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Original contribution

Hydrogen sulfide and endothelial dysfunction: relationship with nitric oxide

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Abstract

The endothelium is a cellular monolayer that lines the inner surface of blood vessels and plays a central role in the maintenance of cardiovascular homeostasis by controlling platelet aggregation, vascular tone, blood fluidity and fibrinolysis, adhesion and transmigration of inflammatory cells, and angiogenesis. Endothelial dysfunctions are associated with various cardiovascular diseases, including atherosclerosis, hypertension, myocardial infarction, and cardiovascular complications of diabetes. Numerous studies have established the anti-inflammatory, anti-apoptotic, and anti-oxidant effects of hydrogen sulfide (H_2S), the latest member to join the gasotransmitter family along with nitric oxide and carbon monoxide, on vascular endothelium. In addition, H_2S may prime endothelial cells (ECs) toward angiogenesis and contribute to wound healing, aside to its well-known ability to relax vascular smooth muscle cells (VSMCs), thereby reducing blood pressure. Finally, H_2S may inhibit VSMC proliferation and platelet aggregation. Consistently, a deficit in H_2S homeostasis is involved in the pathogenesis of atherosclerosis and of hyperglycaemic endothelial injury. Therefore, the application of H_2S-releasing drugs or using gene therapy to increase endogenous H_2S level may help restore endothelial function and antagonize the progression of cardiovascular diseases. The present article reviews recent studies on the role of H_2S in endothelial homeostasis, under both physiological and pathological conditions, and its putative therapeutic applications.

1.0 Endothelial structure and function

The endothelium lines the luminal surface of the entire vascular tree and the cardiac chambers, where it is termed endocardium. As a consequence, the endothelium presents a rather broad extension (300 to 1000 m^2) and weight (1.5 kg), achieving sizes comparable to other vital organs of the human body [1, 2]. The endothelium can, therefore, be regarded as a real "organ spread across the organism" [3] and, as such, not only it is important for the regulation of local tissue functions, but also for maintaining the whole organism homeostasis [4]. Vascular endothelial cells (ECs) possess a polarized architecture: their luminal membrane is directly exposed to hematic constituents and circulating cells, while the basolateral surface is separated from surrounding tissues by a glycoprotein basement membrane – the sub-endothelial basement – which is formed by fibronectin, laminin, and collagen and is secreted by ECs themselves [2]. Heterogeneity is, however, the hallmark of endothelium. ECs differ in morphology, function, and gene expression profile, so that even neighbouring cells from the same organ and blood vessel type exhibit distinct features [4]. For instance, vascular ECs may vary in size, shape, thickness and position of the nucleus. Albeit oriented along direction of blood flow, to attenuate the impact of shear stress forces on the vascular wall, microvascular ECs are rather thin (0.1 μm) and spindle-like, whilst their macrovascular counterparts are thicker (1 μm) and adopt a polygonal shape [5]. Ultrastructural differences have been described in the relative number of Weibel-Palade bodies, endothelial specific organelles that store P-selectin and von Willebrand factor (vWF), and are mostly abundant in pulmonary vasculature as compared to thoracic aorta (where are absent) and myocardial capillaries (where are rare) [6]. Furthermore, caveolae or "small caves", a subset of cholesterol and glycosphingolipid-rich Ω-like shaped invaginations of the plasma membrane, are most numerous in capillaries (up to 10.000 per cell) than in large conduit and resistance vessels [4]. The heterogeneity in caveolae distribution is exacerbated by the notion

that they are far more enriched in lung microvasculature than in microvascular endothelium of brain, retina, and testes [7]. This feature is highly remarkable when considering that caveolae are not only involved in the transport of material across the EC barrier, but serve as a complex signal transduction platform translating extracellular inputs into well defined intracellular responses, owing to their ability to compartmentalize a myriad of signalling molecules [8, 9]. Similarly, the vesciculo-vacuolar organelle (VVO), a recently identified network of interconnected vesicles and vacuoles which provides the main transcellular pathway for macromolecule extravasation, spans venular endothelium from the luminal to the basolateral membrane, whereas it is absent in both arterioles and capillaries [10]. The most striking example of the phenotypic diversity in vascular endothelium is, however, provided by the presence or not of discontinuities, or "*fenestrae*", along the cellular lining of blood vessels [5]. Continuous endothelium is typical of both arteries and veins, where an intact monolayer of ECs is held together by tight junctions and is anchored to the basal membrane. Conversely, the structure of capillary endothelium differs depending on the vascular bed, being tailored to meet the physiological demands of the underlying tissue. A continuous sheet of tight junctions-coupled ECs is characteristic of brain, retina, skin, lung and muscle capillaries, while the endothelium covering the inner surface of exocrine and endocrine gland, intestinal villi, kidney glomeruli and subpopulations of renal tubules presents 70 nm wide pores which are sealed by a 5 to 6 nm non-membranous diaphragm and termed *fenestrae*. This structural organization is essential to sustain elevated trans-endothelial transport and filtration rates [2, 5]. Finally, sinusoidal vascular districts, such as liver, spleen and bone marrow, exhibit larger fenestration (100-200 nm) among ECs which are devoid of diaphragm and associated to a poorly formed membrane basement [2, 5]. The structural heterogeneity of vascular endothelium may be dictated either by the extracellular microenvironment, neighbouring ECs being exposed to subtly different biochemical cues and biomechanical forces, or by epigenetic modifications,

which are independent of the surrounding milieu [4]. The interaction between external factors and individual gene expression profile becomes evident when ECs from distinct organs are harvested from their native tissue and cultured in growing medium: under such "artificial" conditions, they undergo a phenotypic drift that dampen all their differences and is driven by their intrinsic genetic programme [4]. This feature complicates endothelial research, which requires the *in vivo* validation of each result obtained *in vitro*.

Despite of the molecular underpinnings, the remarkable versatility in endothelial phenotype is reflected by the vast repertoire of signalling functions that it accomplishes all along the vasculature [4]. Broadly speaking, ECs exploit their strategic location at the interface between circulating blood and adjoining tissues to perceive the myriad of inputs incoming from both sources and, thereby, produce the most proper output to meet the local needs [4, 5]. This, in turn, exerts a profound effect on the homeostasis of the whole organism. A quiescent, *i.e.* not activated, endothelium serves as anti-coagulant and non-thrombogenic surface, by preserving blood fluidity and preventing platelet activation and clotting. Moreover, it holds the balance between vasodilators and vasoconstrictors, thereby regulating the vasomotor tone, and determines both para- and trans-cellular permeability by changes in the diameter of inter-endothelial clefts and in the luminal expression of cell-adhesion receptors [5]. Vascular EC restores blood perfusion in hypoxic tissues and organs through the process of sprouting angiogenesis, which is entirely sustained by local ECs and does not require the mobilization of circulating endothelial progenitor cells [11, 12]. The endothelium modulates such different functions due to its ability to synthesize and release a number of agents that regulate vascular smooth muscle cell (VSMC) contractility, trigger inflammatory processes, and affect haemostasis [5, 13]. Among the vasodilators produced by ECs are nitric oxide (NO), prostacyclin (PGI_2), carbon monoxide (CO), hydrogen sulfide (H_2S), epoxyeicosatrienoic acids (EETs), and

adenosine [13-15], while vasoconstrictors include endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2, prostaglandin H2 (PGH_2), and reactive oxygen species (ROS) [14, 16]. Furthermore, ECs communicate with VSMCs *via* myoendothelial gap junctions or humoral factors to induce endothelium-dependent hyperpolarization (the so-called endothelium-dependent hyperpolarizing factor, EDHF) caused by the opening of SK_{Ca} and IK_{Ca} channels (small and intermediate conductance Ca^{2+}-activated K^+ channels, respectively) [17], thereby attenuating the vascular tone [18] . The inflammatory response is modulated by H_2S and NO liberation or by expressing a number of proteins involved in leukocyte adhesion, rolling, and extravasation, including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, P-selectin, and junction adhesion molecule-1 [5]. Endothelial-derived H_2S and NO serve an additional role within the vascular wall by inhibiting VSMC proliferation and migration [13], thereby preventing the pathological remodeling of blood vessel structure (see below). ECs regulate coagulation and fibrinolysis by secreting a variety of mediators, such as plasminogen activator, tissue factor inhibitor, von Willebrand factor, NO, thromboxane A2, plasminogen-activator inhibitor-1, and fibrinogen [19]. Additionally, vascular ECs might behave as thermo-sensors and determine peripheral vasoconstriction or vasodilation according to the environmental temperature [20]. A more careful inspection of EC signaling, however, discloses that not all vascular ECs fulfill the same functions, and that some branches of the vascular bed are more prone to carry out a specific task than others. Alternatively, the same process may be regulated through diverse mechanisms by the endothelia of two different vascular districts [4, 5]. For instance, H_2S relaxes small mesenteric arteries much more potently than aortic tissues [21]. NO is the main vasodilator released by ECs in arteries, such as aorta, coronary and brain arteries while EDHF is far more important in smaller arteries and resistance vessels [22]. The identity of EDHFs may vary depending on the vessel type, EETs serve as

EDHF in peripheral muscular and subcutaneous arterioles and H_2O_2 in mesenteric arteries [23]. Another case in point for the functional heterogeneity displayed by vascular endothelium is the leukocyte migration across the vascular wall. The trafficking of white blood cells takes place primarily in post-capillary venules, which also provide the main sites for inducible permeability during acute and chronic inflammation [5]. Angiogenesis is a typical feature of the capillary network all along the vasculature, whilst arteries and veins are unable of sprouting novel vessels towards adjacent hypoxic tissues [24]. However, the wall of arterial conduits contains a complete hierarchy of proliferating cells, ranging from highly replicating clones to non-dividing cells, which are absent in narrower vessels [25]. These cells are those most likely recruited upon a traumatic injury to restore endothelial integrity when the extent of the lesion is limited to a small EC strip [8]. Therefore, endothelium has been described as an input-output device, which utilizes an intricate and non-linear arrays of signal transduction pathways to continuously translate the host of extracellular stimuli detected by its membrane receptors into a specific cellular behaviour (*e.g.* NO synthase, EDHF production, cytoskeletal rearrangement) or phenotype (*e.g.* proliferation or migration). The delicate balance between vasodilation and vasoconstriction, thrombogenesis and fibrinolysis, prevention and stimulation of platelet activation, inhibition and promotion of VSMC proliferation and migration, must be finely tuned to maintain vascular homeostasis at both local and systemic level. As a consequence of the architectural organization of peripheral circulation, ECs indeed establish an intimate relationship (5 μm or less, which is the average distance between ECs and the closest tissue or cells- when peripheral circulation restricts to the narrower and more densely packed capillary tubes) with cells that have direct contact with ECs of the whole organism. Any significant loss in endothelial integrity leads to a loss of function, whereas the hyper-activation of EC signalling results in abnormal function, both being detrimental for the whole body homeostasis and collectively termed as “endothelial

dysfunction". Due to the structural and functional diversification in vascular endothelium described above, these alterations in the innermost lining of vascular wall will produce highly site-specific pathological conditions.

2.0 Endothelial dysfunction

One form of endothelial dysfunctions is EC detachment from the vascular wall, as observed upon balloon catheter angioplasty, intracoronary stent deployment and coronary artery bypass surgery [8, 12]. The de-endothelialisation of arterial vessels exposes the thrombogenic sub-endothelial matrix, thereby inducing platelet activation and aggregation at the site of lesion [1]. Moreover, the following drop in NO and H_2S bioavailability stimulates VSMCs to switch from a contractile quiescent to a proliferative (synthetic) motile phenotype. As a consequence, VSMC migrate from the *media* towards the inner surface of the vessel, where they continue to proliferate and secrete extracellular matrix proteins, leading to neointimal tissue formation [26]. The consequent thickening of the vascular wall, which has been termed in-stent restenosis (ISR), perturbs blood supply to vital and peripheral organs and dramatically affects cardiovascular homeostasis [26]. Another form of endothelial dysfunctions is caused by hyper-activation of EC by a variety of risk factors, including hypercholesterolemia, aging, hypertension, turbulent blood flow, smoking, type I and type II diabetes, glycaemia, and hyperhomocysteinemia, etc [1]. The endothelial sheet reacts to such abnormal environmental conditions by enhancing the production of ROS, among which are $ONOO^-$ and ^-OH, with the consequent increase in the oxidative stress within the vascular wall. The accompanying elevation in lipid permeability causes the accumulation of low density lipoprotein (LDL) in the sub-endothelial space, where they are thus oxidized to oxLDL [27]. At the same time, vascular ECs are induced to express increased

amounts of adhesion molecules (P-selectin and E-selectin) and integrins (ICAM-1 and VCAM-1), which encourage leukocyte adhesion to the vessel wall and their subsequent translocaiton into the intimal layer. This process is favoured by the chemotactic activity of oxLDL and by the reduced bioavailability of NO [1], or H_2S [28]. Once recruited into the nascent atheromatous lesion, monocytes-derived macrophages ingest oxLDL to become foam cells and to form fatty streaks within the artery wall [27]. Foam cells, in turn, release a number of inflammatory mediators and cytokines which induce the phenotypic switch in the neighbouring VSMCs as part of the pathogenic process of plaque formation [27]. This structural remodelling of the vascular wall significantly narrows arterial lumen, thereby causing a stenosis which may lead to local ischemia if the limitation to blood supply is too severe. The vasospasms determined by the shift towards constricting mediators released by dysfunctional ECs exacerbate tissue injury and eventually aggravates the acute consequences of the atherosclerotic process [29]. The pathologic significance of the improper activation of EC signalling is further underscored by the growing number of disorders consequent to excessive blood vessel growth, such as cancer, infantile haemangioma, age-related macular degeneration, pulmonary hypertension, rheumatoid arthritis, peptic ulcer and psoriasis [30, 31]. Among the others, tumoral angiogenesis is drawing particular attention owing to the failure of the most commonly employed targeted therapies to improve overall survival (OS) or progression-free survival (PFS) beyond a few months and to prevent subsequent relapse of the malignancy [12, 32]. Microscopic lesions remain quiescent until they reach to a clinically detectable size (1-2 mm^3), afterword they spread to distant organs and, eventually, kill the host in the absence of a functional vascular network supplying oxygen and nutrients, removing the catabolic waste, and providing the access to peripheral circulation. The hypoxic environment typical of a growing tumour is sensed by neoplastic cells, where the drop in O_2 tension activates the hypoxia-inducible factor-1α (HIF1α), a transcription factor that controls

the expression of vascular endothelial growth factor (VEGF). VEGF is then released in the surrounding tissues to induce adjoining capillaries to sprout towards the micrometastatic tumor, thus pawing the way for its connection to systemic vasculature and progression to a macrometastatic, invasive phenotype [33]. In addition, VEGF activates bone marrow-residing endothelial progenitor cells (EPCs) to emerge from the vascular niche, traffic to the tumour site, and physically engraft within vessel lumen [12, 34]. The most common targeted therapies aim at interfering with the signal transduction pathways downstream VEGFR-2, the receptor isoform whereby VEGF exerts its pro-angiogenic action on both mature ECs and endothelial committed progenitors [32, 35].

These lines of evidence hint at vascular endothelium as the main actor in many human pathologies, portraying either as a victim of iatrogen interventions or as the primary inducer of human disease [36]. Targeting at a dysfunctional endothelium might suppress/activate the up/down-regulated EC-dependent process in the diseased vessel, but bear the risk to perturb the otherwise normal vasculature in nearby or more remote normal organs. For instance, promoting therapeutic angiogenesis to rescue blood supply to ischemic limbs in peripheral arterial disease (PAD) and to the insufficient heart in coronary artery disease (CAD) might lead to the unwanted vascularisation of dormant micrometastasis and subsequent tumour progression. Therefore, any pharmacological approach aiming to restore the proper endothelial signalling should be carefully designed to selectively interfere with the dysfunctional molecular pathway(s) and camouflage among the myriad of endothelium-released mediators in order to avoid any undesired off-target effects "in" and avoid any undesired off-target effects. It is, therefore, not surprising that gasotransmitters stand out among the most promising tools in the clinical practice due to their ability to rescue the endothelial phenotype without grossly altering the normal functioning of healthy vessels.

3. Hydrogen sulfide (H_2S) production and signaling in Endothelium

Vascular ECs produce H_2S by cystathionine ɣ-lyase enzyme (CSE, E.C 4.4.1.1) [15, 37], which is the major H_2S synthesizing enzyme found in endothelium [38]. CSE is distributed within the cytosol and requires pyridoxal-5′-phosphate (vitamin B_6) as co-factor [38]. Two major substrates utilized by CSE to produce H_2S are cysteine and homocysteine [38, 39]. CSE expression is regulated by the transcription factor specificity protein-1 (Sp1) [40-42]. In the vessels, Sp1 regulates CSE expression during VSMC phenotypic switch from a differentiated state (contractile phenotype) to a dedifferentiated one (synthetic phenotype) [42]. MicroRNA-21 (miR-21) repressed CSE mRNA translation and protein expression in human aortic VSMCs by directly targeting at SP1 3'-untranslated regions [41]. MiR-21 overexpression inhibited CSE and SP1 expressions and H_2S production, stimulated VSMC proliferation, and reduced VSMC differentiation genes expression [41]. Similarly, knockdown of Sp1 and Elk1 blunted CSE expression in pancreatic beta cells [40]. The contribution of Sp1 to CSE expression in vascular ECs remains, however, to be elucidated. One study has reported that CSE protein can be *S*-sulfhydrated when liver lysates treated with 100 μM Sodium Hydrosulfide (NaHS) were subjected to LC-MS/MS [43]. A rrecent study also showed that exogenous H_2S treatment can modulate CSE expression in a dose-dependent fashion: downregulation with 10-80 μM H_2S, upregulation with 120 μM, then full inhibition with higher than 160 μM [44].

H_2S stimulates the phosphorylation of many signaling proteins in ECs isolated from different vascular beds and animal species. For example, treatment with NaHS (10 - 200 μM) stimulated Akt phosphorylation in retinal EC line RF/6A [45]. Treatment with NaHS 60 μM promotes Akt, MAPK kinases ERK, p38 and hsp27 phosphorylation in human umbilical vein

ECs (HUVECs) [46]. NaHS (50 - 100 μM) stimulated eNOS phosphorylation in primarily isolated mouse ECs and EAy-926 cells [47]. Treatment of aortic rings with NaHS enhanced phosphorylation on Ser-239 of the vasodilator-stimulated phosphoprotein (VASP) [48]. The molecular mechanisms for H_2S-induced phosphorylation remain unclear and require further investigation.

S-sulfhydration is proposed to be the main mechanism for H_2S bio-signaling as shown in Figure. 1 [43]. *S*-sulfhydration is now an established post-translational modification mechanism in which H_2S modifies cysteine residue of protein by converting sulfhydryl group (-SH) of cysteine to -SSH group [43]. H_2S sulfhydrates many proteins in different tissues or cells to maintain their physiological functions. For example, in liver alone about 10 to 25% of expressed proteins are sulfhydrated under physiological conditions [43]. Among prominent sulfhydrated proteins in liver were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin, and actin [43]. *S*-sulfhydration of these proteins elicits a seven-fold increase in GAPDH activity, enhances actin polymerization and reveals rearrangement of the cytoskeleton [43]. Furthermore, several recent studies have demonstrated the physiological role of sulfhydration modification in multiple cell types. For instance, H_2S-induced *S*-sulfhydration of ATP-sensitive potassium (K_{ATP}) channels hyperpolarizes VSMCs and induces relaxation of the vascular tissues [49]. *S*-sulfhydration of intermediate (IK_{Ca}) and small conductance (SK_{Ca}) calcium-activated potassium channels hyperpolarizes vascular ECs and induces endothelium-dependent VSMC hyperpolarization [50]. *S*-sulfhydration of nuclear factor κB (NF-κB) physiologically determines its anti-apoptotic transcriptional activity [51]. *S*-sulfhydration of protein tyrosine phosphatases (PTP1B) alters endoplasmic reticulum stress response in HEK-293T cells [52]. H_2S-induced *S*-sulfhydration of Kelch-like ECH-associated protein 1 (Keap1) attenuates oxidative stress and delays cellular senescence in mouse embryonic fibroblasts cells [53]. H_2S induces *S*-

sulfhydration of parkin and enhances E3 ligase activity [54]. Collectively, these studies show that H_2S sulfhydrates a large number of proteins in different tissues, and most of them are sulfhydrated under basal levels.

4. Nitric oxide (NO) production and signaling in ECs

NO production in ECs is catalyzed by endothelial nitric oxide synthase (eNOS, EC1.14.13.39) [55]. The eNOS enzyme utilizes L-arginine as substrate to generate NO from the terminal guanidine nitrogen, thereby producing L-citrulline [55]. The eNOS isoform was first purified from bovine aortic ECs (BAECs) [56], primarily localized in particulate subcellular fraction, and found enzymatically active when associated with the plasma membrane [57]. In vascular wall, the expression of eNOS is predominantly restricted to endothelium, whilst it can be detected in cardiomyocytes, hepatocytes, thrombocytes, VSMCs and lung epithelial cells [58]. In the endothelium, eNOS is constitutively expressed and display common features with housekeeping or constitutively expressed gene promoters, such as the truancy of a TATA box and many SP-1 sites [59]. The location of SP-1 binding sites near the starting site for the transcription or other putative binding domains suggests that it may be regulated by a variety of transcription factors-mediated signals [59]. In addition, different stimuli have the ability modulate eNOS expression [59-61]. For example, shear stress modulates eNOS expression in cultured ECs and intact arteries [61, 62]. Estrogen modulates both eNOS expression and phosphorylation in ECs [63]. Insulin treatment (10^{-10} to 10^{-7} mmol/L) causes a time-dependent (2-8 hrs) increase in the expression of eNOS mRNA and protein level in BAECs [64]. Glucagon-like peptide 1(9-36) enhanced the activity and expression levels of eNOS in HUVECs [65].

Post-translational modifications, such as phosphorylation and *S*-nitrosylation, are common mechanisms for eNOS regulation as shown in Table 1 [66]. These modifications can be induced by physiological or pathophysiological stimuli, to inhibit or stimulate eNOS enzyme activity [55]. For example, phosphorylation of eNOS at Ser-114 inhibits eNOS activity [67]. Conversely, the phosphorylation of eNOS at Ser-617 and Ser-635 activates eNOS [68]. Phosphorylation eNOS at Ser-1177/Ser-1179 (human/bovine) activate eNOS [69]. eNOS de-phosphorylation of Thr-495 activates eNOS [70]. Phosphorylation of Try-81 and Try-567 appears to modulate eNOS activity [71].

NO can modify many proteins by *S*-nitrosylation, including eNOS itself, thereby inhibiting eNOS activity [72]. De-nitrosylation increases eNOS activity [72]. Consistently, eNOS has been found to be basally *S*-nitrosylated in BAECs, where it is activated by the de-nitrosylation brought about by agonists, such as VEGF and insulin [73]. The eNOS isoform also presents unique posttranslational modification features among the three known NOS isoforms, being acylated by both the fatty acids myristate and palmitate [74-76]. Other posttranslational modifications can modulate eNOS, such as palmitoylation which occurs at cysteine residue, and myristoylation which occurs at glycine. These modifications affect the subcellular distribution of the eNOS enzyme [77]. Palmitoylation is an irreversible protein modification, whereas myristoylation is reversible and might take place in random sequences [78].

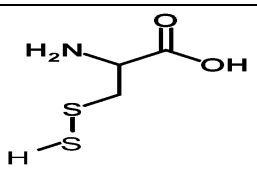
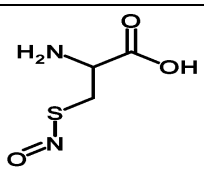
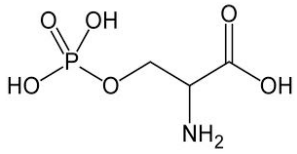
	***S*-sulfhydration**	***S*-nitrosylation**	**Phosphorylation**
Gasotransmitters	H_2S	NO	H_2S and NO
Modification type	Non-catalyzed chemical modification	Non-catalyzed chemical modification	Enzyme driven
Effect on enzyme activity	Increase	Mostly decrease	Increase or decrease depending on the phosphorylated site
Reversible mechanism	Not known	Reversible: *i.e* Thioredioxine, GSNO reductase	Reversible: *i.e* Phosphatases
Energetic mechanism	No ATP needed	No ATP needed	Bio - energetic and need ATP
Bond-dissociation energy	60 kcal/mole	12 - 20 kcal/mole	5 kcal/mole
Detection method	Modified biotin switch assay/ Immunoblot, LC MS/MS	Biotin switch assay + Ascorbate, LC MS/MS	Phsopho-specific antibody/ Immunoblot
Known modified proteins	GAPDH, actin, eNOS	GAPDH, Akt, eNOS	Akt, ERK1/2, p38, eNOS
Bond	Hydropersulfide (-SSH)	S-nitrosothiol (-SNO)	Phosphate ($-PO_4^{3-}$)
Amino acid residue	Cysteine	Cysteine	Serine, theronine, alanine
Modification			

Table 1. Summary of posttranslational modification effects of H_2S and NO in ECs

As mentioned above, NO can regulate many cellular functions through *S*-nitrosylation [79]. *S*-nitrosylation consists of the modification of cysteine thiols (-SH) by NO to form *S*-nitrosothiols (SNOs) [80]. This process influences protein function and protein-protein interaction in different types of tissue [80]. Several endothelial proteins can be *S*-nitrosylated under physiological and pathological conditions. For instance, statins stimulate thioredoxin *S*-nitrosylation, which enhances its enzymatic activity and significantly dampens intracellular ROS levels in HUVECs [52]. SNP (1-2 mM) induces extracellular signal-regulated kinases (ERK) *S*-nitrosylation in MCF-7 breast cancer cells [81]. GSNO treatment (1 mM) stimulated p53 (protein 53) phosphorylation in NIH/3T3 cells [81]. Not all cysteines with free-thiol have the ability to become *S*-nitrosylated [82]. Several factors were proposed to be involved in cysteine reactivity, such as thiol nucleophilicity (pKa), presence of an acid-base motif nearby the cysteine residue, local pH, redox tone and divalent cations, *i.e.* Mg^{2+} or Ca^{2+} [79, 83]. However, the exact molecular mechanism to determine which or how cysteine becomes *S*-nitrosylated is still unclear. Cysteine de-nitrosylation is the reversible mechanism of *S*-nitrosylation accomplished by two enzyme systems, *i.e. S*-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) [84, 85]. GSNOR is involved in SNO removal through metabolism of *S*-nitrosoglutathione [84]. Trx is a 12 kDa protein which has an active site with two redox-active cysteine residues (Cys-Gly-Pro-Cys) [86]. This site binds to SNO (Trx-SNO) through a disulfide bridge and removes SNO groups [86].

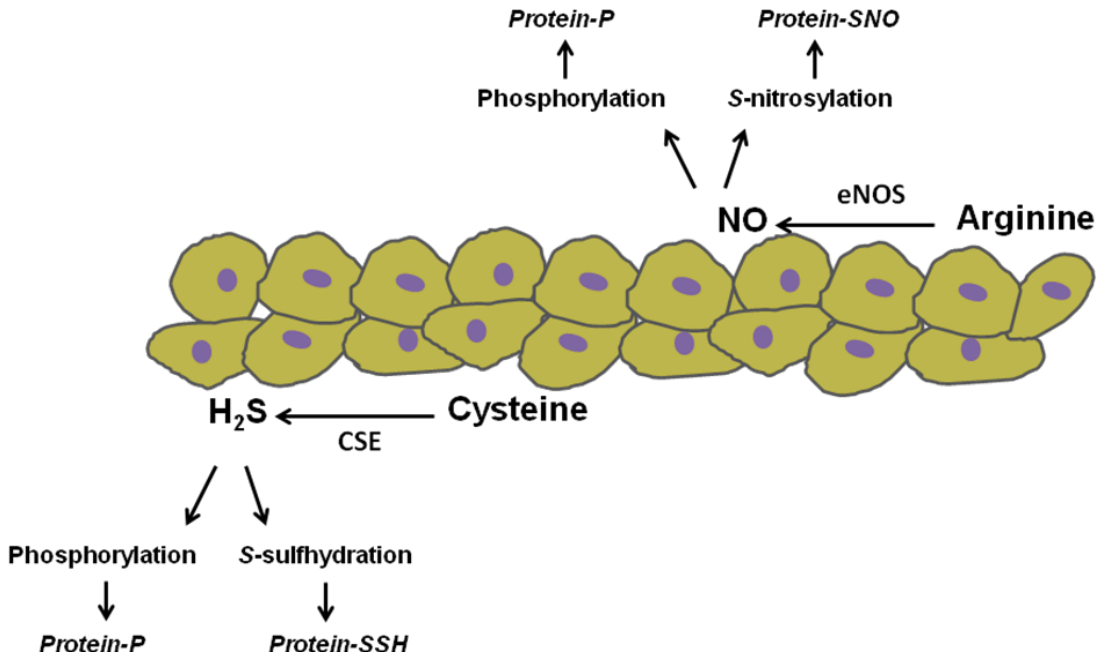


Figure 1. Production and signaling mechanisms of H_2S and NO in ECs

5. CSE and eNOS knockout mice models

A CSE-knockout mouse (CSE-KO) was first generated in 2008 [37]. The deletion of *cse* gene led to significant suppression of endogenous H_2S production [37]. The levels of H_2S in aorta and heart of homozygous CSE-KO mice are reduced by about 80% with a 50% reduction in heterozygous CSE-KO mice [37]. Likewise, H_2S levels in serum of homozygous and heterozygous CSE-KO mice are reduced by 50% and 20%, respectively [37]. CSE is the main H_2S producing enzyme in the vascular system [37]. As a consequence, CSE-KO mice developed hypertension with an onset at 8 weeks of age [37]. Moreover, small resistance arteries displayed a significant reduction in endothelium-dependent vasorelaxation [37]. Aortic rings isolated from CSE-KO mice exhibited markedly reduced neovascularization in response to VEGF treatment when compared to wild-type littermates. Wound healing was also delayed in CSE-KO mice [46].

cGMP levels in the aorta, mesenteric artery and plasma of CSE-KO mice were significantly lower than those from wild-type littermates [48]. Similar to CSE-KO mice, eNOS-knockout mice (eNOS-KO) have been developed as well [87]. These animals lack eNOS mRNA and NOS enzymatic activity, but were fertile and displayed normal anatomy [87, 88]. The phenotypic changes caused by eNOS inactivation include lack of endothelium-derived relaxing factor (EDRF) activity and consequent hypertension [87, 88], an increase in VSMC proliferation [89], platelet aggregation [90], and in leukocyte-endothelial adhesion [91], abnormalities in mitochondrial function and biogenesis [92], insulin resistance [93], and a higher risk to undergo severe strokes [94] and develop atherosclerosis [95]. Up to our knowledge, CSE/eNOS double knockout (DKO) has not been established. Without doubt, developing eNOS/CSE-DKO mice will provide a great animal model to examine the combined effects of H_2S and NO in vascular ECs.

6. Pathophysiological and physiological function of H_2S in ECs

The NO-H_2S balance plays a pivotal role for many pathophysiological and physiological events in the vascular system and contributes to initiation and progression of several forms of EC dysfunction, as shown in Figure 2. Here we briefly reviewed the roles of H_2S and NO in regulation of different EC functions, and their precise role in diseases related to EC dysfunction.

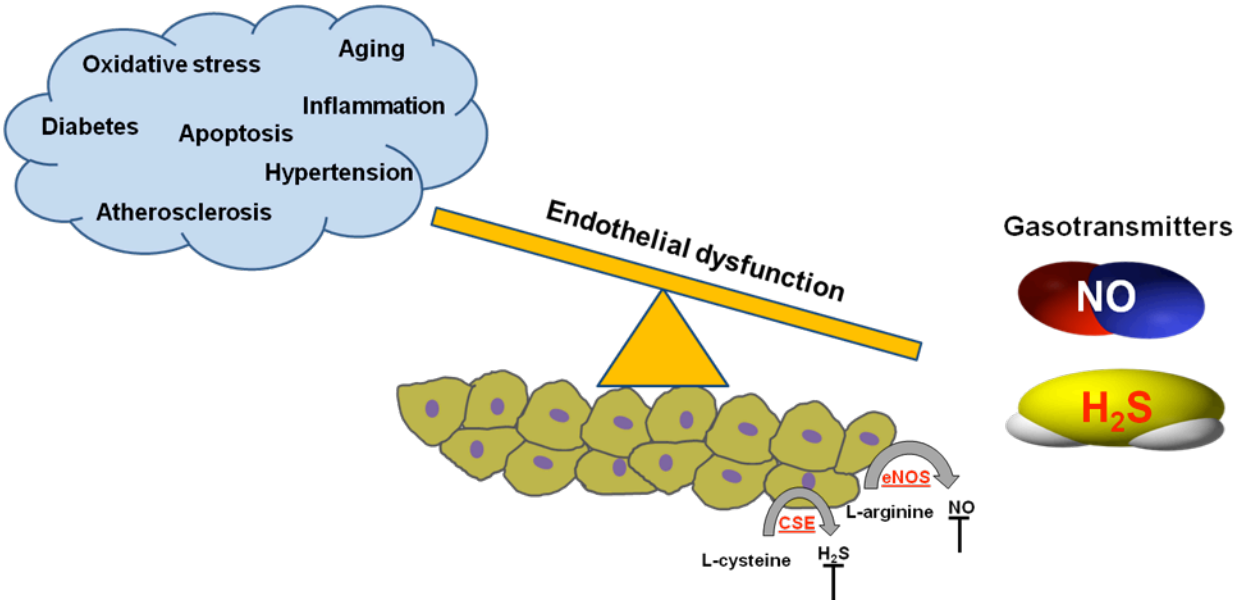


Figure 2. H_2S and NO maintain different EC functions

6. 1. The roles of H_2S and NO in vasodilation

H_2S released from NaHS, dilates blood vessels both *in vitro* and *in vivo*, albeit distinct vascular beds display different sensitivities to the gasotransmitter [15, 38]. The vasorelaxing action of NaHS, administrated at concentrations ranging from 50 μM to 100 μM, has been observed in rat aorta and hepatic artery [96, 97], as well as in resistance mesenteric arteries [21], gastric artery and gastric mucosal circulation [98], cerebral arterioles and artery [99, 100], pulmonary artery [101], and coronary artery [102]. Since NaHS is five-to-nine fold more potent in relaxing mesenteric arteries than thoracic aorta and pulmonary artery (EC_{50} 25 μM *vs.* 125 μM and 233 μM, respectively), it has been proposed as a key regulator of peripheral resistance arteries and, therefore, of blood pressure [15]. H_2S has been shown to dilate other isolated mammalian blood conduits *in vitro*, such as human internal mammary artery [103], mouse aorta and ear microcirculation [104, 105], and newborn pig cerebral arterioles [106]. Consistent with these data, the systemic injection of NaHS at 10-50 μM kg^{-1} causes a transient decrease in mean arterial pressure (MAP) *in vivo* by 12-40 mmHg [96, 107, 108], whereas GYY4137 (26-133 μM Kg^{-1}), a slow H_2S-releasing donor, produces a slowly developed fall in MAP that persists long after drug administration [108]. These observations have been corroborated by the CSE-KO mouse model. CSE-KO mice develop an age-dependent hypertension starting at 8 weeks of age, and peaked at 12 weeks [37]. This significant elevation in blood pressure is associated with the lack of H_2S leading to reduced endothelium-dependent relaxation and elevated resting membrane potential of VSMCs [37], but not those of aorta [17]. These observations are compatible with the notion that H_2S serves as an EDRF [109]. It has been proposed that acetylcholine activates CSE in a Ca^{2+}/CaM-dependent manner in ECs [32, 110]. Once produced, H_2S diffuses to the adjoining VSMCs, where it activates K_{ATP} channels, promoting membrane hyperpolarization and reducing vascular tone by counteracting the activation of voltage-gated Ca^{2+} channels [96, 109].

H_2S may also act as an EDHF. As recently shown in the mesenteric arteries of CSE-KO mice, H_2S causes endothelium-dependent hyperpolarization of VSMCs by stimulating intermediate- and small-conductance K_{Ca} (IK_{Ca} and SK_{Ca}) channels [17, 50]. H_2S has been suggested to mimic NO effect on cGMP level but through different mechanisms. While NO stimulates cGMP production, H_2S decreases cGMP degradation by inhibiting phosphodiesterase-5 (PDE-5) activity in VSMCs, which leads to vasorelaxation in a PKG-dependent manner [48, 111, 112]. In primariy aortic ECs isolated from WT mice, H_2S activates the PI-3K/Akt signaling pathway following increased eNOS phosphorylation in Ser-1177, thereby increasing NO production [47]. Likewise, NO-induced cGMP accumulation and vasorelaxation were attenuated by the genetic knockout of CSE [113], suggesting that both gasotransmitters may act on cGMP to reduce blood pressure.

6. 2. The roles of H_2S and NO in apoptosis

H_2S affects programmed cell death, or apoptosis, in a cell-specific manner. For instance, NaHS has been shown to prevent apoptosis in neurons, cardiomyocytes, colon cancer cells, and 3T3 fibroblasts at concentrations lower than 300 μM [15]. The anti-apoptotic action of H_2S is related to its capability to prevent the mitochondrial membrane potential dissipation by the activation of multiple mechanism, but all through K_{ATP} channels [15]. On the other hand, H_2S stimulates VSMC apoptosis [114]. The increased endogenous production of H_2S derived from CSE overexpression induces apoptosis in human VSMCs by activating ERK1/2 and capsase-3 [114]. Interestingly, VSMCs isolated from CSE-KO mice were more susceptible to apoptosis induced by exogenous H_2S at 100 μM. The pro-apoptotic effects of H_2S are mediated by the phosphorylation of ERK1/2 and expression of cyclin D1 and p21 (Cip/WAF-1) [115]. As for

vascular endothelium, NaHS pretreatment has recently been demonstrated to decrease SA β-gal (cell senescence-associated β-galactosidase) positive rate and cellular apoptosis in HUVECs [116]. Similarly, NaHS triggers an anti-oxidative stress mechanism which protects primary HUVECs from apoptosis challenged with high glucose [117]. The anti-apoptotic action of H_2S in macrophages is mediated by *S*-sulfhydration of NF-kB [51]. H_2S sulfhydrates the p65 subunit of NF-κB at cysteine-38, which promotes its binding to the co-activator ribosomal protein S3 (RPS3) [51]. H_2S inhibits NO production, iNOS gene expression and NF-κB activation in LPS-stimulated macrophages cells *via* a mechanism involving the action of heme oxygenase-1 (HO-1) and CO [118]. Finally, the pro-apoptotic or anti-apoptotic effects of NO depends on the concentration of NO employed, *i.e.* nanomolar range affects Akt phosphorylation and hypoxia inducible factor (HIF)-1α stabilization (pro-survival pathways) and prevent apoptosis, whereas micromolar range triggers phosphorylation of p53 and induces apoptosis [119].

6. 3. The roles of H_2S and NO in oxidative stress

H_2S is a strong reducing agent and may easily interact with oxidative species [15]. Several studies have shown that H_2S displays anti-oxidant activity at 10 - 100 μM [107, 120, 121]. H_2S protects the luminal surface of blood vessel from oxidative stress caused by ischemia/reperfusion injury, as well as from development of atherosclerotic lesions, [28, 122]. H_2S rescues CSE-KO mice from the injury and mortality associated with renal ischemia. Moreover, CSE overexpression reduces the amount of reactive oxygen species (ROS) produced during stress in renal tissues [122]. NaHS has been shown to mitigate the methionine-induced production of free radicals in brain ECs (bEnd3) and to enhance the inhibitory action of reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and L-NAME on ROS

production [123]. Herein, 10 - 500 μM NaHS preserves mitochondrial function by reducing the deleterious effects of oxidative stress on the antioxidants enzymes, SOD, catalase, glutathione peroxidase and glutathione-*S*-transferase [124]. Furthermore, H_2S delays EC senescence by attenuating oxidative stress [116]. A study shows that late passage (e.g. 12) HUVECs have lower SOD activity and higher H_2O_2 level as compared with younger HUVECs (e.g. 4), whereas NaHS pretreatment reverses the changes in SOD activity and H_2O_2 level [116]. Similarly, the expression levels of both xanthine oxidase (XOD) and subunits p67 (phox) of NADPH oxidase are increased in the senescent group relative to the fresh one, whereas manganese-superoxide dismutase (Mn-SOD) expression levels are decreased [125]. NaHS treatment results in the up-regulation of both XOD and p67 (phox) levels but down-regulation of Mn-SOD expression [116], thereby slowing down cell aging. Another study shows that H_2S delays HUVEC senescence and prevents H_2O_2-induced damage *via* sirtuin 1 activation [125]. In addition, H_2S-releasing drugs, NaHS and ACS6 (both at 10 μM), inhibit superoxide formation and gp91 (phox) (a catalytic subunit of NADPH oxidase) expression in porcine pulmonary arterial ECs by increasing cAMP levels and recruiting PKA upon the inhibition of PDE-5 [126]. The cytoprotective effect of H_2S in ECs might involve an increase in the intracellular levels of GSH, one of the most abundant and effective components of the defense system against free radicals [127]. Studies conducted on mouse embryonic fibroblasts isolated from CSE-KO mice (CSE-KO-MEFs) show an increase in oxidative stress and acceleration in cellular senescence compared with MEFs isolated from wild-type mice [127]. Incubation of CSE-KO-MEFs with NaHS significantly increases GSH levels and rescues KO-MEFs from senescence [127]. H_2S *S*-sulfhydrates Keap1, which regulates the antioxidant response, thereby inhibiting Nrf2 activity [127]. *S*-sulfhydration of Keap1 at cysteine-151 induces Nrf2 dissociation from Keap1, thus enhancing the nuclear translocation of Nrf2 and increasing mRNA levels of Nrf2-targeted

downstream genes, such as glutamate-cysteine ligase and glutathione reductase [127]. In addition, H_2S scavenge $ONOO^-$ and suppress tyrosine nitration in VSMCs in the presence of homocysteine (Hcy) [128]. The anti-oxidant properties of H_2S could therapeutically be exploited to prevent lung endothelium damage caused to by oxygen therapy [129], particulate air pollution or tobacco smoke [130]. Similarly, regulation of NO production during conditions of oxidative stress is very important for cell survival. Previous studies have shown that increased oxidative stress is often associated with decreased NO levels [131]. Additionally, as mention before, NO generation together with increased superoxide generation, leads to elevated peroxynitrite formation, which is a powerful oxidant, and related to oxidative injury of blood vessels [131].

6. 4. The roles of H_2S and NO in inflammation

The vascular protective action of H_2S involves its anti-inflammatory effects on the innermost lining of blood vessels. H_2S, released from either NaHS (10-100 μM) or S-propargyl-cysteine (SPRC; 1-10 μM), a novel sulphur-containing amino acid, inhibits the expression of adhesion molecules, including ICAM-1, VCAM-1, P-selectin, and E-selectin, induced by pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), in HUVECs [132, 133]. H_2S prevents the complication of non-steroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid (ASA) therapy [134]. H_2S attenuates gastric mucosal injury, TNF-α, ICAM-1 in rat mesenteric venules challenged with ASA or NSAIDs [134]. Conversely, pharmacological blockade of CSE with β-cyanoalanine (BCA) enhanced leukocyte adhesion and rolling [135]. In agreement with these data, carrageenan-induced paw oedema is suppressed by either NaHS (EC_{50} = 35 μM Kg^{-1}) or Na_2S (EC_{50} = 28 μM Kg^{-1}) and boosted by BCA [134]. The anti-inflammatory action of H_2S involves the activation of K_{ATP} and BK_{Ca} channels [134, 136] and

the up-regulation of HO-1 [133], as well as the inhibition of p38 and NF-kB signalling pathways. Furthermore, H_2S hampers TNF-α-induced monocyte-endothelial interactions by down-regulating the expression of the monocyte chemo-attractant protein-1 (MCP-1) through the inhibition of disintegrin and metalloproteinase metallopeptidase domain 17 (ADAM17) [137]. The ADAM17-dependent TNF-converting enzyme (TACE) activity is, in turn, essential for soluble TNF-α shedding and up-regulation of MCP-1 levels in HUVECs [137]. NaHS administration at 100 μM kg^{-1} restores gastric microcirculation in a K_{ATP} channels-dependent manner [134]. NO also plays a key role in the pathogenesis of inflammation as an anti-inflammatory mediator under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations [138]. Finally, NO inhibits adhesion of inflammatory cells to the endothelial surface as well [139].

6. 5. The roles of H_2S and NO in angiogenesis

H_2S has recently been recognized as an important regulator for angiogenesis both *in vitro* and *in vivo* under physiological conditions [46, 140]. Silencing of CSE gene reduces migration and sprouting of HUVECs in *vitro* [46]. In agreement with these data, the BrdU assay disclosed that the proliferation rate of primarily isolated ECs was dramatically suppressed by knockdown of CSE, but restored by exogenously H_2S treatment [47]. Moreover, *ex vivo* mice aortic ring angiogenesis assay reveals a marked decrease in neovascularization when the aortic tissue from CSE-KO mice was cultured *in vitro* [47]. Conversely, NaHS promotes blood vessel growth of aorta tissues from both WT and CSE-KO mice [47]. Intraperitoneal administration of NaHS for 7 days at 10 - 50 $mmol \cdot kg^{-1} \cdot day^{1}$ increases neovascularization in the mouse *in vivo* [45].

Consistently, treatment with Na_2S increases the vascular length in chicken chorioallantoic membranes (CAMs) at doses of 0.24 - 240 pmol/egg [45]. Blockade of CSE with PPG for 48 hr at increasing concentrations (3, 30, and 300 μmole/egg) decreases vascular network length and branching of CAM [46]. NaHS at a concentration of 100 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ restores blood perfusion in a rat model of hindlimb ischemia by promoting local vessel growth [140]. Therapeutic angiogenesis of occluded peripheral arteries has also been reported in C57BL/6J mice supplemented with Na_2S at 0.5 mg/kg and 1 mg/kg [141]. Likewise, NaHS (30 μM/l in drinking water for 4 weeks) attenuates cardiac remodeling involve in chronic heart failure by promoting *in vivo* angiogenesis in a rat model of myocardial infarction [142]. The signal transduction pathways underlying the pro-angiogenic role of H_2S are not fully unraveled [13, 47]. Activation of PI-3K/Akt by H_2S has been shown to regulate tube-like structure formation in the retinal EC line (RF/6A) by inducing the up-regulation of the adhesion molecules, integrin α2 and β1, and survivin [45]. H_2S increases Akt phosphorylation and improves regional blood flow in a rat model of unilateral hindlimb ischemia [140]. Mitogen-activated protein kinases (MAPK) and ERK1/2 are not activated by H_2S in RF/6A cells [45], but the activation of MAPKs, such as ERK1/2 and p38, by H_2S was shown to play a key role in HUVEC migration [46]. The use of different concentrations of H_2S donors, of multiple EC types, and of diverse assay systems could explain the heterogeneity observed in the molecular underpinnings of the pro-angiogenic effects of H_2S. Importantly, the K_{ATP} channel blocker glibenclamide abolished H_2S-induced p38 phosphorylation and HUVEC motility [46]. Therefore, it can be argued that K_{ATP} channels are located upstream of MAPKs in the signaling pathway contributing to H_2S-induced angiogenesis.

Another putative mechanistic link between H_2S and the molecular decoders of its pro-angiogenic effect is the increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [13, 143]. In fact, many studies have provided the evidence that NaHS affects intracellular Ca^{2+} signals in a variety of

ECs, such as human saphenous vein ECs [144], human microvascular dermal ECs (HMVECs) [145], and rat aortic ECs (RAECs) [146]. The Ca^{2+} response to NaHS in human ECs is shaped by the interaction between intracellular Ca^{2+} release from the endoplasmic reticulum reservoir, which is mainly mediated by inositol-1,4,5-trisphosphate ($InsP_3$) receptors, and store-operated calcium entry (SOCE) across the plasma membrane [144]. Conversely, Ca^{2+} inflow patterned H_2S-evoked elevations in $[Ca^{2+}]_i$ in both HMVECs and RAECs [13, 145]. Ca^{2+} entry in rat aortic endothelium is supported by the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) and sustained by K_{ATP} channels-dependent membrane hyperpolarization [146].

Similar to vasorelaxation, NO may be involved in the pro-angiogenic action of H_2S [112] Accordingly, pharmacological blockage of protein kinase G (PKG) prevented H_2S-induced bEnd3 proliferation and migration *in vitro*, whereas NaHS-elicited neo-vascularization of Matrigel plugs *in vivo* was absent in $eNOS^-$KO mice [113]. In addition, CSE deficiency abolished the pro-angiogenic role of NO donors both *in vitro* and *in vivo* [47]. Treatment with either L-NAME or siRNA to knockdown eNOS attenuates the H_2S-angiogenic effect [47]. Furthermore, L-arginine (the substrate of NOS) induces neovessel growth in cultured ring isolated from wild type mice but not from CSE-KO mice [47]. Therefore, both H_2S and NO are required for optimal angiogenic activity, yet angiogenesis still proceeds in the presence of either H_2S or NO alone albeit to a reduced degree.

Vascular endothelial growth factor (VEGF) is the most powerful stimulator of angiogenesis. The down-regulation of CSE gene led to a decrease in VEGF-induced MAPK activation and HUVEC motility [46]. The *ex vivo* aortic ring angiogenesis assay further showed that VEGF-elicited elevation in microvessel formation is dramatically hampered in CSE-KO mice [46]. Similarly, the genetic suppression of CSE attenuates VEGF-induced proliferation in bEnd3 micro-vascular ECs, whereas it does not impair the mitotic effect of basic fibroblast

growth factor (bFGF) [113]. The influx of Ca^{2+} triggered by VEGF into ECs might serve as a suitable signal to initiate CSE activation and H_2S synthesis [11, 110, 145, 147]. H_2S also stimulates angiogenesis of ischemic tissues. NaHS activated the hypoxia inducible factor 1-α (HIF-1α), the transcription factor driving the expression of numerous growth factors [32], in capillary ECs harvested from mouse skeletal muscle [141]. This process was mediated by H_2S-induced NO and VEGF release and subsequent increase in EC proliferation under hypoxia [141]. These data have been supported by the increase in VEGF expression reported in both ischemic heart and peripheral limbs and related to the pro-angiogenic effect exerted by NaHS treatment in mice [140]. However, NaHS represses hypoxia-induced HIF-1α protein translation in ECs (EA.hy926) [148]. H_2S enhances eukaryotic initiation factor 2-α (eIF2-α) phosphorylation, and the consequent fall in HIF-1α accumulation resulted in the down-regulation of VEGF expression, thus EC proliferation [148]. This discrepancy might be due to the differences in ECs isolated from distinct vascular beds and animal species.

In addition to therapeutic angiogenesis, H_2S might be involved in controlling the neo-vascularisation of growing tumours. NaHS induces Ca^{2+} influx in higher levels in tumour ECs (TECs) harvested from breast cancer (B-TECs) as compared to their healthy counterparts [145]. Importantly, NaHS causes a dose-dependent stimulation of B-TEC proliferation but had little effect on the control healthy cells [145]. VEGF-induced Ca^{2+} entry was prevented by CSE blockade by PPG, albeit the physiological meaning of this process was not further investigated [145]. In consideration of endothelial phenotypic changes observed in ECs isolated from tumour samples, the involvement of H_2S in supporting tumour vascularisation should, therefore, be assessed by focussing on TECs rather on their normal counterparts [145].

6. 6. The roles of H_2S and NO in atherosclerosis

EC dysfunction, hypertension, VSMC proliferation and migration, dyslipidemia, oxidative stress, and recruitment of inflammatory cells are the important factors involved in the development of atherosclerotic lesions [28, 149]. NO and H_2S share several athero-protective actions, including blood vessel relaxation and regulation of vascular tone, EC regeneration, inhibition of leukocyte adhesion, inhibition of platelet clumping to make the blood thinner, and prevention of proliferation and migration of VSMCs [150-153]. The role of NO in atherosclerosis development has been studied in apolipoprotein E knockout (apoE-KO) mice, where L-NAME treatment significantly decreased NO-mediated vascular response and increased atherosclerosis development [154]. NOS gene overexpression reduced adhesion molecules expression, reduced the inflammatory process, and inhibited VSMC proliferation and migration [155, 156]. Another study proved that decreased NO bioavailability in high cholesterol-induced vascular dysfunction of rabbit aortic rings was corrected by eNOS gene transfer, which improved vascular relaxation in response to acetylcholine [157]. Kuhlencordt *et al.* [153] studied whether eNOS deficiency affects atherosclerosis development using apoE/eNOS DKO mice and found that both male and female apoE/eNOS DKO mice showed significantly increased lesion when compared to apoE KO mice [153].

The anti-atherosclerotic role of H_2S was explored in recent years. CSE expression and H_2S production were significantly decreased during the development of balloon injury-induced neointimal hyperplasia in the rat carotid artery, and that exogenous H_2S significantly reduced neointimal lesion formation [158]. Treatment of apoE-KO mice with NaHS decreased, and with PPG increased, atherosclerotic lesion size [159]. NaHS (100 μmol/L for 12 hrs) inhibited ICAM-1 expression in TNF-alpha-induced HUVECs *via* the NF-kB pathway [159]. Moreover, NaHS administration concentration-dependently (50-200 μM) reduced CX3CR1 and CX3CL1

expression in mouse peritoneal macrophages as well as CX3CR1-mediated chemotaxis [160]. In fat-fed apoE-KO mice, NaHS (1 mg/kg, i.p., daily) attenuated, and PPG (10 mg/kg, i.p., daily) exacerbated, the extent of atherosclerotic plaques [160]. H_2S (50 μM) attenuated H_2O_2 and oxidized LDL (oxLDL)-mediated endothelial cytotoxicity in HUVECs [161]. GYY4137 (a slow H_2S releasing molecule) decreased the atherosclerotic plaque formation and partially restored the endothelium-dependent relaxation of apoE-KO mouse aorta [162]. The direct evidence for the role of endogenous H_2S in atherosclerosis development was reported in 2013, demonstrating that CSE-KO mice fed with atherogenic paigen-type diet, but not WT mice, developed early fatty streak lesions in the aortic root, increased aortic intimal proliferation and aortic adhesion molecule expression, and enhanced oxidative stress [28]. These animals also showed increased total plasma cholesterol and LDL-cholesterol levels when compared to WT mice fed with the same atherogenic diet. NaHS supplementation to atherogenic diet-fed CSE-KO mice improved plasma lipid profile and decreased atherosclerotic lesions [28]. Collectively, these studies suggest that NO and H_2S play similar roles in the prevention of atherosclerosis. The crosstalk between NO and H_2S in the development of atherosclerosis, however, has not been explored in details.

6. 7. The roles of H_2S and NO in aging

Aging is related to dramatic structural and functional alterations in both cardiac chambers and blood vessels, which can explain the age-related increase in cardiovascular risk [163, 164]. During aging, ECs undergo senescence, which leads to a reduced angiogenic activity [165, 166]. Cellular senescence, an irreversible arrest of cell cycle [167], can be triggered by the reduced bioavailability of both H_2S and NO. For instance, senescent human aortic ECs (HAECs) exhibited higher ICAM-1 expression and lower eNOS activity [168]. Accordingly, the eNOS

protein isolated from mesenteric arteries in young mice has the dimer configuration, whereas eNOS protein from aged mice was mostly uncoupled to monomers [169]. The same study revealed that eNOS uncoupling contributed to increased superoxide levels in aged vessels and was due to a reduced BH_4 availability [169]. The aging-related EC dysfunction is largely ascribed to oxidative stress and inflammation [170, 171]. An excess of ROS, superoxide, and hydrogen peroxide compromises the vasodilator activity of NO and facilitate the formation of the deleterious radicals [170]. Basal NO production and sensitivity to acetylcholine-mediated vasodilation in thoracic aorta rings were reduced, whereas the pro-inflammatory reaction of ECs was increased in aged mice compared to young mice [172]. In addition, arginase-II (Arg-II) expression/activity was higher in senescent ECs, and Arg-II silencing suppressed eNOS-uncoupling and several senescence markers, such as senescence-associated-β-galactosidase activity, p53-S15, p21, and expression of VCAM1 and ICAM1 [173]. Over-expression of Arg-II in non-senescent ECs promoted eNOS-uncoupling and enhanced VCAM1/ICAM1 levels [173]. Protein nitrotyrosine formation is accompanied with eNOS uncoupling in mesenteric arteries of aged mice [169]. Chronic increase in shear stress in mesenteric arteries of aged rats restored EC function through increasing NO production and antioxidant capacity, and thus decreasing superoxide levels [174]. In soil nematode *Caenorhabditis elegans*, which is prominent model organism for studying aging [175], exposure to H_2S increase life span and thermo-tolerance compared to control animals [176]. This finding is in accordance with data showing that NaHS treatment protects against HUVEC senescence by modulating SIRT1 activity, as mentioned above, and against fibroblast aging *via S*-sulfhydration of Keap1 and Nrf2 activation in association with oxidative stress [127]. A recent study revealed that CSE protein expression was increased in aorta of aging rats maintained on an ad libitum (AL) diet. However, CSE expression was unchanged in rats maintained on a caloric restriction (CR) diet [177]. Furthermore, CR-fed

animals at ages of 18, 29, and 38 months had lower CSE expression than in AL-fed animals, whereas CSE expression at 8 months was not affected by diets [177], suggesting that CR diet may help to stabilize the H_2S signaling during aging.

6. 8. The roles of H_2S and NO in diabetes

H_2S treatment or CSE over-expression protected ECs from the deleterious consequences of hyperglycaemia-induced enhancement of ROS formation and attenuated nuclear DNA injury [178]. H_2S protected against the development of EC dysfunction in aortic tissues incubated in medium with elevated glucose concentration (*in vitro* "hyperglycemia") and reduced the bio-energetic derangements in ECs [178]. On the other hand, CSE knockdown deteriorated hyperglycemia-induced ROS production and led to more severe loss of endothelium-dependent relaxant function [178]. Oxidative stress plays a major role in the development of diabetic microvascular complications [179]. Non-obese diabetic (NOD) mice, which gradually develop type-1 diabetes, exhibited reduced H_2S levels in plasma and aortic tissue [180]. The administration of exogenous H_2S relieved vascular abnormalities by upregulating connexin 40 and connexin 43 and normalizing NADPH oxidase and PKCε in STZ-injected rats [181]. These results have been corroborated by the finding that the circulating levels of H_2S were lower in STZ-diabetic rats [178]. The same study shows that, under hyperglycaemic condition bEnd3, microvascular ECs accelerated H_2S consumption due to the mitochondrial formation of ROS, which severely affected cell viability and caused nuclear DNA damage and switched cell metabolism from oxidative phosphorylation to glycolysis [178]. As expected, these features were associated to the impairment of endothelium-dependent relaxations of rat aortic rings exposed to high glucose *in vitro*. EC functionality was restored either by over-expressing CSE or by supplying NaHS (100 - 300 μM) [178].

The observation that ECs failed to produce sufficient amount of NO in diabetes has been documented in animal model of the disease [182]. Similarly, loss of insulin signalling in the vascular endothelium led to EC dysfunction due to the decrease in NO synthesis, which impaired endothelium-dependent vasodilation and accelerated the progression of atherosclerotic lesions in apoE KO mice [183]. Likewise, hyperglycemia inhibited eNOS activity in cultured BAECs and in the aorta of diabetic rats [184]. All together, these data illustrate the importance of H_2S and NO in protecting ECs from the deleterious vascular consequences of diabetes.

7. Conclusions and perspectives

The endothelial monolayer lines the luminal surface of all blood vasculature, thereby representing the largest input/output device of the whole organism [4]. ECs perceive both chemical and physical stimuli incoming from the extracellular microenvironment, translate them into the appropriate signaling cascade and react by proper changes in protein activity and/or gene expression profile. H_2S, the latest member to join the family of gasotransmitters, has been proven to utilize the endothelial signaling toolkit in the most favorable way for vascular functions without the prerequisite of specific plasma membrane receptors. H_2S provides vascular ECs with important pro-survival signal: it stimulates angiogenesis, protects from apoptosis and senescence, and exerts both anti-inflammatory and anti-atherosclerotic actions. These pro-surviving effects gain much more relevance when considering that low concentrations of H_2S do not lead to the formation of noxious reactive nitrogen species, opposite to what NO does. It is, therefore, perceivable to predict that future therapies of cardiovascular pathologies will benefit from H_2S-based intervention in order to correct endothelial dysfunction involved in the diseases. A growing body of evidence indicates that the administration of H_2S donors might be therapeutically useful in the treatment of chronic heart failure, peripheral artery disease,

hypertension, and atherosclerosis. In addition, CSE might be a potent molecular target to combat tumour growth and vascularization.

The investigation of H_2S signaling in vascular endothelium is, however, in its infancy. The therapeutic potential of this recently discovered gasotransmitter demands for the full comprehension of its downstream decoders before being fully exploited. A number of outstanding questions remain to be solved. The cellular effects of H_2S and the related molecular underpinnings may vary depending on a plethora of factors, such as the types of vascular beds, animal species, and experimental setting. For instance, H_2S inhibits HIF-α expression under hypoxic conditions in HUVECs, whereas it enhances its tissue levels in the ischemic mouse femoral artery. Worth of further studies is also the relationship among the signaling pathways engaged by H_2S in vascular ECs. Taking the calcium signaling as an example, the evidence for a physiological role of Ca^{2+} signaling in H_2S-induced EC proliferation is still missing. Nevertheless, an increase in intracellular Ca^{2+} is sufficient to activate different MAPK members and PI-3K, which are involved in H_2S-driven angiogenesis, EC replication, migration and tubulogenesis [185, 186]. The physiological implications of H_2S-induced sub-cellular Ca^{2+} waves extend to the stimulation of endothelial K_{Ca} channels, including BK_{Ca}, IK_{Ca}, and SK_{Ca} channels, whose gating require a local elevation in Ca^{2+} levels beneath the plasma membrane [187].Therefore, H_2S might provide Ca^{2+} pulse that activates BK_{Ca} channels while inducing the covalent modification that enhance their open probability. It would be interesting to explore the kinetic relationship between H_2S-modulated Ca^{2+} level change and K_{Ca} channel activation in vascular ECs.

The mechanistic relationship between H_2S and NO remains to be untangled. Both NO and H_2S may be generated upon a raise in $[Ca^{2+}]_i$, but whether and how eNOS and CSE are recruited by the same Ca^{2+}/calmodulin signal are not clear. The source of Ca^{2+} leading to eNOS

engagement in large conduit vessels, such as aorta, is provided by store-operated calcium entry (SOCE), a Ca^{2+}-permeable conductance activated on depletion of the intracellular Ca^{2+} reservoir [32]. However, in mesenteric arteries, acetylecholine stimulation results in inositol-1,4,5-trisphosphate ($InsP_3$)-dependent Ca^{2+} release rather than SOCE [188]. Future studies are necessary to understand whether $InsP_3$-evoked Ca^{2+} mobilization and SOCE are selectively associated to CSE and eNOS, respectively. This mechanism would partially explain the differential production of NO and H_2S in conduit and resistance arteries. Moreover, NO may inhibit CSE activity by inducing *S*-nitrosylation of the enzyme [50], which would render larger arteries less sensitive to H_2S. This mechanism, i.*e.* CSE inhibition by NO, would argue against the simultaneous production of both gasotransmitters in the same cells, albeit other studies reported NO-induced H_2S synthesis [189]. Indeed, it is yet to be unraveled how these two gasotransmitters interact when presented at the same time. By the same token, when H_2S and NO donors are simultaneously administrated, their impacts on the vessel tone may be opposite rather than synergistic. This *scenario* is further complicated by the conflicting effects of H_2S on NO levels. At physiological pH, H_2S may react with NO and form nitrosothiols, thereby limiting NO's vasorelaxing (and perhaps pro-angiogenic) activity. Nevertheless, when pH shifts to acidic values, H_2S serves as cofactor of NO-releasers to liberate NO [190], which might be a crucial signalling event during severe vascular disorders and EC dysfunction. It is imperative to understand whether both gasotransmitters serve as parallel signalling pathways in the regulation of crucial EC processes, such as proliferation, control of the vascular tone, resistance to apoptosis, senescence and oxidative stress; or each individual gasotransmitter governs specific EC processes.

Conflicts of interest: None

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