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REVIEW ARTICLE

Nitric oxide and hydrogen sulfide: the gasotransmitter paradigm of the vascular system

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There are several reviews on NO and hydrogen sulfide (H_2S) and their role in vascular diseases in the current relevant literature. The aim of this review is to discuss, within the limits of present knowledge, the interconnection between these two gasotransmitters in vascular function. In particular, the review focuses on the role played by the balance between the NO and H_2S pathways in either physiological or pathological conditions. The distinction between physiology and pathology has been made in order to dissect the molecular basis of this crosstalk, highlighting how and if this balance varies, depending upon the vascular status. Perspectives and possible novel therapeutic approaches are also discussed.

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Abbreviations

3-MST, 3-mercaptopyruvate sulfur transferase; CAV-1, caveolin-1; CBS, cystathionine β synthase; CSE, cystathionine γ lyase; PAG, propargylglycine; sGC, soluble guanylate cyclase; VD, vascular disease

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Introduction

In 1980s the revolutionary concept that a gas, that is, **NO**, produced in our body could act as a mediator surprised the scientific community. It was even more surprising when it was discovered that in the vasculature, NO, a gas, could selectively activate a key enzyme such as the **soluble guanylate cyclase (sGC)** leading to an increase of (c**GMP**) biosynthesis. The first hints of this mechanism were given in 1978 by Murad who demonstrated that nitroglycerin, by activating sGC, increased cGMP levels (Mittal *et al.,* 1978). Then, in 1980, Furchgott hypothesized the existence of an endothelial derived relaxing factor (EDRF) as a mediator of acetylcholine-induced vasorelaxation (Furchgott and Zawadzki, 1980). Finally, Ignarro identified NO as EDRF (Ignarro *et al.,* 1988). All three scientists shared the Nobel Prize in Medicine in 1998.

Nowadays, NO, together with carbon monoxide (CO) and **hydrogen sulfide** (H_2S), constitute a family of endogenous mediators that share the characteristic to be a gas. Among them, H_2S was the last to be identified and it is the only one that has a chemical structure in thre dimesninos, as distict from the planar structure of NO and CO. This discovery of the third gaseous endogenous molecule allowed the scientific community to coin the term 'gasotransmitters' to refer to this growing class of mediators and introducing a novel class of endogenous mediators (Wang, 2002; Szabo, 2007). The gasotransmitter is a small, labile molecule biosynthesized in the body by enzymic reactions with a very short half-life, ranging from seconds to minutes. Being a gas, this transmitter molecule freely permeates the cell membranes without specific transporters, reaching molecular targets relatively far from the site of its biosynthesis. This characteristic unveils a new concept: *The biological effect triggered by the gasotransmitter is not necessarily due to the interaction with a single specific target*. This concept, apparently obvious, has changed radically the classical idea of ligand–receptor interaction in pharmacology. The gasotransmitter, due to its unique chemical nature, once produced, freely travels through the cells, interacting with many different molecular structures.

The gas connection: NO and H_2S in physiology

NO

In the vasculature, NO is generated within the endothelium as a product of the enzymic reaction of **endothelial NOS** (eNOS) with its substrate L-arginine together with molecular oxygen and reduced NADPH as co-substrates. The NO biosynthesis requires the dimerization of two molecules of eNOS and the presence of several co-factors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6R-)5,6,7,8-tetrahydro-L-biopterin (BH4), calmodulin and iron protoporphyrin IX (haem) (see Forstermann and Sessa, 2012; Zhao *et al.,* 2015). Once NO has been generated within the endothelial cells, it diffuses to the vascular smooth muscle cells and binds the haem group of sGC, leading to the conversion of GTP to cGMP. This second messenger, through a **PKG** dependent mechanism, activates a downstream signalling that induces a decrease of the intracellular Ca^{2+} concentration. This decrease prevents myosin phosphorylation by myosin light chain kinase, with consequent vasorelaxation (Word *et al.,* 1994; Mizuno *et al.,* 2008). This pathway is turned off by the catabolic action of **PDE**, enzymes that degrade cGMP to its inactive metabolite, 5′-GMP (Conti and Beavo, 2007).

During its travel through the cells, NO exerts another essential role in modulating cellular signalling, but in a cGMP-independent manner. This alternative signalling role entails the redox reaction between NO and the thiol group of L-cysteine, generating a covalent modification of protein cysteine thiols with a formation of a new chemical entity, called S-nitrosothiols (SNO). However, it has been recently shown that the addition of the NO moiety can be catalysed by a class of enzymes called nitrosylases (Foster *et al.,* 2003; Stamler and Hess, 2010). This reversible, rapid reaction is called S-nitrosylation. The importance of S-nitrosylation is well recognized, and it is now considered as an essential molecular mechanism for modulating cellular function and redox signalling (i.e. enzymic activity, subcellular localization, protein–protein interaction and protein stability). Considering the chemical nature of the S-nitrosylation, it is intuitive that a large number of proteins (many hundreds) may undergo this post-translational modification and this could explain the multiplicity of actions of NO. As with any other mechanism of transduction, S-nitrosylation is a transient phenomenon as other enzymic reactions readily remove the NO group from proteins (denitrosylation), modulating signal transduction (Beigi *et al.,* 2012). So far, two enzymic denitrosylation systems have been identified- the thioredoxin (TRX)/TR system and the GSH/GSNO system. For both of them, an important regulatory function has been demonstrated, under physiological conditions (Benhar *et al.,* 2009; Forrester *et al.,* 2009; Anand and Stamler, 2012). In the blood vessel wall, S-nitrosylation/denitrosylation of connexin plays a primary role in heterocellular communication, regulating the functions of the gap junction between endothelium and smooth muscle cells (Straub *et al.,* 2011). Dysregulation of this system is involved in several diseases such as heart failure, stroke, preeclampsia and pulmonary hypertension (Moya *et al.,* 2002; Bryan *et al.,* 2008; Kunieda *et al.,* 2008; Foster *et al.,* 2009; Gonzalez *et al.,* 2009; Que *et al.,* 2009). These two different signal transduction pathways only appear to be separate. The NO/sGC/cGMP pathway selectively activates PKG and its specific downstream effectors that amplify the signal leading to vasorelaxation. On the contrary, S-nitrosylation generates a wide pattern of nitrosylated proteins, responsible for different actions of NO in vascular tissues. However, an interaction between these two different signalling pathways has been suggested. For instance, the S-nitrosylation/denitrosylation system can act on the NO/sGC/PDE pathway, as the S-nitrosylation of PDE inhibits the catabolic activity of this enzyme (Murray *et al.,* 2008; Lima *et al.,* 2010) leading to an increased level of cGMP.

Hydrogen sulfide

Similar to NO, H_2S is a vasodilating mediator. The first study that showed the vasodilatory properties of H_2S was published

by Hosoki *et al.,* (1997). In this pioneering paper, the authors demonstrated that H_2S donors induce vasorelaxation by themselves and potentiate NO-donor induced vasorelaxation in rat thoracic aorta, giving the first hints of a possible interaction between NO and H_2S (Hosoki *et al.,* 1997).

In mammalian tissues, H_2S can be produced *via* both enzymic and non-enzymic pathways, although the enzymic path (trans-sulfuration pathway) generates the majority of H_2S in the body. Within the vessels, the production of H_2S is provided by different enzymes expressed in both endothelium and smooth muscle cells. These two locations represents the main difference from the biosynthesis of NO which is, instead, generated only within endothelium by eNOS. Cystathionine β synthase (**CBS**) is the best characterized H_2S generating enzyme and this protein is considered to be the main source of H_2S in the CNS (Abe and Kimura, 1996; Eto and Kimura, 2002; Miles and Kraus, 2004). However, it is also highly expressed in peripheral tissues, including the vasculature (Hosoki *et al.,* 1997; Fiorucci *et al.,* 2006; Zhang *et al.,* 2013; Vellecco *et al.,* 2016b; Szabo *et al.,* 2013; Bucci *et al.,* 2014). Another enzyme, cystathionine γ lyase (**CSE** or CGL or CTH), is the main H_2S generating enzyme in the vascular system and is found in both endothelium and smooth muscle cells. However, CSE expression has been detected also in non-vascular smooth muscle and skeletal muscle (Fiorucci *et al.,* 2006; Vellecco *et al.,* 2016a; Bucci *et al.,* 2012; Zhao *et al.,* 2001). Two other H_2S-producing enzymes, 3-mercaptopyruvate sulfur transferase (3-**MST**) and cysteine aminotrasferase (**CAT**) are both expressed in endothelium. However, 3-MST is also found in smooth muscle and other non-vascular tissues, (Nagahara *et al.,* 1998; Shibuya *et al.,* 2009; Módis *et al.,* 2013; Vellecco *et al.,* 2016a). CBS, CSE and CAT all require the aminoacid L-cysteine as substrate and pyridoxal 5′phosphate (PLP) as cofactor. The substrate for 3MST is 3-mercaptopyruvate and this enzyme is not independent on PLP. The substrate L-cysteine can be derived from the diet or can be liberated from endogenous proteins. It can also be synthesized endogenously starting from L-methionine through the trans-sulphuration pathway. In contrast to NO, H_2S is relatively stable in body fluids, but in common with NO, it can be scavenged by haemoglobin. H_2S can be also methylated or oxidised. H_2S is excreted in urine and flatus as free sulfate, thiosulfate or free sulfide, and it is also exhaled in breath (see Wallace and Wang, 2015). This last property means that it is possible to detect exhaled H_2S following intravenous administration of sodium sulfide in healthy human volunteers (Toombs *et al.,* 2010) and in rats (Insko *et al.,* 2009), thereby confirming exhaled H_2S as a way of excretion of H_2S.

The first molecular target identified for vasodilatory activity of H_2S was the **K_{ATP} (K_{ir}6.x) channel** (Zhao *et al.,* 2001). In this study, the H_2S-induced vasodilatation was specifically inhibited by **glibenclamide**, a selective inhibitor of the **K_{ATP}** channels. In vascular smooth muscle cells, a direct action of H_2S on K_{ATP} channel currents and membrane potential has been demonstrated (Zhao *et al.,* 2001). Because, at that time, CSE expression had been detected only in smooth muscle cells and not in endothelium (Zhao *et al.,* 2001), the authors suggested a selective role of H_2S in smooth muscle cells.

In the early years, the scientific results present in the literature did not support the original hypothesis of an interplay between NO and H_2S signalling, as suggested by Hosoki and co-authors, but rather provided an hypothesis of an additive or alternative vasodilating effect. In other words, both gasotransmitters contributed to the vasodilatory process, in which the endothelium-derived NO and the smooth muscle cell-derived H_2S worked in synergy. However, the generation of $eNOS^{-/-}$ mice in the 1990s definitively established the primary role of NO as an endogenous mediator in the control of blood pressure (Stauss *et al.,* 1999).

H_2S/NO interplay in health

As for NO, a role for H_2S as an endogenous mediator was defined by the generation of $CSE^{-/-}$ mice (Yang *et al.,* 2008). In this study, the $CSE^{-/-}$ mice exhibited a hypertensive phenotype and in isolated vessels harvested from $CSE^{-/-}$ mice, methacholine-induced vasodilatation was significantly impaired. More importantly, CSE was located also in the endothelium and its activation shown to be Ca^{2+}-calmodulin dependent. This paper was instrumental in emphasizing the similarity between the NO and H_2S pathways. Indeed, both enzymes, eNOS and CSE, are expressed in endothelium and both require Ca^{2+}-calmodulin binding to be activated (Yang *et al.,* 2008). The second group of H_2S molecular targets to be identified as responsible for H_2S-induced vasorelaxation were the PDEs (Bucci *et al.,* 2010). The PDEs constitute a class of enzymes that hydrolyze cyclic nucleotides cGMP and cAMP to their respective inactive metabolites. In both rat aorta and aortic smooth muscle cells, H_2S donors increase cGMP levels in a concentration-dependent manner. When cells are engineered to over-express CSE, there is an elevation of intracellular cGMP production that is prevented by treatment with **propargylglycine (PAG)**, a CSE inhibitor. Confirmation has been obtained by silencing CSE which causes a marked reduction of cGMP content. Finally, in a cell-free PDE assay, nanomolar concentrations of NaHS significantly inhibited PDE activity, reducing the catabolism of both cGMP and cAMP (Bucci *et al.,* 2010). Thus, NO and H_2S synergistically induce cGMP increase by acting on the same signal transduction pathway but on different enzymes.

As observed for NO, also H_2S exerts post-translational modifications of protein, defined as S-sulfhydration (Mustafa *et al.,* 2009; Lu *et al.,* 2013). Indeed, endogenous H_2S (and in particular CSE-derived H_2S) physiologically S-sulfydrates proteins on the thiol group of L-cysteine residues, in particular of GSH, leading to the formation of the –SSH moiety. Similarly to S-nitrosylation, S-sulfydration is a transient phenomenon as enzymic S-desulfydration of GSSH takes place through a mitochondrial persulfide dioxygenase enzyme (ETHE1) activity, that modulates the GSH status and indirectly the -SSH proteins level (Lu *et al.,* 2013). Much less is known about this S-sulfydration/de-S-sulfydration system compared to our knowledge of the corresponding S-nitrosylation/de-S-nitrosylation system. Also, S-sulfydration involves a large number of proteins (Mustafa *et al.,* 2009), leading to a hypothesis that there could be competition between nitrosylation and S-sulfhydration for the same cysteine residues in a protein, thus allowing the

two gasotransmitters to regulate each other (Hara *et al.,* 2005).

Thus, the gas paradigm in the vasculature starts to take shape. Increased intracellular Ca^{2+} leads to formation of the Ca^{2+}-calmodulin complex that in turn activates both CSE and eNOS, expressed in endothelium, with consequent production of H_2S and NO respectively. Both mediators are gases and diffuse into smooth muscle cells, reaching their own targets: sGC for NO and PDE and K_{ATP} channels for H_2S. Both gases increase cGMP levels but through two complementary, not overlapping, mechanisms: NO activates sGC leading to increased synthesis of cGMP while H_2S inhibits PDE decreasing the degradation of cGMP. Therefore in the normal, healthy vasculature, the two pathways converge on cGMP which represents the link between NO and H_2S, providing an apparently redundant system. In addition, H_2S opens the K_{ATP} channels expressed in smooth muscle cells, allowing the efflux of K^+ ions from the cells. This efflux limits calcium influx into the cells, thereby relaxing vascular smoth muscle (see Wang, 2012). At the same time, S-nitrosylation/de-S-nitrosylation and S-sulfydration/de-S-sulfydration systems continuously regulate a wide range of proteins, modulating the biochemistry of the cell in a dynamic and most likely controlled and coordinated manner.

Another possible molecular event, which has been discussed in several excellent reviews (Fukuto *et al.,* 2012; Olson, 2012; Nagpure and Bian, 2016), is the possible chemical interaction between H_2S and NO. Indeed, the reaction between NO and H_2S may generate nitroxyl, thionitrous acid and other possible compounds which could play roles within the cardiovascular system.

NO and H_2S in inflammatory-based vascular disease

Is the relationship between the NO and H_2S pathways modified in vascular diseases? Vascular diseases represent a heterogeneous group of disorders of the heart and the vascular network, in which many factors differently contribute to the onset, development and severity of the disease. It is now well-established that the combination of genetic predisposition and an unhealthy environment and lifestyle can favour the appearance of vascular diseases. Hyperlipidaemia, hyperglycaemia, cigarette smoking, obesity and hyperhomocysteinemia are among the many risk factors for the development of vascular diseases. All of them could be a cause of vascular diseases, alone or in combination, giving a wide range of these diseases in terms of the symptoms of initiation, the sites affected (heart, kidney, coronary arteries, etc), the severity and the disease progression. All the pathologies included in the vascular diseases, that is, atherosclerosis, heart failure, diabetes and hypertension, are characterized by oxidative stress and inflammatory processes (Ellulu *et al.,* 2016) thereby sustaining the concept of inflammation-based vascular diseases (IBVD). Another group of key players in the IBVD are the ROS. Physiologically, ROS such as peroxynitrite ($ONOO^-$), superoxide (O_2^-), hydroxyl (HO-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), are signal molecules that are involved in vascular homeostasis. They regulate oxygen sensing, apoptosis, cell proliferation, defence from microbial injury and inflammatory reactions (Zhang *et al.,* 2007; Santilli *et al.,* 2015). These molecules are produced within the vessels by endothelium, smooth muscle cells and also in adventitia (Lakshmi *et al.,* 2009) by several enzymic systems including mitochondrial enzymes, xanthine oxidase, lipoxygenase and NADPH oxidase. The vascular tissues have antioxidant systems that continuously operate to counterbalance the ROS generated. These include superoxide dismutase, catalase, TRX peroxidase, GSH peroxidase and , haem oxygenase (Zhang *et al.,* 2007; Santilli *et al.,* 2015). When the ROS production overwhelms the endogenous antioxidant systems, the body undergoes what is called *oxidative stress*. All the IBVD have in common, this condition of oxidative stress and its first target is the endothelium. The impaired biosynthesis of NO by eNOS is the main consequence of oxidative stress in the endothelium, probably because, in presence of high levels of ROS, the eNOS co-factor BH4 is readily oxidised (Antoniades *et al.,* 2007). This induces the uncoupling of eNOS, with consequent generation of O_2^- instead of NO (Antoniades *et al.,* 2007; Dikalova *et al.,* 2016). This process has been confirmed in patients with heart failure with preserved ejection fraction, where the oxidation of BH4 to BH2 is associated with eNOS uncoupling (Yamamoto *et al.,* 2015). The impairment of NO biosynthesis has a wide ranging effect on many aspects of vascular function. In healthy tissues, beyond its capacity to dilate vessels, NO reduces oxidation of LDL, reduces platelet reactivity and decreases leukocyte stickiness, all changes that protect the vasculature. Therefore, the impairment of NO release due to endothelial dysfunction induces vasoconstriction, procoagulability and arterial stiffness that maintain a vicious cycle in IBVD. Another element that contributes to the impairment of NO biosynthesis associated with IBVD is the protein caveolin-1 (CAV-1). In normal, resting conditions, eNOS is bound to CAV-1, a resident protein of caveolae that keeps eNOS in a less active state. When intracellular Ca^{2+} rises, the Ca^{2+}-calmodulin complex binds eNOS and, together with HSP90, displaces eNOS from the negative control of CAV-1, leading to activation of the enzyme (Bucci *et al.,* 2000; Gratton *et al.,* 2000). Several studies have demonstrated that, in IBVD, reduced production of NO is due to an increase of CAV-1 expression that retains the eNOS in a less-active state (Jia and Sowers, 2015). Indeed, CAV-1 overexpression has been found in hyperlipidaemia (Yue *et al.,* 2012; Kamada *et al.,* 2016), diabetes (Bucci *et al.,* 2004; Vellecco *et al.,* 2016a; Carantoni *et al.,* 1997; Clarkson *et al.,* 1996; Ding and Triggle, 2005), atherosclerosis (Fernández-Hernando *et al.,* 2009; Pavlides *et al.,* 2014) and pulmonary hypertension (Gilbert *et al.,* 2016; Mu *et al.,* 2016).

Theoretically, biosynthesis of H_2S should also be impaired in IBVD, as this gasotransmitter is, like NO, mainly produced in endothelium. In line with this hypothesis, there is a progressive reduction of H_2S levels in plasma and vascular tissues of non-obese diabetic mice (Brancaleone *et al.,* 2008). Also, in the spontaneous hypertensive rat (SHR), expression of CSE in blood vessels is decreased, accompanied by a reduction of plasma levels of H_2S (Ahmad *et al.,* 2012; Bucci *et al.,* 2014). Again, in a rat model of chronic kidney disease, CBS, CSE and 3-MST in the kidney are down-regulated (Aminzadeh and Vaziri, 2012) and H_2S content falls. Such

changes have also been shown in the activation of the renin-angiotensin system associated with diabetic nephropathy (Xue *et al.*, 2013).

The protective role of H_2S in the cardiovascular system, and consequently its impairment in IBVD, has been suggested by several studies. Thus, administration of H_2S-donors ameliorates ischaemia-induced heart failure in mice and genetic overexpression of CSE improves left ventricular performance and survival (Calvert *et al.*, 2010). In SHR rats, H_2S-donors reduce systemic blood pressure (Bucci *et al.*, 2014) and myocardial fibrosis (Meng *et al.*, 2015) suggesting a role for H_2S deficiency in the development of these pathologies. Decreased endogenous production of H_2S has also been linked to atherosclerosis, as $CSE^{-/-}$ mice fed with an atherogenic diet for 12 weeks, developed early lesions in aorta, elevated plasma levels of LDL, hyperhomocysteinemia, increased adhesion molecule expression, and enhanced intimal proliferation. Replacement of H_2S with a donor inhibited such accelerated atherosclerosis development (Mani *et al.*, 2013). In patients with acute coronary syndrome, the low concentration of circulating H_2S was associated with increased levels of CCL2 and CX3CL1, two monocyte chemokines involved in the pathogenesis of atherosclerosis (Gao *et al.*, 2015).

H_2S has been recognized also as an endogenous modulator of leukocyte–mediated inflammation (Whiteman *et al.*, 2004). Using intravital microscopy in a murine air pouch model, H_2S-donors inhibited aspirin-induced leukocyte adherence in mesenteric venules and leukocyte infiltration (Zanardo *et al.*, 2006). By using a complementary approach, several studies have shown that inhibition of CSE activity can mimic or worsen some pathological aspects of IBVD. For instance, Zanardo and colleagues show that inhibition of H_2S synthesis promotes leukocyte infiltration (Zanardo *et al.*, 2006). In cultured renal mesangial cells, CSE blockade by PAG increased generation of ROS, similar to that observed in a high glucose environment (Xue *et al.*, 2013). Also in $apoE^{-/-}$ mice fed with a Western diet, PAG treatment enhanced the atherosclerotic lesion area, the content of lipids and macrophages, and was associated with lower plasma NO levels and protein S-nitrosylation, implying that H_2S could modulate atherosclerosis progression (Lin *et al.*, 2016). However, while the involvement of NO in IBVD has been relatively well defined, that of H_2S is not as clear, in terms of the molecular mechanisms involved. This lack of information is most likely due to the lack of selective inhibitors of the different H_2S generating enzymes, along with less knowledge of the mechanisms of activation of H_2S generating enzymes, compared with eNOS. This is an key issue, as all the enzymes are constitutively expressed.

The gas paradigm in IBVD: A central role for cGMP/PKG pathway?

Coming back to the 'gas paradigm', taking in account all the data so far available, the endothelium is the key component in their action in the vasculature. This is particularly true under normal, healthy conditions, where the endothelium synthesizes both gasotransmitters. In IBVD, the endothelial impairment reduces both NO and H_2S production. However, this reduced level of endothelium-derived gasotransmitters exposes a different aspect of the cooperation between NO and H_2S, because, while eNOS is only present in the endothelium, CSE is expressed also in smooth muscle cells (where it was first described by Zhao *et al.*, 2001). Thus, the H_2S biosynthesis in smooth muscle cells is still functional, even when the endothelial synthesis is impaired. In this way, H_2S could provide a backup system for the vasculature that becomes important (or is unmasked) when NO is reduced, that is, in pathological conditions. In other words, it could well be that the limited amount of NO provided by a malfunctioning endothelium, together with the smooth muscle cell-generated H_2S, can still activate the signal transduction pathway. In turn, H_2S, by inhibiting PDE, allows the cGMP to accumulate to the threshold necessary to trigger the downstream signalling. Thus, a new question arises: could H_2S *per se* activate downstream signalling leading to vasodilatation, even in a complete absence of NO? In other words, could smooth muscle-derived H_2S *per se* compensate (even though partly) for the impairment of endothelial-derived gasotransmitters? This is possible as incubation of mouse aorta rings with sildenafil prior to challenge of the tissue with either an NO or H_2S-donor cause opposing effects. While sildenafil potentiates NO-induced vasorelaxation by contributing to cGMP rise by blocking PDE5, this does not apply to H_2S. Indeed, sildenafil and H_2S share the same molecular target, therefore sildenafil prevents and/or competes with H_2S for PDE binding, thereby reducing H_2S-induced vasorelaxation (Bucci *et al.*, 2012). This functional study has been further explored at molecular levels by studying the role of **PKG**. Once synthesized, cGMP binds to PKG, a kinase that activates the downstream signalling that causes vasodilatation (Martinez-Ruiz *et al.*, 2011). NaHS enhanced phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) in aorta tissue in a time-dependent manner, further suggesting that cGMP-dependent PKG is activated, following exposure to H_2S. This was confirmed by the finding that H_2S–induced vasorelaxation was strongly impaired in $PKG^{-/-}$ mice and by using a selective PKG inhibitor DT2 in wild type mice. Inhibition of PKG by DT2 also reduces the hypotension triggered by H_2S administration *in vivo* confirming the role of PKG in H_2S-induced vasorelaxation (Bucci *et al.*, 2012). The concept of cooperation between NO and H_2S signalling and a central role for PKG has been also elegantly demonstrated by Coletta and colleagues (Coletta *et al.*, 2012). In their study, they not only confirm that H_2S activated PKG and its downstream effector VASP (Coletta *et al.*, 2012) but also demonstrate, by alternatively blocking CSE and eNOS, that the cooperative action of the two gasotransmitters on increasing and maintaining intracellular cGMP, is essential for angiogenesis, wound healing and vasorelaxation (Coletta *et al.*, 2012).

Another important point to take in account is that the action of H_2S as an opener of K_{ATP} channels in blood vessels is an action exclusive to H_2S, and is not shared by NO. Therefore, H_2S-induced vasodilatation, due to K_{ATP} channels activation in smooth muscle cells is still functional when endothelial-derived action is impaired. However, the contribution of this vasorelaxant mechanism could be limited, as recent studies have shown that overproduction of ROS in oxidative stress also disrupts vascular K_{ATP} channel

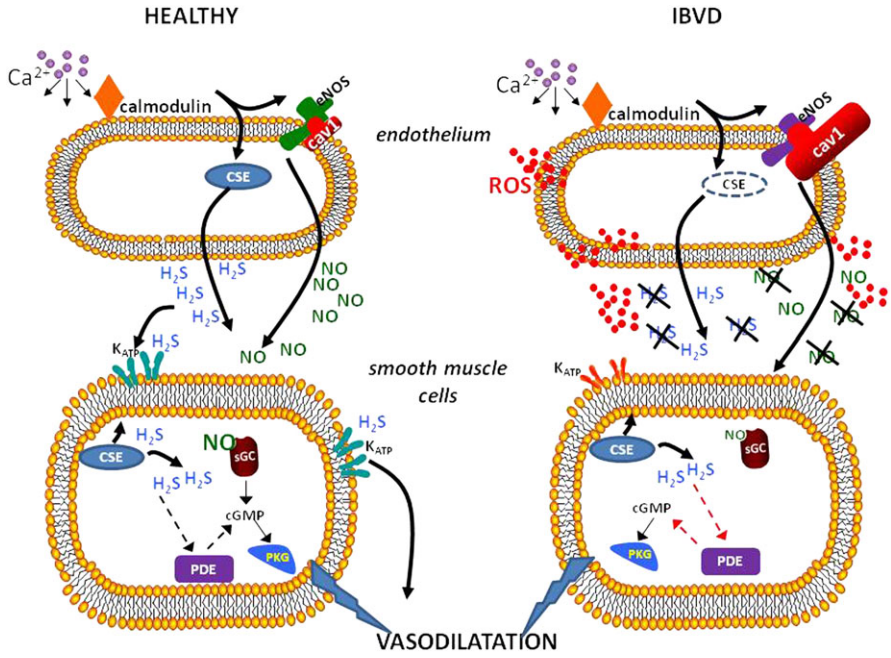

Figure 1

Representative simplified scheme summarizing the main findings discussed. The diagram outlines the interaction between NO and H_2S in physiological (HEALTHY) and pathological (IBVD) conditions. **HEALTHY**: In endothelium, eNOS is bound to CAV-1, a resident protein of caveolae that keeps eNOS in a less active state. When intracellular Ca^{2+} rises, Ca^{2+}-calmodulin complex is formed and binds eNOS. This event contributes to the displacement of eNOS from the negative control of CAV-1, leading to enzyme activation and NO production. At the same time, Ca^{2+}-calmodulin complex also activates CSE, leading to H_2S biosynthesis. Being gases, NO and H_2S diffuse into smooth muscle cells reaching their own targets: sGC for NO and PDE for H_2S. NO activates sGC leading to cGMP increase, while H_2S inhibits PDE reducing cGMP degradation. cGMP, by interacting with PKG, activates the downstream signalling that leads to vasodilatation. In addition, H_2S acts as an opener of K_{ATP} channels, expressed on smooth muscle cells leading to efflux of K^+ ions from the cells. This event reduces calcium influx into the cells contributing to vessel relaxation. CSE is also expressed in smooth muscle cells where it actively produces H_2S. **IBVD**: vascular diseases are characterized by an overproduction of ROS that induce *oxidative stress*. This condition damages the endothelium, impairing both eNOS and CSE activity with consequent reduction of both NO and H_2S biosynthesis. The *oxidative stress* can also affect K_{ATP} channel activity. The limited amount of NO provided by a damaged endothelium, still can activate the signal transduction pathway relying on the contribution of smooth muscle cell-generated H_2S that in turn, by inhibiting PDE, allows a production of cGMP within the threshold necessary to trigger the downstream signal. Solid line: activation; dotted line: inhibition.

activity (Miura *et al.*, 2003; Ross and Armstead, 2003; Erdos *et al.*, 2004; Yang *et al.*, 2010).

Taking all these results into consideration it is clear that the balance between these two pathways is critically altered in IBVD. The molecular basis of this interaction is located within the sGC/cGMP/PKG pathway. Even though NO and H_2S share the same second messenger *i.e.* cGMP, they act at different levels, with NO increasing production of cGMP through stimulation of sGC and H_2S inhibiting cGMP degradation. This evidence taken together with the finding that H_2S can be produced also in the smooth muscle defines the molecular basis of this crosstalk in IBVD. Thus, in IBVD, there is a reduced endothelial function with a decreased production of both NO and H_2S. However, the smooth muscle-derived H_2S can sustain vascular function by preserving the cGMP levels up to the threshold necessary to trigger the cascade of signalling, initiated by activation of PKG (Figure 1). Nevertheless, this alternative source of H_2S will itself fail when the oxidative stress rises further, as the disease increases in severity.

Conclusion and future perspectives

Considering the published results in the field, it is clear that a 'gas paradigm in the vascular system' does exist, and that it has an important role in controlling vascular tone in health and in IBVD. Differences in the relative and interconnected contributions of these two pathways can be assessed by analysing the data available and dividing the results into those obtained in physiological, and those obtained in pathological, conditions. Through this exercise, it is also clear that NO is undoubtedly the primary agent. Its beneficial action protects the vasculature and contributes to the maintenance of the dynamic equilibrium between contraction and dilatation of vessels, thus stabilising blood pressure. However, even when the endothelium is dysfunctional, the body still reacts in order to control the vascular tone. In other words, the endothelium/smooth muscle network must compensate for the reduction in NO signalling. The identification of H_2S as an endogenous vasodilator immediately suggested a possible cooperation between these two gases in controlling vascular homeostasis.

At present, it appears that the H_2S pathway becomes more relevant when the NO pathway is impaired. However, more and extensive investigations are needed to properly understand the exact role of H_2S in this context and, more importantly, its relationship with NO.

There are still several key issues to be clarified. While $eNOS^{-/-}$ mice show an obvious hypertensive phenotype, confirming the relevance of NO as a vasodilating mediator, such a role is not clearly defined for H_2S. Indeed, $CSE^{-/-}$ mice display a less pronounced hypertension and, more importantly, the increase of blood pressure is age-related (Yang *et al.,* 2008). Thus, the rise in blood pressure was similar in homozygous and heterozygous mice until the mice were 10 weeks of age, and only after this point, did the blood pressure of $CSE^{-/-}$ mice rise to about 10 mm Hg higher than that of $CSE^{-/+}$ mice (Yang *et al.,* 2008). Moreover, another group have generated $CSE^{-/-}$ mice and found that both male and female $CSE^{-/-}$ mice are normotensive, but that they display an acute lethal myopathy when fed with a low cysteine diet and show a greater sensitivity to oxidative injury (Ishii *et al.,* 2010). However, it is also important to note that, in the vasculature, H_2S is formed by at least three different enzymes - CSE, 3-MST, CBS - that are present in almost all tissues with different ratios. Therefore, the lack of one of them or its genetic deletion, even if that particular enzyme is the main source of H_2S in the examined cells and/or tissue, does not completely deprive the tissue of this gasotransmitter. Indeed, while an impairment of eNOS function or gene deletion leads to a drastic reduction or total loss of eNOS-derived NO, H_2S production within the $CSE^{-/-}$ endothelium can still be generated by 3MST and/or CBS.

Even though the use in therapy of NO-donors appeared to be the first logical choice, it did not lead to new drugs. Indeed, if we evaluate how much of our knowledge of NO has been translated into therapeutic approaches, it is obvious that very little of the relatively large amount of information about NO has been translated into therapeutically useful agents or procedures. At present, there is still only nitroglycerine, the main drug used for more than two centuries, in acute angina pectoris pain. However, the therapeutic use of this drug is strongly limited by the marked, uncontrolled hypotensive effects and by tolerance. Indeed, these limitations apply to all the new NO-donors developed and they remain as pharmacological, preclinical tools. In this scenario, could the H_2S-donors be developed as a more practical therapeutic approach in IBVD? Being less potent vasodilators than NO, H_2S-donors could be used without affecting systemic blood pressure. Indeed, no changes in blood pressure were observed in human volunteers, receiving intravenous Na_2S, up to a dose of 0.2 $mg \cdot kg^{-1}$ (Toombs *et al.,* 2010).

In this context, in the current literature there are some clinical and preclinical data suggesting that sulphydrylated ACE inhibitors exert additional beneficial effects on the vasculature, apart from ACE inhibition, effects that cannot be explained solely through changes in the NO pathway. For instance, H_2S derived from **S-zofenopril** accounts for the additional beneficial effects on vasculature and systemic blood pressure (Bucci *et al.,* 2014). S-Zofenopril is a pro-drug and, among the four possible diastereoisomers, it is the only isomer active on ACE and used in therapy. The active metabolite of S-zofenopril, namely **S-zofenoprilat**, has a free thiol and, acting as an H_2S-donor, improves vascular reactivity, beyond ACE inhibition (Bucci *et al.,* 2014). This hypothesis has been confirmed by the finding that the inactive diastereoisomer R-zofenoprilat, even though it does not modify blood pressure in SHR, retains the beneficial effect on SHR vascular function and restores plasma and tissue H_2S levels (Bucci *et al.,* 2014). Such protective actions can be ascribed to stimulation of H_2S signalling, rather than to a generic antioxidant property, as it is well-known that antioxidants are not efficacious in this clinical setting (Pasini *et al.,* 2007). Similarly, Monti and co-authors have found that S-zofenoprilat, in a CSE/H_2S-mediated manner, abolished all the inflammatory features induced by IL-1β in human umbilical vein endothelial cells, especially the NF-κB/COX-2/prostanoid pathway (Monti *et al.,* 2016). Again, pretreatment with S-zofenopril significantly augmented both plasma and myocardial H_2S and NO levels in mice and plasma H_2S in pigs, as well as reducing myocardial infarct size and cardiac troponin I levels after I/R injury (Donnarumma *et al.,* 2016). These findings taken together, suggest that it is indeed feasible to develop H_2S-releasing drugs, as therapeutic agents in IBVD.

Another possible approach to increase H_2S content in blood vessels is to stimulate the endogenous molecular machinery and to enhance H_2S biosynthesis (Hine *et al.,* 2015). It is known that dietary restriction (DR) without malnutrition, comprises many alimentary regimens that exert several benefits such as longevity and stress resistance (Fontana *et al.,* 2010., Miller *et al.,* 2005). In this context, Hine and colleagues have shown that, in a mouse model of DR, restriction of sulfur-containing amino acids increased CSE expression, resulting in increased H_2S production and protection from hepatic ischaemia-reperfusion injury. Pharmacological and genetic inhibition of CSE reduced H_2S production and blocked the DR-mediated stress resistance, confirming the role of CSE-derived H_2S in this phenomenon (Hine *et al.,* 2015).

In conclusion, these two gases, NO and H_2S, cooperate in maintaining vascular homeostasis. Under physiological conditions, the key player in the gas paradigm is NO. However, under pathological conditions, as in IBVD, the balance between these two pathways changes and the role of H_2S becomes more relevant as a backup, rescue system, taking advantage from the fact that its biosynthesis is not restricted to the endothelium. Unravelling the molecular mechanisms underlying the interaction of these two gasotransmitters in disease may lead to the development of new therapeutic approaches.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.,* 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.,* 2015).

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Conflict of interest

The authors declare no conflicts of interest.

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