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REVIEW

Gas what: NO is not the only answer to sexual function

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Standard abbreviations conform to BJP's Concise Guide to PHARMACOLOGY (Alexander *et al.*,2013a,b) and to the IUPHAR guidelines.

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The ability to get and keep an erection is important to men for several reasons and the inability is known as erectile dysfunction (ED). ED has started to be accepted as an early indicator of systemic endothelial dysfunction and subsequently of cardiovascular diseases. The role of NO in endothelial relaxation and erectile function is well accepted. The discovery of NO as a small signalling gasotransmitter led to the investigation of the role of other endogenously derived gases, carbon monoxide (CO) and hydrogen sulphide (H_2S) in physiological and pathophysiological conditions. The role of NO and CO in sexual function and dysfunction has been investigated more extensively and, recently, the involvement of H_2S in erectile function has also been confirmed. In this review, we focus on the role of these three sister gasotransmitters in the physiology, pharmacology and pathophysiology of sexual function in man, specifically erectile function. We have also reviewed the role of soluble guanylyl cyclase/cGMP pathway as a common target of these gasotransmitters. Several studies have proposed alternative therapies targeting different mechanisms in addition to PDE-5 inhibition for ED treatment, since some patients do not respond to these drugs. This review highlights complementary and possible coordinated roles for these mediators and treatments targeting these gasotransmitters in erectile function/ED.

LINKED ARTICLES
This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

Abbreviations
CC, corpus cavernosum; ED, erectile dysfunction

Introduction

Erectile function

Erectile physiology is the interplay of vascular, neurological and endocrine factors, which leads to an increase in or facilitates the vasodilatation (tumescence) and/or reduces the contraction (detumescence) of the corpus cavernosum smooth muscle (CCSM) cells. Erection is the final outcome of a complex integration of signals. It is essentially a spinal reflex that can be initiated by recruitment of penile afferents, but also by visual, olfactory and imaginary stimuli and all the stimuli contribute to the increase in vasodilatation of penile tissues (for details, see review by Cirino *et al.*, 2006). Neuronal and endothelial NO are considered as the most important factors for relaxation of penile vessels and CCSM cells.

Erectile dysfunction (ED)

ED is defined as the consistent or recurrent inability to attain or maintain a penile erection sufficient for sexual activity in man (2nd International Consultation on Sexual Dysfunction-Paris, 28 June–1 July 2003). It is interesting to note that ED and cardiovascular disease (CVD) share many of the risk factors that contribute to their development and progression such as age, hypercholesterolaemia, obesity, diabetes, smoking and some less-traditional risk factors including inflammation, hypoxia and homocysteinaemia (Brunner *et al.*, 2005). Moreover it is now well accepted that vascular disturbance of the penile endothelium leads to ED and as a consequence the possibility arises that ED may be an early indicator of systemic endothelial dysfunction and subsequently of CVD. In fact, recognizing ED as a disease marker for CVD may help to identify individuals at risk of having a premature cardiovascular event (Shin *et al.*, 2011).

Nitric oxide

The synthesis and physiological significance of NO in erectile function

Constitutive forms of NOS (see Alexander *et al.*, 2013b), the endothelial (eNOS) and neuronal NOS (nNOS) have a role in erectile process. In contrast, inducible form of NOS, iNOS does not have a direct role but is involved in pathological conditions in the penis (Gonzalez-Cadavid and Rajfer, 2005). nNOS is localized in the pelvic plexus, dorsal penile nerve, cavernous nerve and its branches in the cavernous tissue (Sullivan *et al.*, 1999). eNOS is localized in the arterial and cavernous endothelial cells and also in the CCSM (Andersson, 2001). Penile nNOS variant (PnNOS) has been identified in rat and mouse penis nerves, which is considered to be responsible for the synthesis of NO in the terminal nerve of the penis (Gonzalez-Cadavid *et al.*, 2000).

The role of NO in erectile function as the principal mediator is confirmed by several studies where NOS is genetically or pharmacologically inhibited (Burnett, 1995; Burnett *et al.*, 2002; Cashen *et al.*, 2002; Lasker *et al.*, 2010a).

Depolarization of the cavernous nerves by psychogenic and reflex stimuli leads to rapid nNOS-mediated NO release to initiate tumescence (Burnett, 1995; Burnett *et al.*, 2002; Cashen *et al.*, 2002; Lasker *et al.*, 2010a). NO diffuses the CCSM, activates soluble guanylyl cyclase/cGMP (sGC/cGMP) pathway and causes relaxation which increases blood flow to the penis. Following this, blood flow-induced shear stress causes an increase in sustained NO release via PI3K/Akt/eNOS pathway to supply maintenance of tumescence (Hurt *et al.*, 2012; Lasker *et al.*, 2013). It is believed that eNOS is more significant than nNOS in erectile physiology (Bivalacqua *et al.*, 2007d). However, recently, it has been demonstrated that nNOS also contributes to the maintenance of erectile process via sustained release of NO through PKA activation-induced phosphorylation of nNOS at Ser^{1412} (Hurt *et al.*, 2012). Protein–protein interaction, subcellular localization, phosphorylation and deacetylation (Fleming and Busse, 2003; Mattagajasingh *et al.*, 2007) are the main regulatory mechanisms for eNOS activity. However only a few of eNOS regulatory mechanisms are recognized in penis such as Ca/calmodulin (Ca/CaM), PI3K/Akt-dependent phosphorylation and protein interaction with caveolin or heat shock protein 90 (hsp90; Hurt *et al.*, 2002; Musicki and Burnett, 2006). While hsp90 activates eNOS, caveolin-1 inactivates it by binding to CaM-binding site on eNOS (Musicki *et al.*, 2009). However hsp90 and caveolin-1 are not targets solely for NOS but also for other gaseous molecules and for sGC (see last section of this review). Recently, it has been demonstrated that urotensin II (U-II), an endogenous peptide identified as the natural ligand of a GPCR, physically interacts with eNOS in penis and activates it via phosphorylation (d'Emmanuele di Villa Bianca *et al.*, 2012). Several agonist and stimuli such as shear stress, VEGF, sildenafil, angiopoietin and sphingosine-1-phosphate (S1-P) cause NO production by phosphorylation of eNOS at Ser^{1177} (d'Emmanuele di Villa Bianca *et al.*, 2006; Musicki *et al.*, 2009). There are six specific sites of phosphorylation in eNOS. However only phosphorylation sites at the Ser^{1177} and Thr^{495} residues, activating and inactivating eNOS respectively, were demonstrated in the penis (Hurt *et al.*, 2002; Musicki *et al.*, 2005a).

Pathophysiological significance of NO in ED

Decreased NO bioavailability in vasculogenic ED is caused by decreased NOS activity/synthesis or the inactivation of NO (Musicki *et al.*, 2005a; Jin *et al.*, 2008a; Claudino *et al.*, 2009; Park *et al.*, 2009; Demir *et al.*, 2010; Soner *et al.*, 2010; Saito *et al.*, 2012; Bivalacqua *et al.*, 2013; Dalaklioglu *et al.*, 2013a; Silva *et al.*, 2013; Yang *et al.*, 2013b). Oxidative stress impairing NO bioavailability is a common mechanism for ED. Reactive oxygen species (ROS) result from an imbalance between antioxidant and ROS-generating systems such as NADPH oxidase, myeloperoxidase and even eNOS itself (Zouaoui Boudjeltia *et al.*, 2007; Jin and Burnett, 2008). Oxidation of tetrahydrobiopterin (BH_4) or deficiency in cofactor BH_4, leads to eNOS uncoupling, in which eNOS becomes two monomers and generates superoxide anion rather than NO (Förstermann and Li, 2011; Johnson *et al.*, 2011). The lack of dimerization is responsible for the pathophysiology of ED in hypercholesterolaemia (Musicki *et al.*, 2010). Moreover, oxidative stress increases iNOS expression, but decreases both expressions of nNOS and eNOS and the erectile response in ischaemic rabbit CC (Azadzoi *et al.*, 2004).

It has been shown that eNOS phosphorylation is altered in vasculogenic ED induced by aging, diabetes mellitus and hypercholesterolaemia (Musicki *et al.*, 2009) and has an important role in the prolongation of erection. Thus, inhibiting phosphorylation of eNOS (p-eNOS Thr^{495}) and dephosphorylation of eNOS (p-eNOS Ser^{1177}) appear as new drug targets for the treatment of ED.

Myristoylation, palmitoylation and acetylation are necessary for caveolar localization of the enzyme, which inactivates eNOS; however, the first two mechanisms have not been investigated in the penis yet. Sirtuin-1 (SIRT-1) leads to activation of eNOS through its deacetylation (Arunachalam *et al.*, 2010). Although a direct role of SIRT-1 has not been investigated in the penis, decreased expressions of SIRT-1 expression in CC in androgen depletion (Tomada *et al.*, 2013) or diabetes (Yu *et al.*, 2013)-induced ED has been shown.

S-nitrosylation negatively regulates NOS by inhibition of sGC, eNOS itself and eNOS-regulating proteins including hsp90 and Akt (PKB). Palmer and co-workers have shown that

Table 1

eNOS/NO regulation in ED

Pathology	Molecular mechanisms	References
Hyperlipidaemia/ atherosclerosis	↓p-VASP	Musicki *et al.*, 2010
	↓cGMP	Musicki *et al.*, 2008
	↑NADPH oxidase, ROS, TBARS production	Musicki *et al.*, 2008; 2010; Fraga-Silva *et al.*, 2013
	↓eNOS and nNOS, ⦸ eNOS	Musicki *et al.*, 2010; Fraga-Silva *et al.*, 2013
	⦸ p-eNOS S1177, uncoupled eNOS	Musicki *et al.*, 2008; 2010
	↑ADMA	Park *et al.*, 2009
	↑eNOS binding to Cav-1, ⦸ Cav-1	Musicki *et al.*, 2008
	↑Rho A expression	Dalaklioglu *et al.*, 2013b
Aging	↓p-eNOS S1177	Dalaklioglu *et al.*, 2013b; Silva *et al.*, 2013
	↑p-eNOS T495, ↓ p-Akt	Musicki *et al.*, 2005a
	↑Arginase activity	Sakai *et al.*, 2004; Numao *et al.*, 2007
	↓cGMP	Silva *et al.*, 2013
	↑ROS	Johnson *et al.*, 2011
	↓L-arginine in CC	Sakai *et al.*, 2004
	↓ eNOS and nNOS expression, ⦸ total NOS activity	Numao *et al.*, 2007; Dalaklioglu *et al.*, 2013b
	↑p-AMPK	Labazi *et al.*, 2013
	↓p-eNOS S1177, ⦸ p-eNOS S1177	Saito *et al.*, 2012; Labazi *et al.*, 2013
Hypertension	↓cGMP	Gur *et al.*, 2010; Saito *et al.*, 2012
	↑ROCK mRNA expression, ↑Cav-1	Yono *et al.*, 2009
	↑ROS	Jin *et al.*, 2008a
	↓nNOS and eNOS, ↑iNOS expression, ⦸ nNOS	Gur *et al.*, 2010; Saito *et al.*, 2012; Labazi *et al.*, 2013
	↑ERK1/2 Phosphorylation	Labazi *et al.*, 2013
	↓eNOS, nNOS	Li *et al.*, 2012; Dalaklioglu *et al.*, 2013a; Qiu *et al.*, 2013
	↓p-eNOS S1177	Musicki *et al.*, 2005b; Dalaklioglu *et al.*, 2013a; Yang *et al.*, 2013a
	↑RhoA expression	Dalaklioglu *et al.*, 2013a
Diabetes	↑NADPH oxidase and ROS	Li *et al.*, 2012; Dalaklioglu *et al.*, 2013a; Yang *et al.*, 2013a
	↓p-Akt	Musicki *et al.*, 2005b
	⦸ p-eNOS T495	Musicki *et al.*, 2005b
	↓cGMP	Fukuhara *et al.*, 2011; Yang *et al.*, 2013a
	↑Arginase II	Bivalacqua *et al.*, 2001
	↑Cav-1	Elçioğlu *et al.*, 2010
	↑ROS, uncoupled eNOS, ↑cGMP	Bivalacqua *et al.*, 2013
	↓p-eNOS S1177, ↓ eNOS- HSP90 interaction, ↓ p-AKT, ⦸ p -eNOS T495, ⦸ eNOS, ⦸ Hsp90, ⦸ Cav-1	Musicki *et al.*, 2011

↑, increase; ↓, decrease, ⦸, unchanged; p-VASP, vasodilator stimulated phosphoprotein; Cav-1, caveolin-1; p-AMPK phosphorylated 5′ AMP-activated PK; TBARS, thiobarbituric acid-reactive substances; ADMA, asymmetric dimethylarginine; p-eNOS T495, eNOS phosphorylated on threonine 495; p-eNOS S1177, eNOS phosphorylated on serine 1177; uncoupled eNOS, monomer form of eNOS that is generating ROS rather than NO; ROCK, Rho-kinase.

S-nitrosoglutathione reductase (GSNOR), which catalyses the reduction of S-nitrosothiols (Lima *et al.*, 2010), is co-localized with eNOS in the endothelium of CC. Moreover, S-nitrosylated eNOS levels are increased during detumescence in wild-type mice compared with GSNOR-/- mice (Palmer *et al.*, 2012). The role of S-nitrosylation/denitrosylation of NOS is well documented in erectile physiology but has not been associated with the pathophysiology of erectile function yet.

Beside NO bioavailability, downstream mechanisms of NO, such as increased PDE-5 enzyme and reduced PKG-1 activation by cGMP have also been reported in ED. The pathophysiological significance of eNOS/NO pathway in ED is presented in Table 1.

Treatments targeting NO

Known NO-based therapies include NO precursors, NO donors, NO-based gene therapy, pharmacological NOS

Table 2

Preclinical studies in ED targeting NOS/NO pathway

Drugs targeting NOS/NO pathway	Effects in ED models	References
NOS substrate L-arginine and L-citrulline	↑ICP/MAP in acute arteriogenic rats and relaxation in healthy human	Gur *et al.*, 2007; Shiota *et al.*, 2013
NOS cofactor BH_4	↑ICP/MAP in aged mice and neurogenic relaxation in obese rat CC	Johnson *et al.*, 2011; Sanchez *et al.*, 2012
Arginase inhibitors ABH and BEC	↑Neurogenic and ICP/MAP in aged rats/mice and endothelial relaxation in aged/diabetic mice CC	Bivalacqua *et al.*, 2001; 2007a; Toque *et al.*, 2011; Segal *et al.*, 2012
NADPH oxidase inhibitor apocynin	↑ICP/MAP in hypertensive/diabetic rats/hypercholesterolaemic mice and endothelium-dependent relaxation in aged rat CC.	Jin *et al.*, 2008a; Musicki *et al.*, 2010; Li *et al.*, 2012; Silva *et al.*, 2013
Pharmacological NOS activator resveratrol	↑ICP/MAP in diabetic rats and endothelial relaxation in hypercholesterolaemic rabbit and healthy rat CC.	Shin *et al.*, 2008; Soner *et al.*, 2010; Fukuhara *et al.*, 2011; Yu *et al.*, 2013
NO-releasing agents $NaNO_2$	↑ICP/MAP in healthy and diabetic rats and endothelial relaxation in hypercholesterolaemic rabbit CC.	Shukla *et al.*, 2005; Lasker *et al.*, 2010b; Soni *et al.*, 2013
Gene therapies eNOS/PnNOS/EC-SOD/ iNOS/PKG1α/VEGF gene and angiopoietin-1	↑ICP/MAP in aged/ diabetic rat and in healthy /diabetic rat CC.	Bivalacqua *et al.*, 2000; 2003; 2004c; 2005; 2007b,c; Magee *et al.*, 2002; Chancellor *et al.*, 2003; Ryu *et al.*, 2006; Wang *et al.*, 2013
Inhibition of genes PIN/arginase/RhoA (T19NRhoA)	↑ICP/MAP in healthy/diabetic rat, aged mice and healthy/aged rat CC.	Chitaley *et al.*, 2002b; Bivalacqua *et al.*, 2004b; 2007a; Jin *et al.*, 2006; Magee *et al.*, 2007

MAP, mean arterial pressure; BEC, S- (2-boronoethyl)-L-cysteine; ABH, 2(S)-amino-6-boronohexanoic acid; EC-SOD, endothelial cell superoxide dismutase; PIN, protein inhibitor of NOS.

activators such as resveratrol. See reviews by Decaluwe and co-workers for details (Bryan, 2011; Decaluwe *et al.*, 2013). Although L-arginine substrate of NOS has been found to increase relaxation in human or animal CC (Table 2), clinical studies do not always support the beneficial effects of L-arginine alone. However, it seems successful in combination therapies (Table 3). L-arginine is also a substrate for arginase and its inhibition increases substrate availability of NOS. Arginase inhibitors have been found to increase neuronal and endothelial-dependent relaxation of CC, improve erectile function especially in diabetic ED as well as aging-induced ED. Recently, a protective effect of resveratrol, NOS activator, has been shown in diabetes and hypercholesterolaemia-induced ED (see Table 2). Even though there are many animal studies that indicate the success of NADPH oxidase inhibitors and gene therapies in ED (see Table 2), no clinical trial has been performed with these therapies yet. The importance of the NO/cGMP pathway as drug targets became clear with the discovery of the PDE-5 inhibitors (PDE-5i) and their great success in treating ED. However, there are significant numbers of ED patients with diabetes mellitus and severe vascular disease who do not respond to PDE-5i, suggesting that maintaining physiological levels of NO may not be sufficient in mild or severe ED associated with vascular risk factors. Therefore, drugs targeting the bioavailability or downstream mechanisms of NO or combination therapies have started to be investigated. Clinical and preclinical pharmacological treatments and gene therapies targeting the NOS/cGMP pathway are summarized in Tables 2 and 3 respectively.

Carbon monoxide

CO was known as the 'silent killer' until the 21st century because it is odourless and colourless and it can threaten life by hypoxic intoxication without giving an obvious symptoms or indications. Unlike NO and H_2S, CO elimination is through exhalation by the lungs and does not involve a biochemical modification (Kreck *et al.*, 2001; Motterlini and Otterbein, 2010). CO might exert its effects during longer periods of time and distances compared with NO or H_2S, as it is the most biologically stable gasotransmitter with a half-life of around 3 h (Motterlini and Otterbein, 2010) since it does not have unpaired electrons, and does not chemically dissociate in an aqueous solution to form different chemical species.

The synthesis of CO

The majority of CO is produced by enzymatic haem metabolism (Wu and Wang, 2005) and the remaining fraction of CO arises from other sources that may include lipid peroxidation and xenobiotic metabolism.

Table 3

Clinical studies targeting NOS/NO in ED

Therapy	Drug dose	Patients	Sexual function	References
L-arginine supplementation	L-arginine 3 × 500 mg·day^{-1}	32 patients with mixed-type impotence	No difference	Klotz *et al.*, 1999
	L-arginine (5 g·day^{-1})	50 organic ED patients. A double-blind, randomized, placebo-controlled study.	Significant improvement	Chen *et al.*, 1999
BH4 supplementation	Single oral doses of BH4 200 mg or 500 mg	18 moderate ED patients in a randomized, placebo-controlled, double-blind crossover study.	Increase duration of penile rigidity.	Sommer *et al.*, 2006
Combined therapies with L-arginine	L-arginine aspartate 8 g + adenosine monophosphate 200 mg	Mild-to-moderate ED whose erectile function domain score between 14 and 22	Effective	Neuzillet *et al.*, 2013
	L-arginine + L-carnitine + nicotinic acid + vardanafil	Insulin-dependent diabetic patients	Better than PDE-5 inhibitor alone	Gentile *et al.*, 2009.
	L-arginine 6 g + yohimbine 6 g during 2 weeks or L-arginine 6 g	42 patients mild to moderate ED. Double-blind, placebo-controlled, three-way crossover, randomized clinical trial.	L-arginine do not improve alone but combined therapy improve erectile function	Lebret *et al.*, 2002
	L-arginine aspartate 1 g during 3 months + pycnogenol 3 × 40 mg during 2 months	40 ED patients	Improve sexual function.	Stanislavov and Nikolova, 2003

Haem oxygenase enzymes (HO) exist in constitutive (HO-2 and HO-3) and inducible (HO-1) isoforms (see Alexander *et al.*, 2013b). HO-1, a 32 kDa mammalian stress protein is induced by several stimuli including hypoxia, stress, ROS, inflammatory cytokines and a variety of transition metals and heavy metals (see review by Ryter for a detailed list of HO inducers; Ryter *et al.*, 2006). HO-1 induction leads to the release of iron and the formation of biliverdin and CO, which are able to regulate ROS level and inflammatory processes to a certain extent. HO expression regulates the level of ROS by increasing antioxidant, such as glutathione and bilirubin. HO-1 is expressed less in nerve fibres but is clearly identifiable in the endothelium lining of penile arteries and the sinusoidal walls of the CC and spongiosum (Hedlund *et al.*, 2000b). However, upon stimulation, HO-1 expression in the testes overpowers the expression of HO-2. HO-2 is a constitutively expressed 'haem sensor' in the endothelium and CCSM, engaged in fine-tuning the activity of transcriptional factors and genes that are haem-responsive, including HO-1. HO-2 gene expression has been shown not to be changed by either HO inducers or inhibitors (Abdel Aziz *et al.*, 2005). HO-2 expression is more condensed in the pelvic ganglion and nerve fibres innervating bulbospongiosus muscles in rat and human urogenital system (Burnett *et al.*, 1998; Hedlund *et al.*, 2000b; Ushiyama *et al.*, 2004).

HO-3 has only been found in rat tissues, including brain, liver, kidney and spleen. HO-3 is related to HO-2 and represents pseudo genes originating from HO-2 transcripts (Hayashi *et al.*, 2004).

Physiological significance of CO in erectile function

In isolated vessel preparations, both CO and haem-L-lysinate increase the vasodilatation (Kozma *et al.*, 1997) of which only the latter can be reversed by inhibitors such as HO chromium mesoporphyrin (Kozma *et al.*, 1997). The role of HO/CO pathway in erectile function was first demonstrated by showing HO expression and CO induced relaxation in human CC (Hedlund *et al.*, 2000b). Further, it was confirmed that exogenous CO relaxes the CC dose dependently in rat (Ushiyama *et al.*, 2004). NOS or HO inducers can equally up-regulate expression of both genes and increase the tissue levels of cGMP in CC. Aziz and colleagues suggest that HO/CO system is supervising and dominating NO as a signalling molecule in erectile function (Abdel Aziz *et al.*, 2005). Thus, induction of HO may have therapeutic implications for the management of ED (Decaluwe *et al.*, 2013).

Electrical field stimulation (EFS)-induced relaxations are inhibited by HO inhibitors; tin-protoporphyrin (SnPP) and zinc-protoporphyrin (ZnPP; Ushiyama *et al.*, 2004) and increased by exogenous CO in rat CC (Kim *et al.*, 2010). The suppression of EFS-induced relaxation by SnPP has been found to be specific to HO inhibition and not related to NOS inhibition, as is the case in the hippocampus (Meffert *et al.*, 1994), since the relaxation that remained in HO inhibitor treated group was further inhibited by L-NNA. On the contrary, neurogenic relaxation by EFS is not inhibited in rabbit CC by ZnPP (Kim *et al.*, 1994) or in HO-2 knockout mice

Table 4
Targeting HO/CO in erectile function

HO-1 inducing drug	Model	References
HO-1 cDNA-liposome complex transfer	Aged rats	Abdel Aziz *et al.*, 2009b
Hemine	SHR	Shamloul and Wang, 2006
Losartan	Diabetic rats	Abdel Aziz *et al.*, 2009a
Hemin	Healthy rat	Abdel Aziz *et al.*, 2008
Curcumin	Healthy rat	Abdel Aziz *et al.*, 2010
α-tocopherol	SHR	Ushiyama *et al.*, 2008
PDE-5 inhibitors; sildenafil, tedalafil, verdanafil	Healthy rat	Abdel Aziz *et al.*, 2007a,b,c; Liu *et al.*, 2012

Approaches increasing HO-1 induction and subsequently erectile functions are listed.

(Burnett *et al.*, 1998). Nevertheless, more rigorous investigations need to be performed before suggesting that the role of CO in neurogenic erection may be different in rats compared with other species, since the expression and the biological status of HO-1 are not clear in this HO-2-deficient mice model and HO-1 may also cause neurogenic erection.

In addition to the involvement in penile erection control, CO also plays an important role in regulating ejaculation (Burnett *et al.*, 1998). HO-2 knockout mice have less reflex activity of the bulbospongiosus muscle, where the HO-2 localization is condensed, and substantially reduced ejaculation, without a significant change in erectile function. In the same year, another study reported that prenatal exposure to CO (150 ppm) leads to increased mount/intromission latency, decreased mount/intromission frequency, and a significant decrease in ejaculation frequency, which are associated with changes in mesolimbic dopaminergic function in male rats (Cagiano *et al.*, 1998). The authors speculated that prenatal exposure to CO might influence the development or function of neurons releasing CO locally in the penis and decreases HO-2 expression/activity parallel to the findings in HO-2 $^{-/-}$ animals.

Pathophysiological significance and treatments targeting CO in ED

Drugs targeting activation of HO/CO pathway. A number of studies have suggested that impaired CO-mediated vasodilatation is implicated in ED (Abdel Aziz *et al.*, 2009c; Shamloul, 2009). Ushiyama and colleagues clearly showed that NO- and CO-dependent relaxation of the CC in response to EFS is diminished in spontaneously hypertensive rats (SHR) and suggested that this may be due to decreased activity of HO-2, since the HO-2 gene expression was unchanged (Ushiyama *et al.*, 2004). This study for the first time showed that the impairment of neurogenic relaxation induced by HO/CO systems may, to a certain degree, be involved in the diminished erectile responses in SHR (Ushiyama *et al.*, 2004). Two studies suggest that HO inducers may ameliorate the erectile function in SHR by showing that; (i) a potent HO-1 inducer, haemin, increased both intracavernous pressure (ICP) and HO-1 level, but not HO-2, as well as HO-1 downstream molecule sGC expression in SHR (Shamloul and Wang, 2006); and (ii) the improved erectile function by the antioxidant α-tocopherol in SHR could be blocked by an HO inhibitor (Ushiyama *et al.*, 2008).

An HO-1 inducer reversed the decreased erectile function, gene expression and enzymatic activity of HO-1 in CC of diabetic rats (Abdel Aziz *et al.*, 2009a). This study suggests that the decline in erectile function in diabetic rats may be attributed to a down-regulation of the HO/CO pathway and indicates that stimulating this pathway could be an effective treatment for ED in diabetic patients. In addition, HO-1 induction also restores decreased eNOS expression and vascular responses as well as reversing the increased iNOS expression in diabetic rat aorta (Ahmad *et al.*, 2005). It has been suggested that the antioxidant effects of HO might also contribute to its endothelial protective effect in diabetes (Kruger *et al.*, 2006).

In addition, chronic administration of the HO-1 inducers in hypertensive and diabetic rats and an *in vivo* gene therapy using HO-1 cDNA-liposome complex transfer have been found to be beneficial for ED induced by aging (Abdel Aziz *et al.*, 2009b). HO-1 inducers, which have been reported to augment HO-1 expressions and/or cGMP concentrations together with subsequent relaxation in CC, are listed in Table 4. Several studies show that HO-1 induction by losartan and/or CoPP (Abdel Aziz *et al.*, 2009a), hemin (Abdel Aziz *et al.*, 2008), curcumin (Abdel Aziz *et al.*, 2010) restores ED through the up-regulation of the local tissue levels of cGMP. The erectile function induced by HO-1 induction was found to be as effective as up-regulating NOS by L-Arg. (Abdel Aziz *et al.*, 2005), complementary to and even dominating NO in mediating erectile function (Abdel Aziz *et al.*, 2009a).

Interestingly, NO itself has been shown to induce HO-1 to produce CO (Durante *et al.*, 1997). Thus if the HO/CO pathway is involved in the mechanism of the NO targeting, drug-induced beneficial effects in relaxation should be investigated. Moreover, the effect of sildenafil on ED has been attributed to interactions between CO and NO (Abdel Aziz *et al.*, 2007a). α-tocopherol has been shown to enhance erectile function in both a NOS- and HO-dependent manner in ED in SHR (Ushiyama *et al.*, 2008). Some of the cardiovascular drugs targeting HO/CO pathway are listed in Table 5 as well as losartan and sildenafil, which are listed in Table 4. Among those drugs, losartan, α-tocopherol and PDE-5i are shown to

Table 5

Cardiovascular treatments targeting HO/CO in the vascular system

Cardiovascular drug	Explanation	References
Atorvastatin	Activates HO-1 to get compensatory anti-inflammatory and vasorelaxant effect in hypercholesterolaemic rabbit aorta	Muchova *et al.*, 2007; Fujita *et al.*, 2010; Ong *et al.*, 2011
Atorvastatin-clinical study	Decreases inflammation and oxidant stress via mechanisms associated with HO-1 induction and CO, but not bilirubin	Ong *et al.*, 2011
Angiotensin II	Regulates HO-1 in rat vascular smooth muscle cells	Ishizaka and Griendling, 1997
Resveratrol	Induces HO-1 in human aortic smooth muscle cells in a concentration-dependent manner	Juan *et al.*, 2005
NO donors (SpermineNONOate, SNAP)	Inhibits HO activity in vascular smooth muscle cell	Durante *et al.*, 1997; Hartsfield *et al.*, 1997

induce HO/CO in CC but HO-related effects of statins have not been investigated in the penis yet. However, there is increasing knowledge concerning the significance of the HO/CO pathway in the pathophysiology, which has led to the development of CO-releasing molecules, known as CORMs, a safe therapeutic strategy, releasing CO with controllable kinetics (Motterlini *et al.*, 2002). Tayem and colleagues indicated that CORMs can induce HO-1 and thus have a dual action, releasing CO and increasing HO-1 (Tayem *et al.*, 2006). This is not surprising since the ability of CO to induce HO activity has already been shown (Kim *et al.*, 2007). *In vivo* delivery of CORM-3 increases blood flow in penile arterioles and sinuses (Abdel Aziz *et al.*, 2008). CORM-2 also induces relaxation in mice CC but differently from CO, CORM-2-induced corporal relaxation was not affected by sGC inhibition. (Decaluwe *et al.*, 2012b) Readers interested in CO targeting molecules as therapeutics in specific pathological conditions are referred to a recent review by Motterlini and co-workers (Motterlini and Foresti, 2014).

Drugs leading to inhibition of HO/CO pathway. Priapism represents a 'medical emergency' with a persistent, usually painful erection that lasts for more than 4 h and occurs without sexual stimulation. It may lead to permanent ED and penile necrosis if left untreated and occurs in approximately 40% of patients with sickle cell disease (SCD; Kato and Gladwin, 2008). HO-1 expression is increased in SCD patients (Nath *et al.*, 2001; Jison *et al.*, 2004) in transgenic sickle mice (Belcher *et al.*, 2006) and in artificially- induced veno-occlusive, low-flow priapism (Jin *et al.*, 2008b).

The question arising is this; should we try to prevent HO-1 activation before it appears? Or inhibit the HO-1 activity in late priapism? Prompt treatment for priapism is usually needed to prevent tissue damage that could result in ED. The evidence to recommend medical prophylaxis is sparse but based on a consensus of experts and small phase 2 or 3 clinical trials (Olujohungbe and Burnett, 2013). It has been shown that HO inhibition by ZnPP reversed the apoptosis induced by ischaemic priapism in rats and seems promising for preserving erectile function in late priapism (Karakeci *et al.*, 2013).

Hydrogen sulphide

This molecule, now considered to be the third gaseous transmitter, shares many characteristics with the other gaseous transmitters: NO and CO (Wang, 2002). The role of H_2S in the homeostatic control of our body is now consistently supported by the literature (Wang, 2012).

H_2S presence in mammalian tissues was known even in the eighties but it was considered a metabolic waste product, and its potential physiological activity was ignored. Kruszyna and co-workers in 1985 described an influence by cyanide and sulphide compounds in the relaxation induced by nitrogenous compounds (Kruszyna *et al.*, 1985). The first evidence indicating this gas as an endogenous mediator was in 1996 by Abe and Kimura (1996) and it was in the brain.

Solid evidence demonstrated that H_2S acts as a potential neurotransmitter (Gadalla and Snyder, 2010) and exerts many activities in mammalian cardiovascular and respiratory systems (Hosoki *et al.*, 1997; Zhao *et al.*, 2001). Regarding the physiological significance of H_2S, a turning point has been achieved by the development of the knockout (KO) strain for both cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes. CBS is essential for life since in homozygous KO of CBS mice the lifespan would only be (about) 4 weeks (Watanabe *et al.*, 1995) and CSE-KO mice develop hypertension (Yang *et al.*, 2008).

Synthesis of H_2S

H_2S is generated within the mammalian cells via both enzymatic and non-enzymatic pathways, although the major contribution comes from the enzymatic one. CBS and CSE use L-cysteine (L-Cys) as the substrate to produce H_2S, while CBS can also use homocysteine to produce cystathionine that is metabolized by CSE to H_2S. Both CBS and CSE use pyridoxal 5′-phosphate, as a cofactor. The main H_2S-producing enzyme in the CNS is CBS while in the cardiovascular system, it is CSE (Zhao *et al.*, 2001; Eto *et al.*, 2002). Moreover, it has been suggested that H_2S could exert a negative feedback effect on the enzyme activity to regulate its synthesis and release (Kredich *et al.*, 1973). Other enzymes mainly localized in endothelial cell have been proposed to synthesize this gas,

the 3-mercaptopyruvate sulphurtransferase and the cysteine aminotransferase. For more details, see review by Wang (2012).

Physiological significance of H_2S in erectile function

In 2006, it was shown that intracavernosal injection of sodium hydrogen sulphide (NaHS) resulted in a significant increase in penile length and cavernous pressure in primates. Administration of DL-propargylglycine (PAG, CSE inhibitor) to rats resulted in a significant reduction in cavernous nerve stimulation-evoked perfusion pressure. On the basis of these results, a possible role for endogenous H_2S in erectile function has been suggested (Srilatha *et al.*, 2006).

In 2009, d'Emmanuele and co-workers clearly demonstrated that the L-Cys/H_2S pathway is present in human CC tissues. In particular, it was shown that both CBS and CSE are present and are active in human CC since tissue homogenates efficiently convert L-Cys to H_2S. CBS and CSE are localized within muscular trabeculae and the smooth-muscle component of the penile artery. Conversely, CSE but not CBS is also expressed in peripheral nerves. Moreover, both H_2S and L-Cys cause a concentration-dependent relaxation of human CC strips. This relaxation effect was inhibited by the CBS inhibitor, aminoxyacetic acid (AOAA), glibenclamide, a K_{ATP} ($K_{ir}6.1$-6.2) channel (see Alexander *et al.*, 2013a) inhibitor, and was only slightly reduced by L-NG-nitroarginine methyl ester (L-NAME), a NOS inhibitor. EFS of human penile tissue, under resting conditions, caused an increase in tension that was significantly potentiated by either PAG or AOAA. The role of this pathway in erectile function was also confirmed *in vivo*, in fact, NaHS and L-Cys increased the ICP in rat, and the response to L-Cys was blocked by PAG (d'Emmanuele di Villa Bianca *et al.*, 2009).

Pathophysiological significance and treatments targeting H_2S in ED

The altered expression of CSE and H_2S levels are involved in some acute inflammatory processes (Zanardo *et al.*, 2006; d'Emmanuele di Villa Bianca *et al.*, 2010) in atherosclerosis (Wang, 2009b; Wang *et al.*, 2009), diabetes (Wu *et al.*, 2009), hypertension (Yang *et al.*, 2008), hyper-homocysteinaemia (d'Emmanuele di Villa Bianca *et al.*, 2013) and obesity (Elshorbagy *et al.*, 2012), which are pathological conditions associated with ED. A link between male sexual hormones and H_2S has been suggested by Bucci and co-authors, who demonstrated that testosterone (T) causes an increase in the H_2S concentration acting on K_{ATP} channels. Thus, H_2S contributes to the vasodilator effect of testosterone (Bucci *et al.*, 2009). Testosterone induces relaxation by activating smooth muscle K_{ATP} channels in human CC strips (Yildiz *et al.*, 2009) and in horse penile resistance arteries (Ruiz Rubio *et al.*, 2004). It has been demonstrated that aging significantly reduces NO and H_2S levels both in plasma and CC and a reduction of the ICP was countered by NaHS or sildenafil treatment for 10 weeks. To confirm that there is a link between T and H_2S, Syrilatha and co-authors have shown a marked increase in T or oestradiol after NaHS supplementation. These data support the idea that ED related to aging may be also linked to a derangement in the H_2S pathway accompanied by low T levels (Srilatha *et al.*, 2012).

If T can modulate H_2S production, the decline in T level with aging or hypogonadism may also affect H_2S biosynthesis. All these data suggest the involvement of the L-cys/H_2S pathway in penile erection mechanisms of T (for details, see review by d'Emmanuele di Villa Bianca *et al.*, 2011). This very interesting issue needs to be addressed more accurately to translate this preclinical data to humans.

The efficacy of PDE-5i, the mainstay in the treatment of ED, seems to be tightly associated with the integrity of nerves and endothelium in CC and in several pathologies such as CVD, diabetes, obesity and post-prostatectomy state, this integrity is severely compromised leading to lack of the NO/cGMP pathway. Thus, there is a pressing need to discover new therapies for targeting other pathways not totally dependent on endothelium integrity. In this regard, the H_2S pathway could offer one opportunity since CBS and CSE are mainly localized within muscular trabeculae and in human penile tissues and the H_2S-induced relaxation is only partially reduced by L-NAME treatment. A tentative move towards developing a drug working on H_2S and cGMP pathways (i.e. not totally dependent on endothelium integrity) was performed by Shukla and co-workers who synthesized and characterized an H_2S-donating derivative of sildenafil (ACS6; Shukla *et al.*, 2009). Surprisingly, ACS6 had a similar efficacy to sildenafil and this result can be explained by the fact that H_2S and PDE-5i share the same target (e.g. PDE-5). Most probably, the development of drugs that either deliver H_2S directly or stimulate the enzyme activity responsible for its synthesis might be more efficacious.

While the inorganic forms of H_2S-releasing molecules, NaHS or Na_2S, are basic tools used to understand the H_2S role in the body, they are not eligible for treatments due to the rapid H_2S donation because of high solubility. For instance, we need the H_2S long-term releasing molecule. The best way to obtain a controlled gas release is to induce its synthesis endogenously by using L-Cys and/or N-acetylcysteine, but this approach could not work in a condition where a down-regulation of the enzyme CBS and CSE occurs. Until now, no studies have addressed the potential effect of L-Cys on human ED.

Concerning natural plant-derived compounds, the S-allyl cysteine, a bioactive component derived from garlic, can restore erectile function in diabetic rats by preventing ROS formation through modulation of NADPH oxidase subunit expression (Yang *et al.*, 2013b). However, whether it plays a role as a H_2S precursor or a modulator of H_2S-related enzymes is controversial (Jacob *et al.*, 2008). Other garlic-derived molecules, generally considered as precursors of H_2S metabolized in blood, have been studied for their potential anti-inflammatory and anti-cancer effects such as diallyl trisulphide, diallyl sulphide, diallyl disulphide and diallyl tetrasulphide but no data concerning their efficacy on CC are available.

In contrast, the synthetic H_2S donor that is attracting most interest is GYY4137. It inhibits lipid accumulation exhibiting anti-atherosclerotic activity both *in vitro* and *in vivo* (Yang *et al.*, 2013b). However, there is no data available on the effect of GYY4137 in ED.

Recently, it has been shown that H_2S can elicit vasoprotection by both scavenging O_2^- and by reducing vascular NADPH oxidase-derived O_2^- production in vascular tissues

(Vacek *et al.*, 2010; Hamar *et al.*, 2012; Al-Magableh *et al.*, 2014), Since ROS is the common cause of the ED and when eNOS is uncoupled it can produce ROS, the beneficial effects of drugs targeting H_2S in ED is not surprising.

The three gases – is there a convergence point?

The three gasotransmitters share similarities as modulators of physiological processes (Wang, 2002). CO, NO and H_2S are all able to induce SM relaxation contributing to penile erection. The common mechanism of these gases to cause erectile function is 'increasing the cGMP level' (Figure 1). The enzyme sGC is accepted as the most important target for NO to increase cGMP, which contributes to penile erection. Besides NO, CO can also bind to the enzyme sGC for its activation. However, sGC is not always associated as the target molecule for the three of them. H_2S increases the cGMP level without stimulating sGC. In this section, we will discuss the relationship between he gasotransmitters (NO, CO and H_2S) and their molecular mechanism in erectile function.

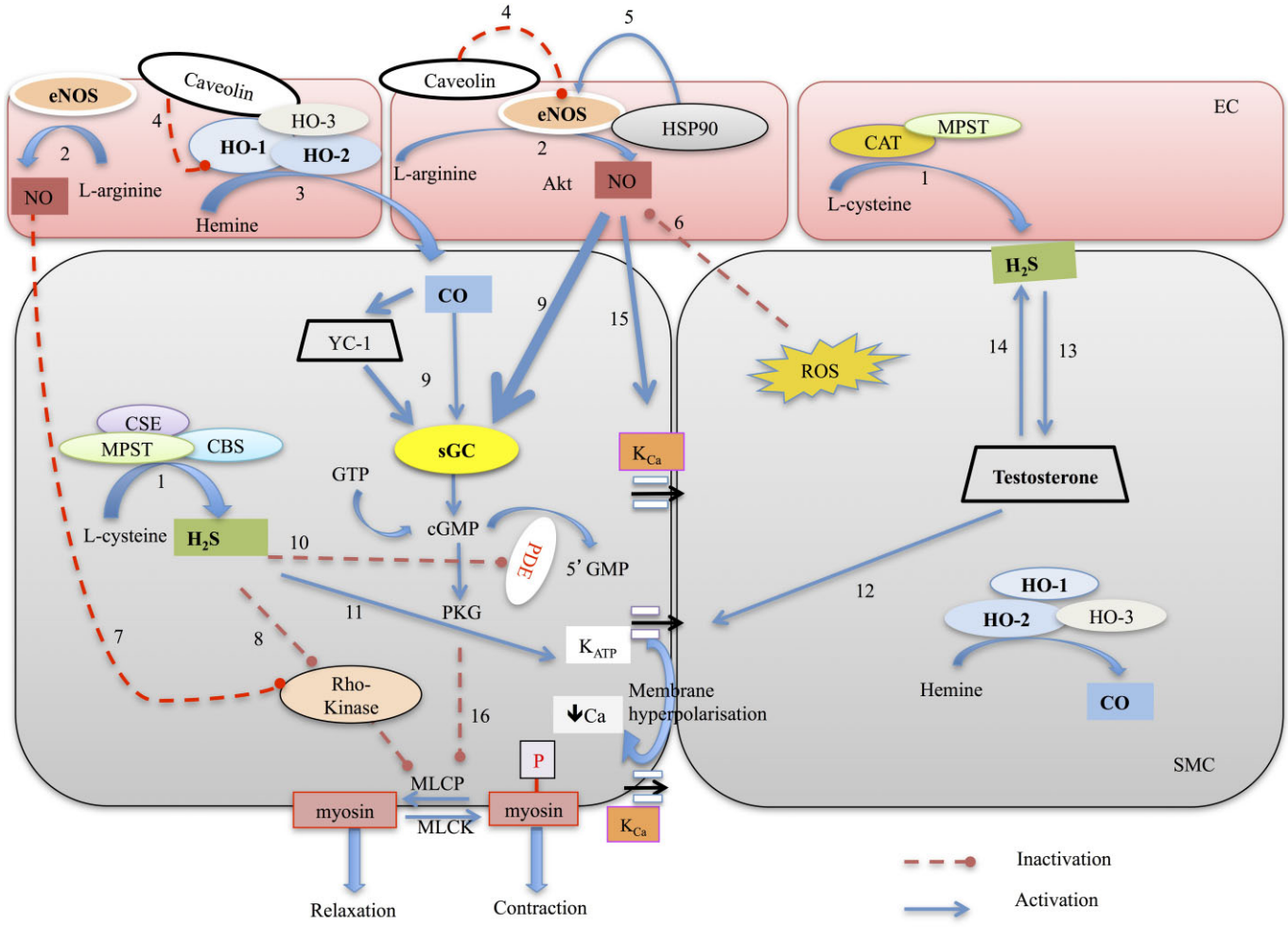

Figure 1

Synthesis and mechanisms of gaseous neurotransmitters in the relaxation of penile or other vascular tissues. Unbold fonts indicate evidence obtained in other vascular tissues rather than the penis. Dashed red lines indicate inhibition, whereas straight lines indicate activation. Endothelial cells (EC) are shown as pink boxes and smooth muscle cells (SMC) are shown as grey boxes. 1: CBS, CSE and MPST synthesize H_2S from L-cysteine. (CBS, CSE and MPST are expressed in smooth muscle cells in the penis. MPST can be also expressed in the endothelium of some vascular tissues). 2: eNOS synthesizes NO from L-arginine. 3: CO is synthesized from hemine by constitutive (HO-2 and HO-3) and inducible (HO-1) haem oxygenases. 4: Caveolin interacts and inactivates both eNOS and HO-1. 5: Hsp90 (HSP90) activates eNOS. 6: ROS decreases the availability of NO to act on sGC. 7: NO induces relaxation via inhibition of Rho-kinase (ROCK) signalling in the penile tissue. 8: H_2S-induced relaxations are increased in CC precontracted with endothelin, indicating a possible involvement of the RhoA/ROCK pathway in H_2S-induced erectile function. 9: Both NO and CO activate sGC to produce cGMP. CO-induced activation of sGC is lower than NO-induced activation of sGC. However, CO favours YC-1-induced haem-independent activation of sGC. 10: H_2S inhibits cGMP breakdown by PDE-5. 11: H_2S activates K_{ATP} and leads to membrane hyperpolarization, which decreases intracellular calcium level via K_{Ca} channels and consequently causes relaxation. 12: Testosterone induces relaxation by activating smooth muscle K_{ATP} channels in human CC strips. 13: NaHS treatment increases testosterone level in aging rats. 14: Testosterone causes an increase in H_2S level. 15: NO activates large conductance K_{Ca} ($K_{Ca}1.1$ also known as BK_{Ca}) in horse penile resistance arteries. 16: PKG can cause relaxation through activation of MLCP and reduce Ca^{2+} sensitivity in the penis.

sGC

sGC is a heterodimer and it is similar to other nucleotide-converting enzymes. Two different subunits with two isoforms of each have been identified: α1, α2, β1 and β2. The most abundant form of the heterodimer sGC is α1/β1 in CC (Behrends *et al.*, 1995). Both show sensitivity towards NO-releasing substances and to sGC activators. CC from sGCα1-/- mice showed significantly less or no relaxation in response to bradykinin (BK) and ACh, respectively, emphasizing the requirement of sGCα1 subunit for the erectile function of endothelium-derived NO (Nimmegeers *et al.*, 2008). The absence of EFS-induced relaxation in these mice indicates sGCα1β1 as the predominant target for neuronal NO. The minor contribution of sGCα2β1 isoform in erectile function has been suggested in this study since some responsiveness to exogenous NO (SNP and NO-gas) and sGC stimulator (BAY 41-2272) remains in the sGCα1-/- mice CC (Nimmegeers *et al.*, 2008).

Activation of sGC by NO involves binding to the enzyme's prosthetic haem group since its removal abolishes NO-induced activation (Stone and Marletta, 1995). After binding to the sGC haem, NO increases sGC activity by several hundred-fold (Derbyshire and Marletta, 2009) promoting the conversion of GTP to cGMP. In contrast, CO causes only a few fold increases in sGC activity, whereas this enzyme is unlikely to be activated by H_2S (Zhao and Wang, 2002). Despite the lower ability of CO to activate sGC compared with NO, it was reported that the vasodilator and erectile effects of CO are mediated by sGC activation (Friebe *et al.*, 1996; Nakane *et al.*, 2002; Decaluwe *et al.*, 2012a).

Besides the well-established NO/haem-mediated stimulation, other mechanisms for sGC activation have been identified. The activation induced by 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) and 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) involves binding to a site different from the haem group (Stone and Marletta, 1995; Friebe *et al.*, 1998). In vascular SM cells, YC-1 sensitizes sGC to NO and CO (Liu *et al.*, 2009). These compounds together with the amino dicarboxylic acid substance, BAY 58-2667 can evoke erectile responses, enhance cGMP formation and/or CC relaxation synergistically with NO (Mizusawa *et al.*, 2002; Nakane *et al.*, 2002; Stasch *et al.*, 2002; Baracat *et al.*, 2003; Hsieh *et al.*, 2003; Teixeira *et al.*, 2007; Frey *et al.*, 2012).

Although CO stimulates purified sGC very poorly, only 3–4-fold (Schmidt *et al.*, 2001), in the presence of sGC activators such as YC-1, sGC activation by CO is drastically enhanced, near to that stimulated by NO (Friebe *et al.*, 1996; Lee *et al.*, 2000; Ma *et al.*, 2007). CO relaxes CC through activation of sGC, indicated by the inhibiting effect of (1H-[1,2,4] oxadiazolo[4,3,-a]quinoxalin-1-one) and potentiating effect of YC-1 on the CO-induced responses in mice CC (Decaluwe *et al.*, 2012a).

The findings that (i) CO activates sGC in a similar way to NO, and (ii) it can activate sGC in nNOS-deficient mice (Zakhary *et al.*, 1997), suggest that endogenous CO might serve as a backup system when constitutive enzymes for NO are not functional or available.

Hsp90

We have previously observed that the relaxation mediated by sGC is regulated by the molecular chaperone 90-kDa heat shock protein hsp90 (Yetik-Anacak *et al.*, 2006). Inherent ATPase activity of hsp90 helps to protect cells against stressors through the control of maturation, trafficking, stability and activity of client proteins, such as the enzymes NOS and sGC (Garcia-Cardena *et al.*, 1998; Venema *et al.*, 2003; Yetik-Anacak *et al.*, 2006). Hsp90 is important to drive haem insertion and maturation of sGC (Ghosh and Stuehr, 2012). We demonstrated hsp90 and eNOS interaction and functional significance in mice CC (Yetik-Anacak *et al.*, 2013). Musicki *et al.* also showed the decreased complex formation of hsp90-eNOS in sickle cell anaemia-induced ED (Musicki *et al.*, 2011). There is evidence that it also regulates CO and H_2S activities in myocardial cells and astrocytes (Choi *et al.*, 2010; Yang *et al.*, 2011). Whether hsp90 interacts with CO and H_2S in the CC and contributes to penile erection remain to be elucidated.

cGMP

The product formed following sGC activation from GTP is the second messenger cGMP, that modulates the activity of several effector proteins leading to vasorelaxation (Schmidt *et al.*, 1993). NO and CO induce an increase in cGMP levels in CC (Priviero and Webb, 2010; Decaluwe *et al.*, 2012b). H_2S is also able to induce an increase in cGMP levels; however, it does not seem to directly activate sGC as mentioned above (Coletta *et al.*, 2012). As sildenafil, H_2S has been implicated as an inhibitor of PDE-5 delaying cGMP degradation (Bucci *et al.*, 2010; Coletta *et al.*, 2012). Furthermore, although it has been suggested that cGMP or cAMP analogues cause an increase in H_2S production in human bladder (Fusco *et al.*, 2012), this issue has to be confirmed in penile tissue.

PKG

Once formed, the principal intracellular mediator of the cGMP is the PK dependent on cGMP, PKG, which is a key step in the signal cascade leading to penile erection (Hedlund *et al.*, 2000a). PKG plays a role in mediating NO-, CO- and H_2S-dependent signalling in vascular tissue and BP control (Lohmann *et al.*, 1997; Lincoln *et al.*, 2001; Schlossmann *et al.*, 2003; Leffler *et al.*, 2005; Bucci *et al.*, 2012; Burgoyne *et al.*, 2012).

PKG can cause vascular relaxation through activation of myosin light chain phosphatase (MLCP) and reduce Ca^{2+} sensitivity in the penis (Mills *et al.*, 2002). Additionally, it has been shown that PKG phosphorylates and inhibits RhoA in the aorta (Sauzeau *et al.*, 2000).

RhoA/Rho-kinase pathway

Rho-kinase (ROCK) phosphorylates and inhibits MLCP thus promoting the binding of actin and myosin for contraction of CC (Chitaley *et al.*, 2002a; Wang *et al.*, 2002; Jin and Burnett, 2006) Chitaley and colleagues (2001) were the first to demonstrate the involvement of RhoA/Rho-kinase signalling in erectile response (Chitaley *et al.*, 2001). This signalling pathway is increased in the CCSM of several models of ED in rats, such as those associated with hypertension, diabetes and aging (Bakircioglu *et al.*, 2001; Chitaley *et al.*, 2001;

Bivalacqua *et al.*, 2004c). There is evidence that NO induces relaxation via inhibition of ROCK signalling in the penis (Mills *et al.*, 2002) and CO in aorta (Awede *et al.*, 2010). Furthermore, co-localization of eNOS and Rho-kinase was found in sinusoidal endothelium of CC (Mills *et al.*, 2002; Bivalacqua *et al.*, 2004a) CO inhibits the production of the potent vasoconstrictor, endothelin-1, which has been shown to activate RhoA (Morita and Kourembanas, 1995). H_2S may also interfere with the contractile mechanism mediated by the RhoA/ROCK pathway. In fact, in human CC strips pre-contracted with either U46619 or h-ET1, there was a marked increase in the H_2S vasorelaxant effect compared with that observed in strips pre-contracted with 1-agonist (d'Emmanuele di Villa Bianca *et al.*, 2009).

Caveolin-1

The enzyme sGC that was believed to be present only at the cytosol has been also detected in association with the plasma membrane (Zabel *et al.*, 2002; Venema *et al.*, 2003). sGC translocates to caveolar domain to be sensitized by NO (Zabel *et al.*, 2002; Venema *et al.*, 2003). In the CC of caveolin-1 knockout mice, the relaxation induced by EFS and by the NO donor is impaired compared with wild-type mice (Shakirova *et al.*, 2009), supporting a role for caveolae and caveolin-1 in erection. Previously, we observed that the relaxation induced by the sGC activator YC-1 is impaired in both the endothelium-intact aortic rings and CC after treatment with methyl-β-cyclodextrin, a compound that depletes plasma membrane cholesterol and disassembles caveolae (Linder *et al.*, 2005; 2006). In the aortic and sinusoidal endothelium, we observed colocalization of sGC and the major coat protein of caveolae, caveolin-1 (Linder *et al.*, 2005; 2006). These findings establish the association of sGC to caveolae in the endothelium introducing a potential therapeutic strategy for CVDs related to endothelial dysfunction, such as ED.

The well-established association of the enzyme eNOS with the plasma membrane protein, caveolin-1, maintains the enzyme in an inactive state (Feron *et al.*, 1996) and an increase in intracellular calcium concentration in the endothelial cell is a key step for the dissociation of these proteins and, consequently eNOS activation (Gratton *et al.*, 2000).

Similar to eNOS, HO-1 also appears in caveolae and physically interacts with caveolin-1 (Jung *et al.*, 2003; Kim *et al.*, 2004). HO enzyme activity increases in the absence of caveolin-1. In contrast, caveolin-1 causes inhibition of HO induction (Taira *et al.*, 2011). The negative regulation of both eNOS and HO-1 activity by caveolin-1 give rise to the hypothesis that caveolin-1 may serve as a molecular brake on signalling mechanisms involving small gaseous second messengers. H_2S-producing enzymes are also expressed in endothelium (Chertok and Kotsyuba, 2012; Baragatti *et al.*, 2013). Recent studies showed that H_2S is produced in adipose tissue, which is enriched by caveolin-1, but it is not known yet if H_2S-producing enzymes are located at caveolae and if H_2S interacts with caveolin-1. The only study addressing H_2S-caveolin relation demonstrated the lack of effect of H_2S donor (NaHS) on caveolin-1 expression in the CC (Meng *et al.*, 2013) but it remains to be investigated whether caveolin-1 regulates H_2S producing enzymes or H_2S-induced relaxations in penile tissue.

Alterations in caveolin-1 expression were reported in different animal models such as decreased caveolin-1 expression in diabetic, aged and nerve injured rats penis (Becher *et al.*, 2009) or increased caveolin-1 mRNA expression in SHR and protein expression in hypercholesterolaemic rat penis (Bakircioglu *et al.*, 2000; Yono *et al.*, 2009). Investigating the role of caveolar domains in erectile function of these gasotransmitters may bring new targets for ED treatment.

ATP-sensitive potassium channels: K_{ATP} channels

Activation of K_{ATP} channels leads to subsequent membrane hyperpolarization, which causes closure of voltage-dependent calcium channels resulting in smooth muscle relaxation. With respect to the physiology of erection, K channels in corporeal smooth muscle cells are accepted to represent a critical modulator of the flow of blood to and from the penis and, thus, an important determinant of erectile capacity (Spektor *et al.*, 2002). NO activates K_{ATP} channels via a cGMP-dependent mechanism in vascular smooth muscle cells (Kubo *et al.*, 1994) but not in horse penile resistance arteries (Simonsen *et al.*, 1995) or horse corpus cavernosum (Recio *et al.*, 1998). Glibenclamide inhibits CO-induced relaxation in vascular tissue (Foresti *et al.*, 2004) but not in mice CC suggesting that CO-induced erectile function does not involve K_{ATP} channels (Friebe *et al.*, 1996; Nakane *et al.*, 2002; Decaluwe *et al.*, 2012a). It has been proposed that H_2S causes opening of K_{ATP} channels by a protein S-sulphydration (Mustafa *et al.*, 2009; Jiang *et al.*, 2010). The role of these channels in H_2S-induced relaxation of human CC has also been confirmed (d'Emmanuele di Villa Bianca *et al.*, 2009). These studies show that both NO- and CO-induced relaxation mechanisms in the penis are different from those in other vascular tissues.

Calcium-activated potassium channels K_{Ca}

The endothelium-dependent vasodilatation evoked by ACh is resistant to blockade of NOS in penile small arteries (Prieto, 2008). The relaxant effect of NO is due in part to activation of large-conductance K_{Ca} ($K_{Ca}1.1$ also known as BK_{Ca}, see Alexander *et al.*, 2013a) in horse penile resistance arteries, (Simonsen *et al.*, 1995) but not in horse CC (Recio *et al.*, 1998) suggesting the diameter of the vessel may determine the involvement of K_{Ca} in the relaxation. ACh is the most common agonist that causes relaxation mediated by endothelial-derived hyperpolarizing factor (EDHF). Muscarinic cholinergic receptor activation causes CSE activation and in turn H_2S production and there are data supporting H_2S as an EDHF (Wang, 2003; 2009a). The exact nature of EDHF is still unknown but many hypotheses have been proposed. (Feletou and Vanhoutte, 2009). It is believed that K_{Ca} channels are the main mediator for vasodilator effects of the EDHF. The combination of K_{Ca} blockers, charybdotoxin and apamine significantly reduces the H_2S-induced endothelial-dependent relaxation, underlining that K_{Ca} channels are targets for H_2S and as it is well known, these channels are also the targets of EDHF (d'Emmanuele di Villa Bianca *et al.*, 2011; Mustafa *et al.*, 2011).

CO also leads to stimulation of K_{Ca} channels in several vascular tissues (Dubuis *et al.*, 2005; Decaluwe *et al.*, 2012a);

however, CO-induced relaxation in mice CC does not involve K_{Ca} channels (Decaluwe *et al.*, 2012b).

The interactions among the three sister gases

The interactions among these gases are mostly shown in other vascular tissues rather than the penis. The traffic between these gasotransmitters and downstream molecules and their implication in erectile function/dysfunction represent a very complicated but intriguing issue. There is evidence that the effects induced by CO and H_2S are partially mediated by NO/cGMP (Wegiel *et al.*, 2010; Coletta *et al.*, 2012; Fusco *et al.*, 2012; Meng *et al.*, 2013). In other words, H_2S and CO potentiate the stimulating action of endogenously synthesized NO. Additionally, Meng and colleagues have shown that H_2S enhances NOS expression in endothelial cells of CC leading to NO production (Meng *et al.*, 2013). The crosstalk among the gases was summarized in a representative figure (Figure 2).

H_2S–CO interaction

Recently, the data showing inhibition of H_2S producing enzyme CBS by constitutive CO suggests an H_2S-HO-2/CO

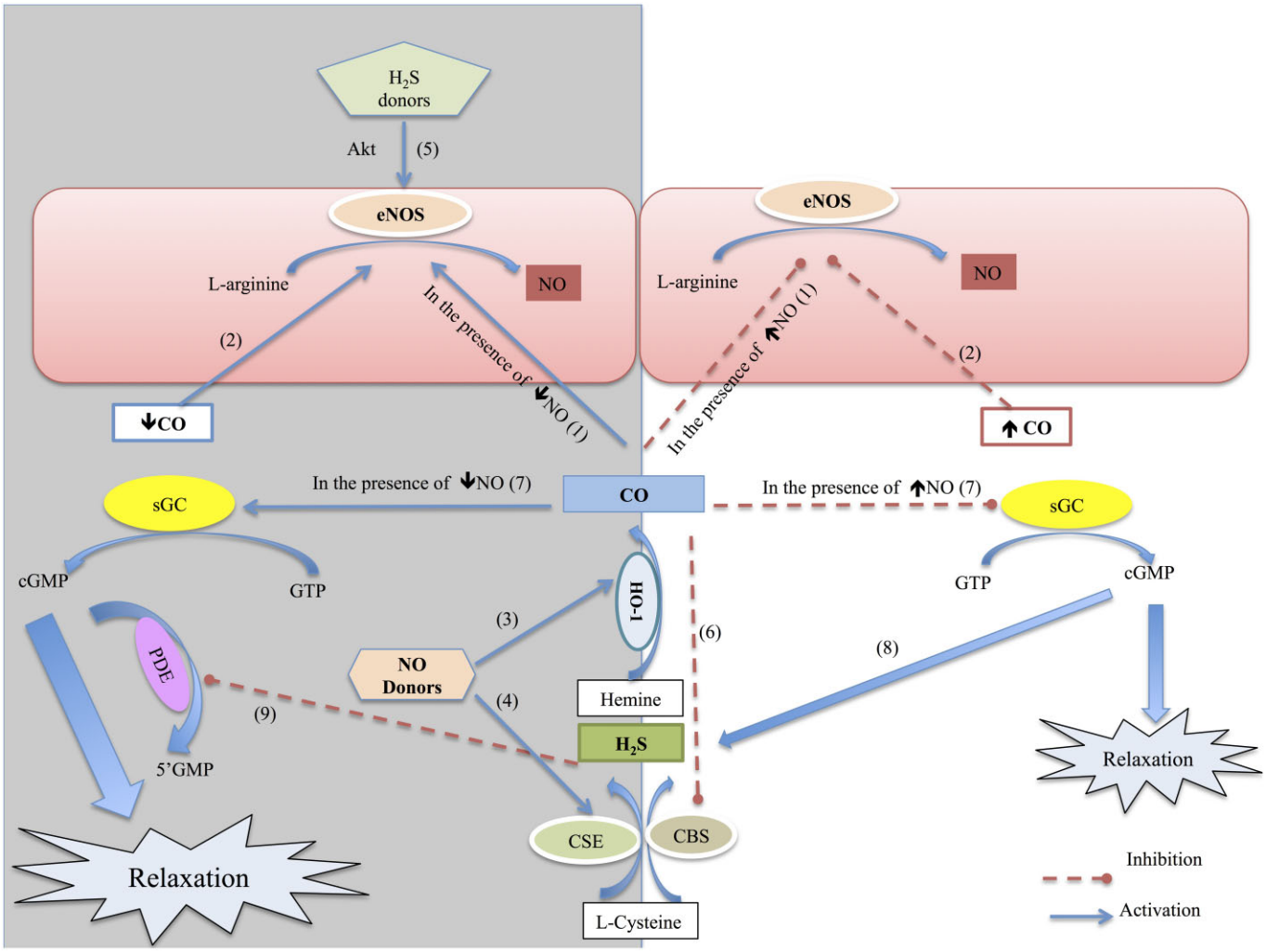

Figure 2

Crosstalk among NO/CO/H_2S/sGC pathways in vascular tissues including the penis. Bold fonts indicate the evidence obtainded in the penis. 1: CO inhibits eNOS in the presence of higher amounts of NO. However, CO activates eNOS when there is a low amount of NO (renal arteries; Botros and Navar, 2006). 2: High levels of CO inhibit NOS activity and NO generation, lower concentrations of CO induce release of NO (Thorup *et al.*, 1999). 3: NO donors activate HO-1 (Foresti and Motterlini, 1999). 4: NO donors up-regulate the expression and activity of CSE in vascular tissues and cultured aortic smooth muscle cells (Leffler *et al.*, 2005 and Zhao *et al.*, 2001). 5: H_2S cause eNOS activation in aorta through Akt. Coletta *et al.*, 2012, and directly increase the expression of eNOS in CC (Meng *et al.*, 2013). 6: CO inhibits CBS sensor (Taoka and Banerjee, 2001). 7: CO modulates NO-stimulated sGC activation dependent on NO concentration. In that, in the presence of low concentrations of NO, CO stimulates, otherwise CO inhibit sGC activation (Kajimura *et al.*, 2003). 8: cGMP causes an increase in H_2S production in vasculature (Bucci *et al.*, 2012). 9: H_2S acts as an endogenous inhibitor of PDE activity (Bucci *et al.*, 2010).

interaction to coordinate cerebral vasodilatation (Morikawa *et al.*, 2012). Whereas, it has been shown that H_2S up-regulates HO-1 expression in HUVEC (Pan *et al.*, 2011). However, the interactions between these gasotransmitters have not been studied in the penis yet.

NO–CO interaction

The NO–CO crosstalk seems dependent on the concentration of gasotransmitters; such that low concentrations of CO induce release of NO and, therefore, may mimic the vascular effects of NO. (Thorup *et al.*, 1999). In contrast, supra-physiological high levels of CO or HO-1 gene over-expression inhibit NOS activity and NO generation (Abdel Aziz *et al.*, 2009c). Supporting this, it has been found that elevated levels of endogenous CO contribute to arteriolar NO dysfunction in Dahl salt-sensitive rats (Johnson *et al.*, 2003). This CO-induced preconditioning conforms with a defence mechanism to inhibit iNOS-induced higher concentration of NO in pathological conditions.

In the same way, CO inhibits the NO-cGMP pathway under high NO concentrations, but compensates for NO to prevent excess vasoconstriction when insufficient NO is available (Botros and Navar, 2006). This study also suggests that the effect of CO on modulating sGC activity is also not static but dynamic. Supporting this low tissue availability of NO makes CO a stimulating modulator of sGC, while high tissue availability of NO causes the opposite (Kajimura *et al.*, 2003). Thus, it is believed that CO regulates NOS and sGC activity in a way that the HO/CO pathway is compensatory for NOS.

In contrast, NO donors cause HO-1 induction (Durante *et al.*, 1997; Foresti and Motterlini, 1999). Since CO inhibits NOS, under high concentrations of NO as in the case of exogenous NO administration, NO-induced HO-1 induction controls itself later by inhibiting NOS, representing a negative feedback mechanism. For further information on how the two systems are interrelated, readers are referred to the review by Foresti (Foresti and Motterlini, 1999).

H_2S–NO interaction

In 1997, a physiological role for H_2S in the vasculature and a link between NO and H_2S (Hosoki *et al.*, 1997) were suggested. Studies showing that H_2S enhances cGMP levels in isolated aortic rings, and inhibits both cGMP and cAMP breakdown in a cell-free system provide direct evidence that H_2S acts as an endogenous inhibitor of PDE activity (Bucci *et al.*, 2010). In line with this evidence, it has been demonstrated that exposure of endothelial cells to H_2S increases intracellular cGMP in a NO-dependent manner; H_2S activates PI3K/Akt and increases eNOS phosphorylation, demonstrating the requirement of NO in vascular H_2S signalling. NO and H_2S are mutually required for the physiological control of smooth muscle tone and function in the aorta (Coletta *et al.*, 2012). A contribution of NO/cGMP pathway in NaHS-induced human CC relaxation has also been addressed (d'Emmanuele di Villa Bianca *et al.*, 2009). NO donors up-regulate the expression and activity of CSE in vascular tissues and cultured aortic smooth muscle cells (Zhao *et al.*, 2001; Leffler *et al.*, 2005). Recently, it has been shown that H_2S promotes NO production in CC by enhancing he expression of eNOS (Meng *et al.*, 2013). However, NO–H_2S interactions have not been investigated in-depth in penile tissue.

Future directions

The evidence showing beneficial effects of CO-producing approaches in diabetes, hypertension or aging-induced ED as well as H_2S donors in aging-induced ED are encouraging the development of drugs that target H_2S or CO pathways and clinical studies. In addition, NO donors have been shown to increase both the H_2S level and HO-1 activity in vascular tissues, thus drugs acting on the NO pathway may also be further beneficial in ED treatment because of their pleiotropic effects on other gasotransmitters. As a consequence when the endothelium is disrupted, a compound that supplies NO and increases both HO/CO and the H_2S pathways could be beneficial in ED. Interestingly, PDE-5i have been shown to increase the activity of the HO/CO pathway in penile tissues (Abdel Aziz *et al.*, 2007a) and H_2S production in human bladder (Fusco *et al.*, 2012) as well as limiting myocardial infarction through H_2S signalling (Salloum *et al.*, 2009). Moreover, our preliminary study suggests that H_2S signalling may represent a new mechanism involved in the effect of sildenafil on erectile function (Dikmen *et al.*, 2013). Thus, a specific study needs to be performed to clarify the H_2S-related mechanisms of PDE-5 inhibitors in CC as well. Furthermore, an in-depth investigation into the close relationship among the testosterone, H_2S and cGMP pathways will help urologists to decide the best therapeutic approach to counteract or prevent ED. More importantly, the trafficking among these gasotransmitters and downstream molecules and their implication in erectile function/dysfunction represent a very complicated but intriguing issue.

Conclusion

Although the role of the NOS/NO pathway in erectile function and dysfunction is fundamental, the clinical studies targeting the NOS pathway in ED have not been successful to reach full erectile response recovery. Besides NO, the role of both CO and H_2S in erectile function has been well established in preclinical studies. The finding that CO can activate sGC in nNOS-deficient mice (Zakhary *et al.*, 1997), and can compensate for NO to relax the vessel, when the NO level is low, may be important from bench to bedside translation to find a compensatory alternative therapy for ED. On the contrary, since H_2S is mainly produced by smooth muscle in human CC, this pathway may complement NO signalling in ED especially in conditions associated with endothelial dysfunction. Moreover, since ROS is the main cause of ED and when eNOS is uncoupled it can be converted to ROS-producing enzyme, the antioxidant effects of H_2S and the HO/CO pathway, as well as haem-independent activation of sGC by CO or endothelium-independent erectile effects of H_2S, may have additional benefits in ED when NO-dependent cGMP formation is impaired because of either decreased synthesis/bioavailability, ROS-induced disruption of NO or the inability of haem-dependent activation of sGC in

vasculogenic ED. Thus, targeting the other sister gases, H_2S and CO, may represent new therapeutic potentials in ED.

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Conflict of interest

None.

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