may have beneficial properties in several pathologies. Although high level of H_2S is toxic, slightly elevated H_2S levels have been shown to mediate protective cellular processes [7–9]. Endogenous H_2S in the body is generated by enzymatic and non-enzymatic reactions, or it is produced by the gut microflora. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) mainly generates H_2S from L-cysteine [10], while 3-mercaptopyruvate sulfurtransferase (3-MPST) produces H_2S from 3-mercaptopyruvate [11]. Non-enzymatic H_2S production involves H_2S production from sulfur-containing molecules found in herbs, such as garlic [12], or H_2S production from thiosulfate [13]. Hine and

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https://doi.org/10.1016/j.redox.2022.102504
Received 18 August 2022; Received in revised form 6 October 2022; Accepted 7 October 2022
Available online 8 October 2022

colleagues have also demonstrated another non-enzymatic H_2S -producing mechanism *in vivo* catalyzed by iron and vitamin B6 [14].

Today many publications provide information on physiologic functions of H_2S in vascular homeostasis [15,16], oxygen sensing [17], inflammation, immunomodulation [18,19] and oxidative stress [20]. Recent studies have shown that decreased H_2S production and tissue concentration promote vascular disorders, atherosclerosis, oxidative stress [21], and chronic inflammation [22,23]. The beneficial effects of sulfide in these processes are mostly associated with its interactions with the iron centers of heme proteins [24,25] or with the sulfhydryl side chain of protein cysteine residues to give persulfide derivatives [26,27]. Although their biological formation is still debated, cysteine persulfides are intracellularly abundant modifications, which are largely accepted to be major players in cellular protection against oxidative stress and in redox signaling [28,29]. The interactions of sulfide with heme iron centers and protein cysteine persulfidation are not independent, but rather intimately interlinked processes. For example, heme-mediated oxidation of sulfide can trigger protein cysteine persulfidation *via* the generation of inorganic polysulfide species [30] and cysteine persulfides can effectively reduce heme iron centers [31].

Animal models have demonstrated that decreased endogenous H_2S production predisposes accelerated atherosclerosis development in CSE-knockout mice that is compensated by exogenous NaHS treatment [32]. Moreover, CSE expression has been shown to control flow-dependent vascular remodeling in disturbed flow regions of the vasculature [33]. In human studies, it has been shown that patients with vascular disease have significantly lower circulating H_2S production capacity and sulfide concentrations compared to controls without the disease [34,35]. In addition, patients undergoing surgical revascularization with lower H_2S-producing capability have increased mortality probability within 36 months post-surgery [35].

Microbiomes also influence H_2S metabolism in humans. Imbalances in the gut microbiota triggered by environmental factors are called as gut dysbiosis, which plays an etiologic role in some pathologies [36], among them cardiovascular diseases [37]. H_2S is also produced by gut microbiota, including *Desulfovibrio, Desulfobulbus, Enterococci*, and *Enterobacteria* [38,39]. However, information on the importance of gut microbiome-derived H_2S on cardiovascular diseases is still lacking. Interestingly, it has been found that intracolonic H_2S lowers blood pressure in rat models suggesting that gut microbiome-derived H_2S could be a possible therapeutic target in hypertension [40]. Considering that there is a link between the gut microbiome and atherosclerosis [41], further studies need to be performed to assess the potential connection between gut microbiome-derived H_2S and vascular diseases.

In the present review, we summarize the latest findings about the effects of H_2S on atherosclerosis and CAVD as a potential therapeutic application with a special emphasis on the oxidation of hemoglobin (Hb)/heme in plaque development and vascular calcification (VC).

2. Proposed protective pathways of H_2S on vascular plaque development provoked by hemoglobin and heme. Modification of lipid oxidation and vascular inflammation

Pathologic intimal thickening is a common characteristic of atheroma formation resulting in reduced oxygen diffusion through the vessel wall [4] that triggers neovascularization from the vasa vasorum through the tunica adventitia into the plaque [3]. Red blood cells (RBCs) often infiltrate atherosclerotic lesions during atheroma progression due to plaque hemorrhage or leakage from immature neovessels [42–44]. Damaged RBCs are lysed, releasing free Hb allowing direct interaction of Hb with plaque lipids and vascular cells, among them macrophages [45,46]. In the highly oxidative milieu of plaques [47], Hb interacts with plaque lipids and oxidants derived from macrophages, inducing the formation of various oxidized Hb derivatives, among them metHb (Fe^{3+}) and ferryl-Hb (Fe^{4+}) with the concomitant production of globin-centered free radical-based cross-linking [45,46,48].

2.1. Mechanisms of Hb oxidation

It was shown as early as 1956 that free radicals are formed when metHb (Fe^{3+}) reacts with hydrogen peroxide (H_2O_2) [49]. The reaction is fast (within seconds) and results in metHb oxidation at two sites; heme is oxidized to the ferryl form, and a free radical is formed on the globin chain [49,50]. The spectral fingerprints of the transient ferryl-Hb can be captured by a spectrophotometric method [51]. This method has been applied to quantify the oxidative states of heme-iron in Hb within complicated atherosclerotic plaques of carotid arteries showing that 55% of the total Hb is ferryl-Hb, 39% is metHb, and 1.4% is oxyHb [46].

Solid evidence shows that the free radicals observed in frozen blood samples and the free radicals derived from oxidation of purified metHb by H_2O_2 are identical [52]. Redox cycling of human metHb by H_2O_2 has been revealed by Rakesh et al. to yield persistent ferryl iron and protein based radicals [53]. Redox reactions of Hb can be summarized as follows [52,53]:

Autoxidation of Hb is associated with formation of the superoxide anion radical.

$$Hb\ (Fe^{2+})\text{-}O_2 \rightarrow O_2^{\cdot-} + Hb(Fe^{3+})$$

Superoxide radicals dismutate to produce H_2O_2.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

The generated metHb reacts with H_2O_2 or lipid hydroperoxides (LOOH) to give ferryl-Hb species.

$$Hb(Fe^{3+}) + H_2O_2 \rightarrow \text{Ferryl-Hb}(Fe^{4+})$$

$$Hb(Fe^{3+}) + LOOH \rightarrow \text{Ferryl-Hb}(Fe^{4+})$$

Peroxide can also react directly with deoxyHb, resulting in a ferryl form.

$$Hb(Fe^{2+}) + H_2O_2 \rightarrow \text{Ferryl-Hb}(Fe^{4+})$$

Ferryl forms can comproportionate with ferrous Hb to give metHb:

$$Hb(Fe^{4+}) + Hb(Fe^{2+}) \rightarrow Hb(Fe^{3+})$$

Ferryl-Hb and ferryl-myoglobin can also be reduced back to metHb and metmyoglobin by exogenous reductants [55–57]. The radical on the globin chain is presumably centered on the αTyr-42 close to the heme groups [54]. Ferryl heme can be reduced directly by interactions of reducing agents with the heme pocket [57] or *via* intramolecular electron transfer through one or more amino acids including the tyrosine at the α42 position [58]. It was proposed that intramolecular electron transfer on the β chain of Hb is not favoured; however, mutation of a specific phenylalanine residue at β41 to a tyrosine enhances ferryl removal [58]. Interestingly, introducing this redox-active tyrosine mutation to the β chain improves electron transfer from plasma antioxidants that decreases heme-mediated oxidative reactivity, and enhances NO bioavailability [59]. A novel oxidative pathway was observed during the exposure of Hb to H_2O_2 leading to oxidation of βCys-93 and βCys-112 [60].

2.2. Oxidation of Hb in atherosclerotic lesion

Strong evidence exsist that ferryl-Hb, as a strong oxidant, can shuttle oxidizing equivalents from the iron center to the globin chain to induce intramolecular damage of amino acid residues *via* the formation of a ferric iron center within the artery wall [46]. Migrating protein radicals have been shown to affect primarily the βCys-93, β1Cys112, β2Cys112 and α1Cys104 residues in complicated atherosclerotic lesions [46], which induces Hb dimerization [61,62]. In addition, ferryl-Hb produces free radicals in the α (αTyr-24, αHis-20, αTyr-42) and β(βTyr-36, βTyr-130) chains of globins [63–65]. Termination reactions of globin-centered radicals yield globin-globin crosslinking and the

formation of Hb dimers, tetramers, and multimers that are present in human complicated atherosclerotic lesions [45,48]. Hb oxidation has been shown to generate globin-derived peptides in ruptured plaques and intraventricular hemorrhage of the brain [66]. Hb is also a known Fenton-reagent facilitating hydroxyl-radical generation from activated oxygen species [67].

2.3. Interactions between Hb and H_2S

At the low physiological H_2S:heme ratios, metHb reacts with H_2S according to the followings [68]. H_2S rapidly binds to metHb forming a $Hb(Fe^{3+}) - H_2S$ complex, and H_2S can either dissociate and regenerate ferric heme in a reversible process (Reaction 1), or it can reduce slowly the bound Fe^{3+} heme to the Fe^{2+} form and dissociate as $HS^{\cdot}$ (Reaction 2).

$$Hb(Fe^{3+}) + H_2S \rightleftarrows Hb(Fe^{3+}) - H_2S \quad \text{(Reaction 1)}$$

$$Hb(Fe^{3+}) - H_2S \rightarrow Hb(Fe^{3+}) - HS^- + H^+ \rightarrow Hb(Fe^{2+}) - HS^{\bullet} \rightarrow Hb(Fe^{2+}) + HS^{\bullet} \quad \text{(Reaction 2)}$$

Alternatively, H_2S may also reduce Fe^{3+} heme without prior iron coordination according to Reaction 3.

$$Hb(Fe^{3+}) - H_2O + H_2S \rightarrow Hb(Fe^{2+}) + H_2O + H^+ + HS^{\bullet} \quad \text{(Reaction 3)}$$

H_2S also inhibits oxidative cross-linking of Hb subunits exposed to peroxide [25], the hallmark of ferryl-Hb formation. Hb cross-linking proceeds *via* ferryl-Hb intermediate species, which are found to be scavenged rapidly by sulfide [25], hence providing a potential molecular model for sulfide-mediated inhibition of Hb oligomerization. The measured rate constants for the reactions of ferryl-Hb species with sulfide represent at least 1–3 orders of magnitude faster reactions [25] (for the slower reactions recorded at 425 nm and the faster ones at 620 nm) compared to those with ascorbate or urate [69] that are the previously proposed primary heme-redox-intermediate-scavenging antioxidants (because thiols in most cases have no access to the metal center at the active site). Also, the relatively high stability of the ferryl-Hb species implies that Hb oligomers must form in much slower reactions. In this Special issue, a review will be devoted to the detailed molecular mechanism of interaction between heme proteins and H_2S.

2.4. Hb oxidation results in polarization of macrophages towards a proatherogenic phenotype

Ferryl-Hb is taken up *via* CD163 receptor-mediated endocytosis and phagocytosed by macrophages, then transported to lysosomes triggering the pro-atherogenic polarization of macrophages in human atherosclerotic plaques [46]. Ferryl-Hb activates proinflammatory programs in macrophages by inducing the expression of inflammatory cytokines such as IL-1β, TNF-α, and CXCL8 and by decreasing the expression of anti-inflammatory cytokines such as CD209 and IL27RA. The proinflammatory gene expressions induced by ferryl-Hb in macrophages is driven by the activation of PI3K/HIF-1α/VEGF pathway. A 39% overlap in the differential gene expression profiles of human macrophages exposed to ferryl-Hb and those of complicated lesions is considered as a signature of ferryl-Hb in the progression of atherosclerosis. Among these 547 genes, inflammatory, angiogenesis, iron metabolism, calcification, tissue remodeling, and cellular damage related gene clusters that are regulated in macrophages were found to indicate a proatherogenic phenotype swich [46].

Similar to ferryl-Hb, chemically modified Hb is also taken up by macrophages *via* CD163 scavenger receptor in the absence of haptoglobin [70].

2.5. Haptoglobin-mediated protection against Hb toxicity

While the binding of haptoglobin to intact Hb dimers is well characterized and its protection against Hb toxicity has been revealed [71], the binding of ferryl-Hb to haptoglobin hasn't been studied in details. Haptoglobin binding of Hb decreases both the ferryl iron and free radical reactivity of Hb during peroxide challenge [69]. Oxidative fragmentation of the globin chains is also inhibited by the binding of Hb to haptoglobin [66]. The significantly higher excess of free Hb in ruptured atherosclerotic lesions [46] over haptoglobin eliminates haptoglobin-mediated beneficial functions in this pathology.

Haptoglobin-realated alternative pathways have been shown in macrophage polarization to mitigate atherosclerosis owing to haptoglobin. Hb-haptoglobin polarizes macrophages into a Hb-clearance phenotype with reduced antigen presentation [72]. Importantly, the Hb-haptoglobin complex results in a phenotype characterized by elevated heme oxygenase 1 (HO-1), CD163, and interleukin-10 expression [73,74]. This adaptation of macrophages to intraplaque hemorrhage is mediated by heme oxygenase 1 regulated via activating transcription factor 1 (ATF-1) [75]. This special macrophage phenotype may have an essential protective role in hemorrhaged atherosclerotic plaques to mitigate Hb-induced tissue injury.

2.6. Protective effect of H_2S on heme- and Hb-mediated oxidation of low-density lipoprotein

Oxidative modification of low-density lipoprotein (LDL) plays a fundamental role in atherogenesis [76]. In addition, to its direct role in atherogenesis, Hb also aggravates atherosclerotic plaque progression *via* catalyzing LDL oxidation. Hb liberated from RBCs is the main source of labile heme, a pathophysiologic catalytic agent of LDL oxidation [77,78]. Heme and Hb is also involved in the oxidative crosslinking of LDL promoting LDL protein aggregation that facilitates foam cell formation [79,80]. Hb-induced LDL oxidation is catalyzed by globin radicals as well [81]. Heme is easily released from Hb after its oxidation depending on the redox states of heme-iron, and the release is inhibited by haptoglobin [82]. The liberated heme is captured by hemopexin [83] and alpha-1-macroglobulin [84]. MetHb loses its heme moieties at substantially higher rates than ferryl-Hb [85]. The abundance of metHb and ferryl-Hb in complicated lesions [46] over heme binding proteins found in plasma demolishes its cytoprotective function against Hb toxicity in ruptured lesions.

Translocation of heme to lipoproteins in plasma [78,86] and in atherosclerotic plaques after rupture is observed with subsequent oxidative reactions, degradation of porphyrin ring and liberation of iron [45]. Globin radicals formed upon Hb oxidation also contribute to Hb-induced LDL oxidation [81]. In addition, LDL oxidation occurs in the blood due to the oxidative cross-linking of ApoB100 [87]. Given the pivotal role of oxidized LDL (LDLox) in atherosclerosis, it is reasonable to assume that decreased LDL oxidation might be a promising therapeutic approach in vascular diseases. In line with this, H_2S has been shown to decrease lipid hydroperoxides in LDLox [88]. H_2S is reported to delay the accumulation of lipid peroxidation products, such as thiobarbituric acid reactive substances, conjugated dienes, and lipid hydroperoxides (LOOH) during hemin-mediated oxidation of LDL, and H_2S also lowers the LOOH content of both plaque lipids and LDLox as well as the subsequent cell death [89]. Furthermore, the rapid amplification of lipid peroxidation products in atherosclerotic lesions catalyzed by heme can be inhibited by H_2S [89]. Based on in vitro kinetic analyses, we proposed that these processes are likely to be related to sulfide-mediated rapid reduction of ferryl-Hb derivatives [25].

2.7. Effects of LDLox on CSE/CBS expression

Interestingly, several reports have shown that LDLox can influence CSE and CBS expression. LDLox triggers the hypermethylation of the CSE promoter that decreases CSE expression as well as H_2S production in mice and murine macrophages [90,91]. In contrast, others have found that LDLox up-regulates CSE expression in murine macrophages and

human ECs and SMCs [25]. CSE expression is up-regulated in early and complicated human carotid artery plaques, especially in macrophages, foam cells, SMCs, and myofibroblasts [25]. A similar pattern has been observed in aortic lesions of apolipoprotein E-deficient (apoE−/−) mice on a high-fat diet [25]. Identification of triggers for inducing CSE expression during the development of atherosclerosis have clinical relevance. Ferryl-Hb, heme, LDLox catalyzed by heme-iron, plaque lipids were found to induce the expression of CSE in human macrophages and smooth muscle cells (SMCs) *in vitro* [25]. However, it is worth mentioning that high CSE expression is not necessarily associated with increased H_2S production in these lesions. This notion is underlined by a recent study demonstrating that in human aortic valve specimens derived from patients diagnosed with CAVD and in aortic valves of apoE−/− mice presenting valvular calcification, a high level of CSE expression has been detected in the valvular interstitial cells associated with low H_2S levels within the tissues compared to normal valves without mineralization [92]. One explanation may arise from an observation that revealed a posttranslational modification of CSE *via* Ser377 phosphorylation leading to decreased activity [93]. Protective pathways of H_2S on vascular plaque development provoked by hemoglobin and heme are summarized in Fig. 1.

A limitation of these works is that data on hemoglobin/H_2S interactions derive from *ex vivo* test tube experiments which showed that H_2S inhibits Hb oxidation. The local in vivo H_2S concentrations in intact arteries and atherosclerotic lesions have remained unexplored yet. Employing different animal models future studies should reveal the effective concentration of H_2S generated endogenously or released by H_2S donors for the control of atherosclerotic plaques development.

2.8. Other protective mechanisms mediated by H_2S in atherosclerosis development

In vascular endothelial cells (ECs), ferryl-Hb activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) [94] and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome [66] supporting its pro-inflammatory effect. Notably, well-known pathogenic factors of atherosclerosis including oxidized Hb and heme induce CSE expression in human macrophages and SMCs *in vitro* [25]. Considering that Hb oxidation plays a crucial role in atherosclerosis development, it is reasonable to expect that the inhibition of this pathologic process might attenuate atherosclerosis.

In murine model, H_2S mitigates diabetes-accelerated atherosclerosis *via* the activation of Nuclear factor erythroid 2-related factor 2 (Nrf2)-related antioxidant defense by the sulfhydrylation of Kelch-like ECH-associated protein 1 (Keap-1), which induces HO-1 expression and reduces superoxide formation as well as the up-regulation of adhesion molecules [95]. In addition, H_2S counteracts high glucose-induced cell death, ROS production, and pro-inflammatory cytokine production *via* the activation of PI3K/Akt/eNOS pathway that could counterbalance EC dysfunction involved in atherosclerosis [96].

Chronic kidney disease markedly accelerates both arterial calcification and atherosclerosis [97,98]. In murine model of uremia accelerated atherosclerosis (UAAS), uremic mice develop early atherosclerosis that is counteracted by NaHS administration [99]. In UAAS mice, the

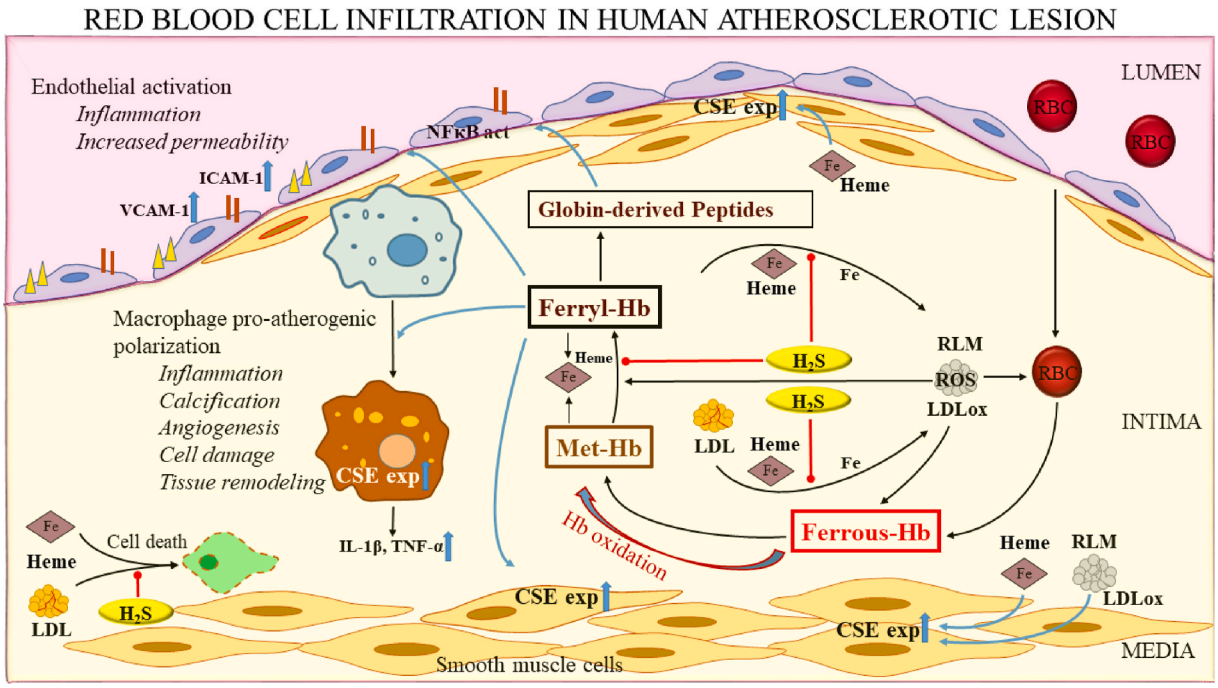

Fig. 1. Proposed protective pathways of H_2S on vascular plaque development provoked by hemoglobin and heme. Modification of lipid oxidation and vascular inflammation

Infiltration of red blood cells (RBCs) into atheromatous plaques is common during atherosclerosis progression. Hemoglobin (Hb) released from RBCs readily oxidizes to met- (Fe^{3+}) and ferryl-Hb (Fe^{4+}), followed by release of heme from globin and iron from heme moiety. Hb oxidation facilitates protein radical formation damaging the globin structure that yields globin-globin crosslinking, Hb dimers, tetramers, multimers, and globin-derived peptides. Free heme and iron catalyze the oxidation of low-density lipoprotein (LDL) and plaque lipids resulting in oxidized LDL (LDLox) and the formation of reactive lipid mediator (RLM), releasing reactive oxygen species (ROS). Both ferryl-Hb and globin-derived peptides provoke endothelial cell activation and induce adhesion molecule expressions, such as vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), leading to increased endothelial cell permeability. Notably, ferryl-Hb and globin-derived peptides trigger inflammatory response *via* nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Ferryl-Hb also drives the pro-atherogenic polarization of macrophages in the vessel wall, thereby affecting diverse processes, among them inflammation, calcification, angiogenesis, cell damage, and tissue remodeling. Heme, oxidized lipid mediators, and ferryl-Hb induce cystathionine γ-lyase (CSE) expression in the resident cells of atherosclerosis. Significantly, H2S inhibits the oxidation of Hb and plaque lipids and prevents LDLox-induced cell death. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expression of CSE and transforming growth factor-β (TGF-β) are lowered, Smad3 phosphorylation decreases and all these effects are compensated by NaHS. Importantly, the CSE inhibitor propargylglycine accelerates atherosclerosis in UAAS mice [99] suggesting the protective role of CSE/H_2S and the downstream TGF-β/Smad3 signaling in UAAS. Others have found that the protective function of H_2S in UAAS is, at least partly, mediated by the novel protein kinase-δ/Akt pathway [100] and by the conventional protein kinase C βII/Akt signaling [101].

Another regulatory point of H_2S in atherosclerosis development is protein S-nitrosylation [102]. Protein S-nitrosylation involves the covalent attachment of a nitrogen monoxide group to the thiol side chain of Cys that regulates protein-protein interactions and ubiquitylation-dependent protein degradation (reviewed by Ref. [103]). In the murine model of atherosclerosis, H_2S reduces aortic atherosclerosis and increases plasma NO and protein S-nitrosylation in aortic SMCs. In contrast, the CSE inhibitor propargylglycine has the opposite effect suggesting that H_2S attenuates atherosclerosis by inhibiting SMC migration and proliferation *via* up-regulating plasma NO and protein S-nitrosylation [102].

Angiotensin-converting enzyme 2 (ACE2) and angiotensin 1-7 (Ang 1–7) has been reported to protect against atherosclerosis [104,105]. In the partially ligated carotid artery model of atherosclerosis in ApoE−/− mice, H_2S attenuates atherosclerosis by increasing ACE2 expression and Ang 1–7 formation, while the anti-atherosclerotic potential of NaHS is abolished by an ACE2 inhibitor [106]. Interestingly, propargylglycine increases the atherosclerotic burden and down-regulates ACE2 expression in atherosclerotic plaques suggesting that H_2S protects against atherosclerosis *via* increasing ACE2 expression [106].

2.9. H_2S donors in atherosclerosis research

The physiologic concentrations of bioavailable H_2S within the artery wall and heart valves have not been measured. Substantial effort was devoted to synthesize H_2S donors for research as well as for therapeutic utilization. Sulfide donors are designed to release sulfide *via* hydrolysis or other triggering mechanisms. Many inorganic sulfide salts have been utilized to administer H_2S to biological systems, including Na_2S and NaHS, which produce dissolved sulfide in aqueous media upon dissolution. Utilization of inorganic sulfide salts in animal experiments could produce misleading results. Intravenous or intraperitoneal injections may introduce inordinately high local concentrations of H_2S at the injection site, which may provoke adverse effects. Therefore, inorganic sulfide salts -although suitable for in vitro experiments-are less preferred for *in vivo* studies.

A new generation, slow-releasing sulfide donor compound (GYY4137) was synthesized from Lawesson's reagent and morpholine [107]. More stable H_2S-releasing compounds such as AP67 and AP72 exhibit extremely slow H_2S generating rates in biological systems especially compared to sulfide salts [107–109]. These donors have been used for both *in vitro* and *in vivo* studies in which they were administered intraperitoneally. Thiol- and perthiol-triggered hydrogen sulfide donors also have the potential for clinical application [110–113]. Clinical use of H_2S donors as therapeutical drugs in vascular diseases largely depends upon their site-specific activities in the vicinity of the lesions.

It is important to note that the concentrations of donors do not represent the total amount of released sulfide, and all slow donors have different sulfide-releasing potentials. In addition, it is increasingly recognized that slow-releasing H_2S donors are likely to more closely mimic the effects of the endogenous H_2S buffer system, because the slow release does not provoke steep elevation in local concentrations of sulfide, as it was observed with sulfide salts [108,114]. The effective concentration depends upon the targets of H_2S donors at subcellular level. In particular, the mitochondria-targeted H_2S donor, AP39 requires 3 orders of magnitude less administered concentrations (10^{-8} mol/L) to obtain a beneficial effect as compared to AP72 (10^{-5} mol/L). Each H2S donor has a specific and narrow therapeutic range, which also depends on the experimental settings.

Exceeding such concentrations leads not only to the loss of benefit but also to adverse effects or even toxicity. H_2S toxicity is caused by the reaction of H_2S with trivalent iron in oxidized cytochrome oxidase, which inhibits cellular respiration and leads to cellular hypoxia [115].

Overall, H_2S is protective against many different processes that contribute to atherogenesis in various models, however, these models seem to have limitations. Although different H_2S donors with different H_2S-releasing kinetics are used in these models, the local H_2S concentrations in the atherosclerotic plaques are not measured which makes local effective H_2S concentrations difficult to define. Considering that H_2S is toxic at higher local concentrations, the minimally effective in vivo concentration locally in the plaques needs to be precisely measured. Other issues in the human application could be to find the optimal mode of administration since the majority of studies administers H_2S donors intraperitoneally. Otherwise, vascular diseases in humans are chronic and animal studies are generally short-term compared to human applications which necessitate research on the effects of long-term, chronic applications of H_2S in humans to find effective local concentrations with limited toxicity. To overcome this issue, a feasible approach could be to target H_2S donors in an organ- or tissue-specific manner.

3. Inhibitory pathways of H_2S on vascular/valvular calcification by the modification osteochondrogenic *trans*-differentiation of vascular smooth muscle cells and valvular interstitial cells

Vascular SMCs (VSMCs) and valvular interstitial cells (VICs) are critical for maintaining the physiologic functions of the vasculature, and their osteo-chondrogenic transformation triggered by calcifying stimuli such as high plasma phosphate (Pi) [116], Lipoprotein(a) [117], or, lipid oxidation products including LDLox [118,119] play an etiologic role in atherosclerosis and CAVD development [120]. Atherosclerosis is commonly associated with intimal calcification, while medial calcification mainly occurs in chronic kidney disease [121] and diabetes mellitus [122]; both dramatically aggravate plaque development and progression. Osteo-chondrogenic *trans*-differentiation of VSMCs and VICs involves the downregulation of SMC- and VIC-specific genes, such as smooth muscle α-actin [123]. Parallel with that, genes involved in osteoblast differentiation such as Krüppel-like factor 5 (KLF-5) [124], Runt-related transcription factor 2 (Runx2) [125], and Bone morphogenetic protein-2 (BMP-2) are induced or activated [126]. Inhibitory pathways of H_2S on vascular/valvular calcification are summarized in Fig. 2.

3.1. Protective effect of H_2S on vascular calcification via regulating phosphate-dependent Runx2 activation

Multiple studies suggest that H_2S can ameliorate VC *via* various mechanisms. Given that increased Pi uptake by VSMCs and VICs plays a crucial role in their osteo-chondorgenic transformation, from a therapeutic aspect, lowering Pi uptake might represent an ideal therapeutic approach to decrease VC. The sodium-dependent phosphate cotransporter, Pit-1, plays an essential role in VC [127]. H_2S has been shown to lower Pi uptake *via* reducing the expression of phosphate channel Pit1, thereby decreasing the activity of alkaline phosphatase and the secretion of osteocalcin by inhibiting the nuclear translocation of Runx2 [34,92].

3.2. Protective effect of H_2S on vascular calcification via regulating pyrophosphate metabolism and Stat3/Cathepsin S pathways

Regulation of pyro-phosphate (PPi) generation by H_2S represents a novel, additional mechanism to control VC [92]. PPi acts as an endogenous inhibitor of VC that is supported by the finding that PPi plasma levels in end-stage renal disease patients and tissue samples of CAVD patients are reduced [92,128]. Increased production of PPi is maintained by endogenous and exogenous H_2S *via* upregulating

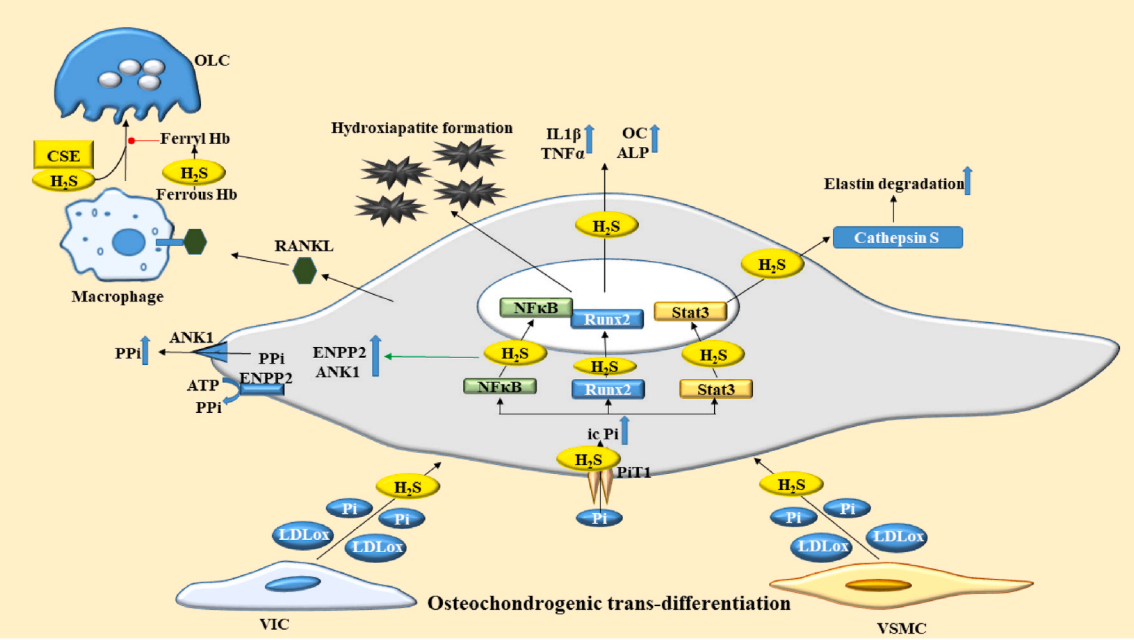

Fig. 2. Inhibitory pathways of H_2S on vascular/valvular calcification by the modification osteochondrogenic *trans*-differentiation of vascular smooth muscle cells and valvular interstitial cells.

Osteochondrogenic *trans*-differentiation of vascular smooth muscle cells (VSMCs) and valvular interstitial cells (VICs) induced by high phosphate (Pi) and oxidized low-density lipoprotein (LDLox) is a hallmark of vascular calcification. Pi taken up by the sodium-dependent phosphate cotransporter, PiT-1, triggers the nuclear translocation of Runt-related transcription factor 2 (Runx2), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and Stat3. Runx2, the master regulator of calcification, induces the expression of calcification-specific markers, such as osteocalcin (OC), alkaline phosphatase (ALP), and receptor activator of nuclear factor kappa-B ligand (RANKL), promoting hydroxyapatite formation. NFκB induced by high Pi or LDLox activates inflammatory response by triggering interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression that aggravates Runx2-mediated calcification. In contrast, Stat3 induces cathepsin S expression that facilitates elastin degradation and mineralization in the vessel wall. Significantly, H_2S induces ectonucleotide pyrophosphatase/PDE family member 2 (ENPP2) and Ankyrin G1 (ANK1) expression, thereby promoting the synthesis of the anti-calcification product pyrophosphate (PPi). H2S also lowers Pi uptake, the nuclear translocation of Runx2, NFκB, and Stat3, and ameliorates the expression of OC, ALP, IL-1β, TNF-α, and cathepsin S. RANKL secreted by VSMCs and VICs underwent osteochondrogenic *trans*-differentiation promotes osteoclastogenic *trans*-differentiation of macrophages to limit calcification in the vessel wall. Ferryl-Hb counteracts RANKL-mediated OLC formation that impairs calcium resorption in calcified atherosclerotic lesions. H_2S facilitates OLC formation by directly increasing OLC formation or, in an indirect manner, by inhibiting ferryl-Hb formation in hemorrhaged plaques.

ectonucleotide pyrophosphatase/PDE family member 2 and Ankyrin G1 [92].

Cathepsin S is involved in the VC of ApoE−/− mice with chronic kidney disease [129]. However, exogenous H_2S attenuates VSMC calcification aggravated by high glucose *via* suppressing Stat3/Cathepsin S signaling, thereby up-regulating elastin expression [130].

3.3. Protective effect of H_2S on vascular calcification via regulating oxidative stress

Another point where H_2S might ameliorate VC is that it mitigates oxidative stress generated by reactive oxygen species (ROS). Oxidative stress provokes VC by increasing Runx2 expression and transactivation *via* Akt signaling [131]. In addition, focal ROS production increases in rat aortas by laser treatment with subsequent vascular degeneration and calcification [132]. In calcifying aortas from rat CKD model, where VC is induced with high calcium-Pi diet and vitamin D, significant ROS generation can be detected in the aortas [133]. H_2S can mitigate oxidative stress through multiple mechanisms: by inducing antioxidant response through Nrf2 signaling [134], or, *via* interacting with ROS inducing metalloprotein centers {Dóka et al. Redox biol this special issue}, or *via* protecting protein thiol residues from overoxidation *via* persulfidation [28]. H_2S-mediated antioxidant protection is supported by a recent study demonstrating that it inhibits circulating calciprotein particles-induced VSMC calcification by activating KEAP1-Nrf2 enhances NAD(P)H dehydrogenase [quinone] 1 (NQO1) expression [135].

3.4. Protective effect of H_2S on vascular calcification via regulating inflammatory response

Given that atherosclerosis is not only a lipid oxidation process but also a chronic inflammatory disease, several studies underline the link between inflammation and VC [136,137]. In the process of VC, pro-inflammatory cytokines such as interleukin-1β (IL-1β) [138] and tumor necrosis factor-α (TNF-α) are released from pro-atherogenic M1-type macrophages [139]. It has been revealed that (L-1β and TNF-α are involved in CAVD. Nuclear translocation of NF-κB, induced by TNF-α, promotes Pi-induced calcification in VSMCs [140]. Activation of NF-κB accompanied by increased expression of IL-1β and TNF-α has been observed in patients with CAVD and in the calcified aortic valve of ApoE−/− mice on high-fat diet [141]. Notably, it has been shown that the activation of Runx2 is dependent on NF-κB, indicating that inflammation and mineralization are linked at the transcriptional level [141]. Importantly, H_2S prevents activation of NF-κB and the induction of IL-1β and TNF-α as well as mitigates Runx2 activation and subsequent VIC mineralization both in vitro and in vivo [141].

3.5. Protective effect of H_2S on vascular calcification via regulating osteoclast-like cell formation

Another checkpoint where H_2S may ameliorate VC is the regulation of osteoclast-like cell (OLCs) formation in vascular tissues. OLCs differentiation from the monocyte/macrophage lineage requires macrophage/monocyte colony-stimulating factor-1 (M-CSF) and the receptor activator of nuclear factor kappa-B ligand (RANKL) [142,143], both of

which are expressed in calcifying vessels. OLCs in atherosclerotic plaques differentiate from infiltrating macrophages and colocalize with mineralization and cholesterol deposition [144]. Arterial calcium deposits are suggested to form a unique environment favoring OLC formation to limit calcification in atherosclerosis [145,146]. Recently, it has been found that OLCs are abundantly present in calcified plaques and colocalize with calcium deposition, while the number of OLCs is decreased in hemorrhagic lesions with ferryl-Hb accumulation [147]. Ferryl-Hb but no ferrous Hb inhibits RANKL-mediated osteoclastogenic gene expression [147]. In addition, heme has been shown to inhibit osteoclastogenesis *via* the induction of HO-1 [148]. The decreased bone resorption activity suggests that accumulation of ferryl-Hb and heme might impair calcium resorption in calcified atherosclerotic lesions [147]. Notably, CSE has been reported to boost OLC differentiation and calcium resorption, while CSE silencing or inhibition weakens bone resorption [149]. Others have found that H_2S promotes RANKL-induced osteoclastogenesis *via* activating the PI3K/AKT/mTOR pathway that down-regulates autophagy [150]. These suggest that H_2S facilitates OLC formation by directly increasing OLC formation or, in an indirect manner, by inhibiting ferryl-Hb formation in hemorrhaged plaques.

Overall, these suggest that impaired H_2S production can be involved in both vascular and valvular calcification, and restoring H_2S levels in the vascular tissues may prevent pathologic calcification by reducing the osteo-chondrogenic *trans*-differentiation of VSMCs and facilitating OLC formation. Important points in this protective effect could be to inhibit key points of vascular/valvular calcification. These mechanisms involve the inhibition of the osteoblastic transformation of VSMCs and VICs by blunting the master regulator of calcification Runx2 and, diminishing inflammatory response through NFκB. These effects could inhibit or, at least, slow down calcification processes. Otherwise, H_2S could be beneficial by enhancing natural protective pathways in plaques by activating OLC formation which might counteract tissue mineralization in the vascular tissues.

4. Beneficial effects of H_2S on atherosclerosis through endoplasmic reticulum (ER) stress, epigenetic regulation, and hypoxic states

ER stress, epigenetic modifications, and hypoxia play a significant role in atherosclerosis [151–154]. Given that physiologic ER stress plays a pivotal role in bone development [155,156], it is reasonable to assume that ER stress might be involved in VSMC calcification. Several studies have shown that ER stress is involved in VC [157,158].

4.1. Effects of H_2S on ER stress in atherosclerosis development

ER stress plays a significant role in atherosclerosis [151,159]. Considering that H_2S protects against ER stress in many conditions [160], it is reasonable to hypothesize that H_2S might protect against pathologic ER stress associated with VC. To support this, in rat model of VC involving vitamin D3 plus nicotine, H_2S alle*via*tes VC by inhibiting ER stress [161]. Others have recently reported that H_2S mitigates hyperhomocysteinemia-induced atherosclerosis by enhancing the activity of protein disulfide isomerase, a key enzyme of oxidative protein folding in the ER *via* sulfhydration [162]. These studies underline that H_2S effectively mitigates atherosclerosis-related ER stress. However, it is essential to note that studies in this field are still scarce.

4.2. Epigenetic regulation of endogenous H_2S production

Epigenetic alterations play a pathogenic role in atherosclerosis [152]. Evidence shows that epigenetic regulation exists in CSE expression. In ApoE−/− mice, DNA methyltransferase (DNMT) activity is induced while CSE expression and H_2S production decrease in peritoneal macrophages compared to those from control mice [90]. Similar to this, LDLox-treated RAW264.7 macrophages showed increased DNMT and enhanced DNA methylation of the CSE promoter. However, inhibition of DNMT has reversed CSE mRNA decrease suggesting that hypermethylation of the CSE promoter and decreased CSE expression contribute to the development of atherosclerosis [90]. In addition to DNA modifications, CSE expression is also regulated by microRNAs (miRNAs), short non-coding RNAs targeting mRNAs, resulting in mRNA silencing [163].

MiRNAs are involved in several diseases, among them atherosclerosis. ATP-binding cassette transporter A1 (ABCA1) plays a vital role in promoting cholesterol efflux, thereby inhibiting its accumulation in macrophages. ABCA1 deficiency results in impaired cholesterol efflux that increases atherosclerosis development [164]. CSE/H_2S has been reported to induce ABCA1 expression in THP-1 macrophages, while miRNA-216a targeting the 3′-untranslated region of CSE markedly reduces CSE and ABCA-1 expression leading to decreased cholesterol efflux and increased intracellular cholesterol level in THP-1 macrophage-derived foam cells [165]. Evidence suggests that miRNA-216a is involved in atherosclerosis development by promoting M1 pro-inflammatory macrophage formation [166]. In addition, miRNA-216a level is increased in patients with vulnerable coronary plaques [166]. Interestingly, H_2S is involved in histone modifications [167]. In ApoE−/− mice, H_2S treatment induces Sirtuin 1 (SIRT1) mRNA expression in the aorta and liver [168]. SIRT1, an NAD+-dependent class III histone deacetylase, has an anti-atherogenic effect [169]. Sulfhydration of SIRT1 by H_2S enhances SIRT1 activity that reduces endothelial inflammation and macrophage-derived foam cell formation which may reduce atherosclerotic plaque formation [168]. These studies strongly support that epigenetic regulation of H_2S producing pathways plays a pivotal role in atherosclerosis development, and targeting these regulatory checkpoints might represent promising directions in atherosclerosis therapy (Fig. 3).

4.3. Cross-talk between hypoxia and CBS/CSE/H_2S-mediated signaling

A body of evidence suggests that hypoxia is involved in atherosclerosis development [153,154]. However, interactions between CBS/CSE/H_2S and hypoxic signaling in atherosclerosis are still ill-lit.

Hypoxia-inducible factor-1 (HIF-1) has a pivotal role in cellular responses to hypoxia-regulated genes involved in angiogenesis, energy metabolism, and cell death. Under normal conditions, the von Hippel-Lindau (VHL) tumor suppressor gene product pVHL targets HIF-1α for proteasomal degradation [170], while hypoxia induces alterations in well-conserved prolyl- and asparaginyl residues of HIF-1α resulting in its stabilization and HIF-dependent activation of target genes [171]. Evidence suggests that hypoxia and CSE/H_2S systems might interact in several ways. In human VSMCs, the transcription factor Specificity protein 1 (Sp1) tightly regulates CSE gene expression [172]. In VSMCs, miRNA-21 down-regulates Sp1 and subsequent CSE expression [173]. Importantly, hypoxia stimulates miRNA-21 expression that down-regulates CSE in hypoxic SMCs [174]. SMCs derived from CSE-knockout mice are characterized by redox imbalance and impaired mitochondrial activity compared to wild-type cells [175]. In addition, CSE-knockout cells are more susceptible to hypoxia-induced cell death suggesting an important role for the CSE/H_2S pathway in protecting against hypoxic stress-induced damages [175]. In the glomus cells, which are responsible for oxygen sensing in the carotid body, hypoxia increases H_2S generation in a stimulus-dependent manner. At the same time, CSE-deficient mice are characterized by impaired carotid body response and ventilatory stimulation in response to hypoxia and a loss of hypoxia-evoked H_2S generation [176]. Others have found in human liver cancer SMMC-7721 and lung adenocarcinoma A549 cells that during a short hypoxic period (4 h), CSE protein levels show a somewhat increased expression that declined back to the baseline [177]. These results indicate that H_2S production under hypoxia, particularly in VSMCs (Fig. 4), is not well understood, requiring further research to reveal the interaction between hypoxia and endogenous H_2S generation.

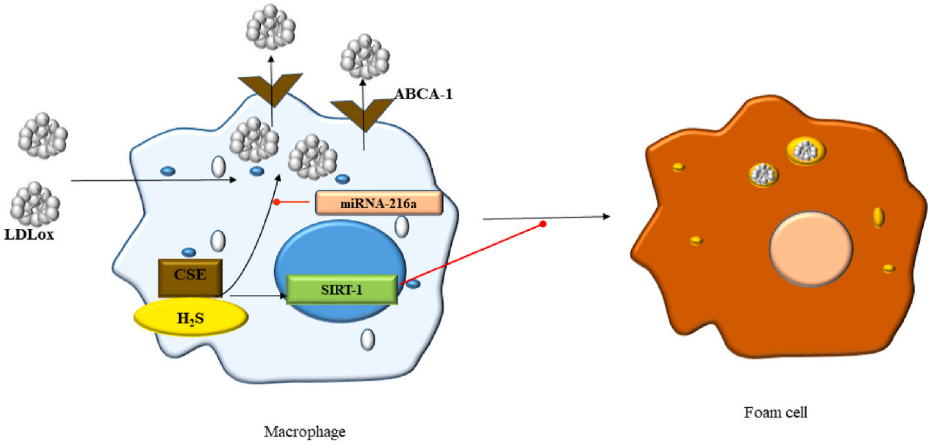

Fig. 3. Epigenetic reprogramming of foam cell formation by H_2S
Cystathionine γ-lyase (CSE) and H_2S protect against LDLox-mediated foam cell formation by promoting cholesterol efflux through the ATP-binding cassette transporter A1 (ABCA1), which is inhibited by MicroRNA-216a. In addition, CSE/H_2S promotes Sirtuin 1 (SIRT1) sulfhydration increasing its histone deacetylase activity that lowers foam cell formation.

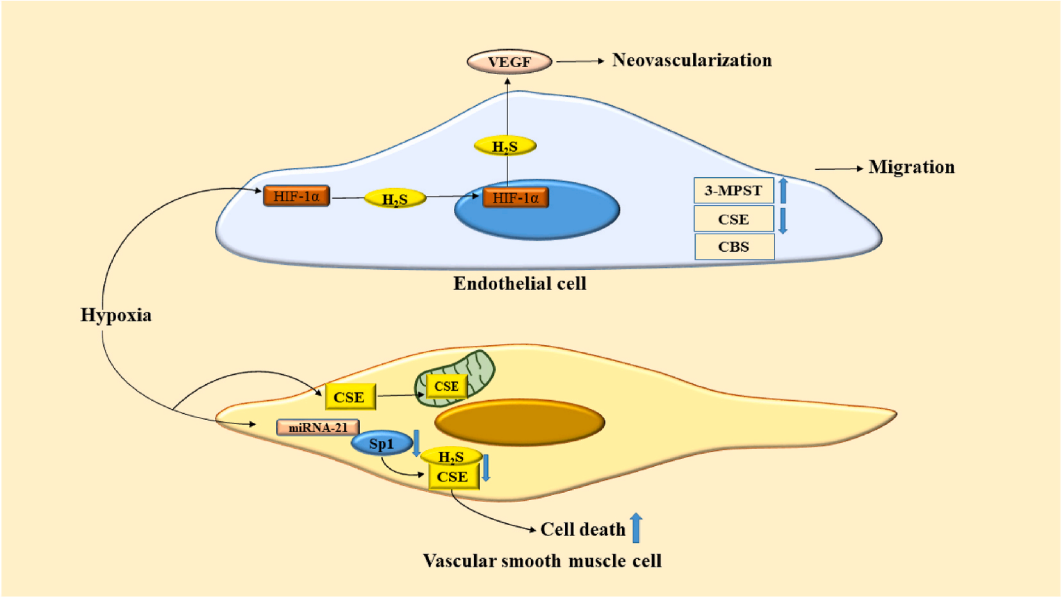

Fig. 4. Cross-talk between hypoxia and H_2S/endogenous H_2S synthesis
Hypoxia and hypoxia-mimetic drugs induce the accumulation and nuclear translocation of hypoxia-inducible factor-1α (HIF-1α) followed by an increase in the expression of hypoxia-responsive genes, among them vascular endothelial growth factor (VEGF), which promotes neovascularization. In endothelial cells (ECs), H_2S reverses HIF-1α accumulation and subsequent VEGF production that might counteract hypoxia-induced neovascularization and tube formation. In addition, hypoxia differentially regulates the expression of cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MPST), and cystathionine β-synthase (CBS). Hypoxia induces 3-MPST, decreases CSE, and has no effect on CBS levels. In vascular smooth muscle cells (VSMCs), hypoxia down-regulates Specificity protein 1 (Sp1), the master regulator of CSE in VSMCs, thereby lowering overall CSE expression, leading to increased cell death. In addition, hypoxia induces the translocation of CSE to the mitochondria.

Alternatively, hypoxia also regulates mitochondria-specific H_2S production. In SMCs from mesenteric arteries of mice, the hypoxia mimetic calcium ionophore A23187 induces the expression and translocation of CSE from the cytosol into the mitochondria, enhancing H_2S production inside the mitochondria, followed by increased ATP production that sustains mitochondrial ATP production under hypoxic conditions [178].

Considering that hypoxia-induced Vascular endothelial growth factor (VEGF) production and EC migration/tube formation are essential in the neovascularization and hemorrhage of atherosclerotic plaques, from a therapeutic perspective, it is vital to reveal the possible effects of H_2S on neovascularization. In immortalized human vascular endothelial cells (EA.hy926), HIF-1α accumulation, in response to hypoxia (1% O2) and hypoxia-mimetics, is reversed by NaHS administration *via* enhancing eIF2α phosphorylation. Significantly, NaHS also inhibits VEGF expression and in vitro tube formation under hypoxia [179]. In primary human umbilical vein endothelial cells (HUVECs), moderate hypoxia (10% O2) increases, while lower pO2 (8%) does not affect ECs migration [180]. This work has shown that CBS, CSE, and 3-MPST, the three endogenous enzymatic H_2S-producing enzymes, differently respond to hypoxia: hypoxia significantly induces 3-MPST, decreases CSE, but does not affect CBS levels. In addition, CBS promotes the migration of HUVECs in both normoxic and hypoxic conditions, CSE has an inhibitory effect on migration. In contrast, 3-MPST promotes the migration of HUVECs in hypoxia but has no effect in normoxia suggesting that 3-MPST governs the hypoxia-induced migration of HUVECs [180]. Importantly, it has been shown that hypoxia and H_2S differently affect migration and tubulogenesis of normal and tumor-derived ECs [181]. Overall, these results suggest that the variability of cell types and hypoxic conditions should be carefully considered for accurate interpretation of the vascular effects of H_2S in hypoxia in human aortic ECs (Fig. 4), which necessitates further research in atherosclerosis.

5. Conclusions

The incidence of vascular diseases is continuing to grow worldwide. Vascular/valvular calcification, hemorrhage and Hb oxidation play a significant role in atherosclerosis by influencing inflammation, LDL oxidation, ER stress and foam cell formation. These play a pivotal role in atherosclerosis development and death associated with vascular disease. Growing evidence suggests that H_2S has remarkable protective properties in atherosclerosis. Thus, the successful application of H_2S donors or restoring the impaired endogenous H_2S production to reduce vascular diseases in humans in the future could both decrease the burden of atherosclerosis and improve the vascular status of patients for a desirable clinical outcome.

6. Future directions

We lack substantial knowledge regarding the molecular mechanisms of the regulation of various cellular functions *via* H_2S including mineralization of tissues, polarization of macrophages, vascular resident cells and stem cells. Further research is required to determine the levels of H_2S in various sites of the vasculature along the development of disease states and to reveal the etiology of altered production and metabolism of H_2S. H_2S donors represent therapeutic potential to treat or prevent cardiovascular diseases, a body of evidence shows that H_2S-based drugs can be applied a wide range of disorders. A major challenge in these therapeutic applications involves the targeting of H_2S and expanding our knowledge on the toxic versus protective concentrations of H_2S.

Author contributions

T.G., P.N., G.B., and J.B. wrote the manuscript, T.G., P.N., D.G., L.P., J.G.B., G.B., and J.B. reviewed and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The research group is supported by Eötvös Loránd Research Network (11003). This work was supported by Hungarian Government grants, OTKA-K 132828 (J.B.). The project was co-financed by the European Union and the European Social Fund: GINOP-2.3.2-15-2016-00043 (IRONHEARTH) and EFOP-3.6.2-16-2017-00006 (LIVE LONGER). Project no. TKP2020-NKA-04 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the 2020-4.1.1-TKP2020 funding scheme. Project no. TKP2021-EGA-18 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme. The work was carried out at University of Debrecen, Kálmán Laki Doctoral School of Biomedical and Clinical Sciences.

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