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Interactions Between Gasotransmitters

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SUMMARY

It is well established that nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S) have signaling roles in the body. There are important similarities among them in their actions and generation, but there are also intriguing differences. The mechanism of action of H₂S still has not been fully elucidated. It is becoming increasingly clear that there are important interactions among the gasotransmitters. There is clear evidence of links between the NO- and CO-generating systems. So far, this is most apparent in the control of the cardiovascular system, and knowledge of the function of NO has led to new therapeutic interventions. There is also a suggestion of synergy between NO and H₂S that is not yet fully understood. Interactions between CO and H₂S have not yet been explored, and more research is required in this area. Interactions in the immune system also require more research, and increased understanding of this area could lead to novel therapies.

Key Words: Nitric oxide; carbon monoxide; hydrogen sulfide; gasotransmitters; interactions; signal transduction.

1. INTRODUCTION

Research in the field of gas signaling molecules has increased exponentially since Palmer et al. (1) published their seminal article. It is now well established that nitric oxide

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Table 1
Comparison of Metabolism and Function of Gasotransmitters^a

	<i>H₂S</i>	<i>CO</i>	<i>NO</i>
Main substrates	L-cysteine	Heme	L-arginine
Generating enzymes	CBS, CSE	Heme oxygenases	NO synthases
Inducer	NO	Free radicals	Acetylcholin, endotoxin
Scavenger	Hemoglobin	Hemoglobin	Hemoglobin
Inhibitor	D,L-Propargylglycerine	Zinc-PPIX	L-NAME
Protein targets	K _{ATP} channel cAMP (?)	cGMP, K _{Ca} channel	cGMP, K _{Ca} channel
Amino acid targets	?	Histidine	Cysteine
Half life in solution	Minutes	Minutes	Seconds
Production tissue source	SMC, not in EC	EC < SMC	EC > SMC

^a Only examples, not a complete list, are given. SMC, smooth muscle cell; EC, endothelial cell; zinc-PPIX, zinc protoporphyrin-IX; L-NAME, N^G-nitro-L-arginine methyl ester.

(Reproduced from ref. 2.)

(NO), carbon monoxide (CO), and hydrogen sulfide (H₂S) have signaling roles in the body. There are some similarities in their structure, properties, and actions. For example, because of their structural similarity to molecular oxygen (O₂), they all bind to heme groups in key protein molecules. It is not surprising, then, that they all relax smooth muscle and that there are interactions among them. Some of the similarities and differences among NO, CO, and H₂S are summarized in **Table 1** (2). It is important to state that this chapter covers interactions among NO, CO, and H₂S at physiological levels and that the remit does not include toxicological effects at higher concentrations.

2. COMPARISON OF CELLULAR EFFECTS OF NO, CO, AND H₂S

It is well established that NO is a neurotransmitter in the central and peripheral nervous systems, a smooth muscle relaxant, and an inflammatory mediator. The main signaling target for NO is the enzyme guanylate cyclase, which converts guanosine *S'*-triphosphate to cyclic guanosine *S'*-monophosphate (cGMP). High levels of intracellular cGMP are known to cause relaxation of smooth muscle. Because NO is also a free radical (*NO) and can react with oxygen to produce peroxynitrite (ONOO⁻), it also has an important role as an inflammatory mediator. Similarly to NO, CO has been described as a gaseous muscle relaxant and a neuronal messenger (3). Like NO, exogenously administered CO relaxes isolated blood vessels and inhibits platelet aggregation, presumably by increasing intracellular cGMP levels (4). Alternatively, CO may dilate blood vessels by interference with a cytochrome P450-based constrictor mechanism as described by Coceani (5). His team demonstrated CO-induced vascular relaxation that remained unchanged after treatment with methylene blue, a guanylate cyclase inhibitor, which suggests that guanylate cyclase did not have a role in the relaxation. He found the involvement of a cytochrome P450 hemoprotein, which limited the effect of the vasoconstrictor endothelin (5).

The CO produced in the body originates mainly from the breakdown of hemoglobin (6). This degradation is catalyzed by the group of enzymes known as heme oxygenases (HOs), which oxidize the α -methine bridge of the heme porphyrin structure and thereby yield CO and biliverdin. HOs resemble mixed function oxidases as they require the

reducing cofactor NADPH and oxygen for the oxidation of their substrate (7). Two isoforms of HO have been found, an inducible form (HO-1) and a constitutive form (HO-2). HO-1 is induced by oxidative stress and is abundant in spleen and liver tissue, where it decomposes heme-containing proteins. HO-2 activity is mainly found in the brain and testes (3,4). All HOs are inhibited by zinc- and tin-containing porphyrin analogs (3,4,7).

The mechanisms of action of H₂S have not yet been fully elucidated. There is evidence that some of the effects of H₂S result from an increase in intracellular cyclic adenosine monophosphate (cAMP) and activation of the protein kinase A pathway (8). Increased levels of cAMP are known to relax smooth muscle. H₂S has been shown to enhance *N*-methyl-D-aspartate receptor-mediated responses in neurons and neuronal cell lines (9), which appears to be a specific action of H₂S compared to NO and CO. Some published studies have used *S*-nitroso-L-cysteine (10,11) or other sulfur-containing agents as NO donors; however, it is not clear whether these can also act as H₂S donors and perhaps cause relaxation of smooth muscle via this mechanism.

Large-conductance K_{Ca} channels are a common target for NO and CO; however, the exact mechanism of interaction at the molecular level is not known. These gasotransmitters excite K_{Ca} channels leading to opening, which increases K⁺ conductance, causing hyperpolarization in smooth muscle cells and thus relaxation. New evidence has suggested that the interaction between NO and CO with K_{Ca} channels may be different. The effects of CO on K_{Ca} channels have been shown to be mediated via interactions with histidine residues and the α -subunit of the channel protein (12). By contrast, NO modifies sulfhydryl groups and interacts with the β -subunit (12).

CO is much more stable than NO, and, therefore, its effects may be longer lasting and it could act at a distance from its site of production. The metabolism of sulfur-containing compounds in cells is highly complex and H₂S is probably rapidly metabolized after being produced. Alternatively, various groups, such as heme groups, bind H₂S, so its effects may be truncated by being taken up.

One difference between NO and the other gas signaling molecules is that its redox state varies and this changes its biological effects (13). Different NO donors release different redox state forms of NO in biological systems; thus, (+)*S*-nitroso-*N*-acetylpenicillamine releases the free radical [•]NO, 3-morpholino-sydnonime forms NO and superoxide, and sodium nitroprusside generates the nitrosonium ion NO⁺ (4). A further complication is that exogenously applied NO can be converted from one redox form to another depending on the local conditions (13). Similarly, it has been found that inducible nitric oxide synthase (iNOS) generates different redox forms of NO depending on the intracellular conditions (14). This makes it difficult to determine exactly which form of NO is responsible for which actions, and perhaps this is not always fully taken into account.

3. INTERACTIONS BETWEEN THE CO AND NO SYSTEMS

Because there are some similarities between the production and effects of NO and CO, interactions between the two systems might be expected. In the vasculature, exogenously administered CO, like NO, relaxes blood vessels by increasing intracellular cGMP levels in vascular smooth muscle cells (VSMCs). Unlike HO-2, HO-1 is found in VSMCs, and NO selectively induces HO-1 gene expression and CO release in these cells (4). Recently, it has been shown that NO triggers the release of free heme from heme proteins, and unbound heme is known to induce HO-1 expression. Unfortunately, the exact mechanism of this upregulation is not entirely clear. The ability of NO to induce CO production in

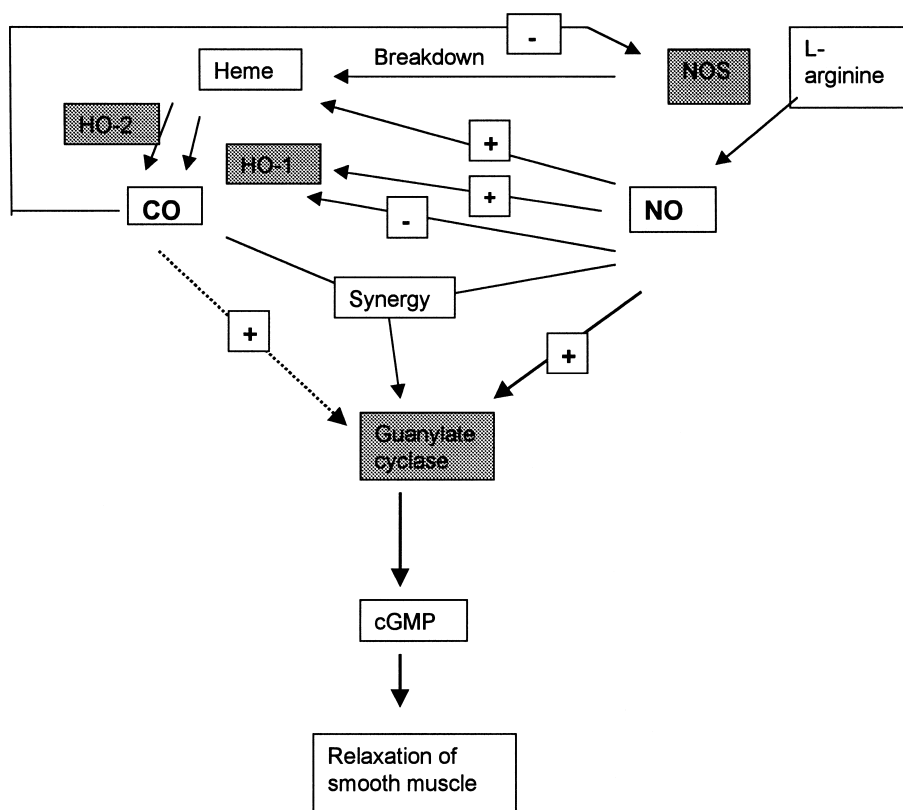


Fig. 1. Summary of interactions between NO and CO systems.

VSMCs may provide another mechanism by which NO activates guanylate cyclase and regulates vascular tone. Interestingly, CO directly inhibits iNOS activity by binding to the heme moiety of the enzyme. Thus, CO might act as a cytoprotector by limiting excessive NO synthesis, such as because of oxidative stress (4).

Ingi et al. (3) showed that CO produced via HO-2 stimulates guanylate cyclase, such as in olfactory neurons, which do not show NOS activity. The findings of this study on a possible CO–NO interaction in cerebellar cells are controversial. HO-2 activity seems to peak in immature cerebellar cells, whereas NO output increases as these cells mature. CO obviously does not affect the NO–cGMP system in immature cells; however, Infi et al. (3) report that HO activity suppresses cGMP levels in later culture development, when NO is present. Furthermore, they found that CO inhibits purified NOS in vitro. They conclude that rather than CO interfering with the NO–cGMP system at the stage of NO synthesis, it interferes at the level of NO activation of guanylate cyclase. Here, CO may function as a partial agonist or inhibitory modulator of the enzyme, whereas NO acts as a full agonist of guanylate cyclase. Presumably, CO may bind to the enzyme and thereby induce conformational changes that may affect NO-mediated activation (3). Interactions between the CO and NO systems are illustrated in **Fig. 1**.

3.1. Interactions of the CO- and NO-Generating Systems

There is increasing evidence of a link between the regulation of HO activity and NO production, but the purpose of this link is not fully clear. NO is a highly reactive free radical

as well as a signaling molecule, and, therefore, its production must be controlled in cells. It seems that the HO system is one way in which this control is exerted (15). NOS is a heme-containing protein, and binding of CO to the heme groups could inactivate the enzyme. The neuronal isoform of NOS, nNOS, has been shown to bind CO (16). The heme groups in NOS could also act as a substrate for HO, thus decreasing the production of NO (15).

NO has been shown to inhibit as well as stimulate HO enzyme. Incubation with the NO donors L-arginine and sodium nitroprusside has been found to both reduce HO activity (17) and to increase HO activity (18). Maines (15) has proposed an explanation for this apparent paradox: NO as a free radical could inactivate HO by attacking cysteine residues in the protein, but it can also induce HO-1 expression, and by displacing O₂ from heme groups, NO could inhibit HO activity. In addition, iron is known to be involved in gene expression and iron metabolism can be influenced by NO (15).

NO donors were found to selectively increase mRNA and protein expression for HO-1 in rat VSMCs in culture and to increase the production of CO, although a nonspecific bioassay for CO was used (4). The mechanism by which NO induces the expression of HO-1 is not yet clear, although it does not seem to involve the cGMP signaling pathway (4). It may involve the liberation of free heme, which is known to induce HO-1 expression (4,15).

Hemin, a CO donor, has been found to potentiate L-arginine-stimulated insulin secretion from mice islet cells, suggesting a link between the CO and NO systems (19). It has also been demonstrated that HO-1 expression and activity can be increased by both hemin and sodium nitroprusside in a rat skeletal muscle cell line (20); however, the mechanism involved has not yet been fully elucidated.

In a study using cerebellar granule cell cultures, Ingi et al. (3) found that exogenously applied CO blocked an increase in cGMP mediated by NO. They also showed that inhibitors of endogenous CO production potentiated the increase in cGMP mediated by NO. By comparison, an inhibitor of endogenous NO production, *N*^ω-nitro-L-arginine, significantly inhibited dilation caused by CO in porcine pial arterioles in vivo, and the addition of an NO donor, sodium nitroprusside, restored vasodilation to CO (21). This suggests that NO is essential for the vasodilatory effect of CO. Maines (15) has previously summarized interactions between NO and CO (**Fig. 2**).

4. INTERACTIONS BETWEEN H₂S AND NO

There is some published evidence to suggest that there is some synergy between H₂S and NO in the relaxation of vascular smooth muscle. Indeed, the relaxant effect of H₂S in vascular smooth muscle may be at least partially dependent on NO. In some elegant experiments, Zhao et al. (22) showed that *N*^ω-Nitro-L-arginine methyl ester (L-NAME), an inhibitor of endogenous NO production, significantly shifted the H₂S dose-response relaxation curve to the right, decreasing the potency of H₂S, in rat aortic rings (**Fig. 3**). Similar effects were obtained by removing the endothelium from the aortic rings. These findings suggest that H₂S stimulates the endogenous production of NO and this is at least partially responsible for the vasorelaxant effect of H₂S. These investigators also showed that the addition of a specific inhibitor of soluble guanylate cyclase (sGC), 1 H-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one, significantly increased the relaxant effect of H₂S, showing that it could not be working directly via stimulating guanylate cyclase. However, Teague et al. (23) found that L-NAME had no significant effect on relaxation of guinea pig ileum by H₂S. Interestingly, they also found that a combination of NaHS (an H₂S donor) and sodium nitroprusside (an NO donor) produced a significantly greater

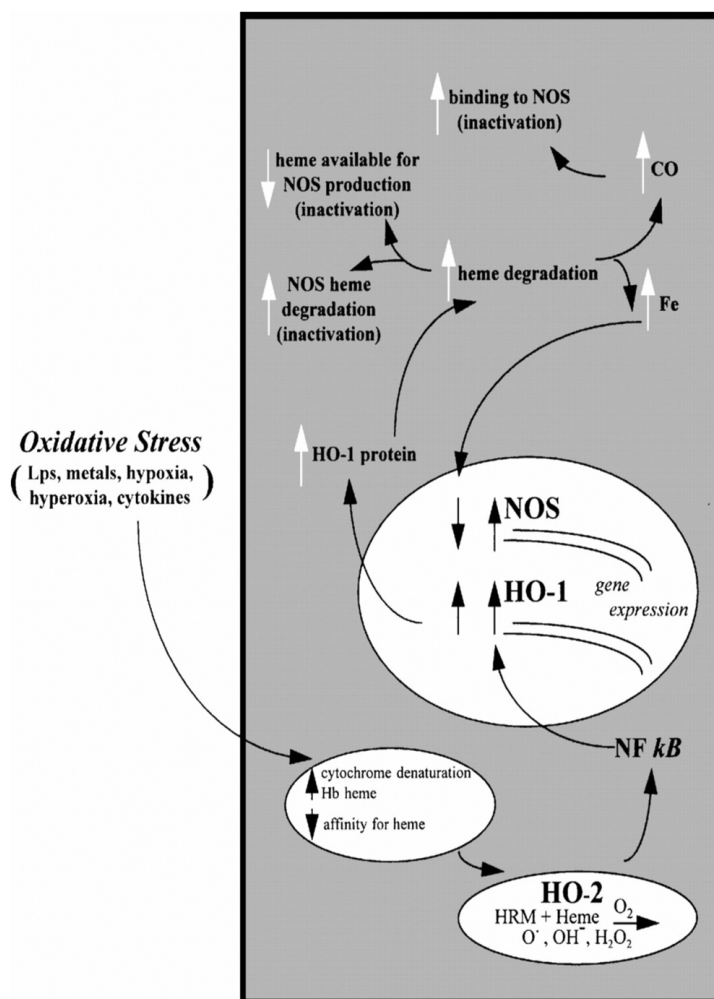


Fig. 2. Schematic presentation of regulatory interactions between HO and NOS systems proposed by Maines (Reprinted with permission from the *Annual Review of Pharmacology and Toxicology*, Volume 37 ©1997 by Annual Reviews www.annualreviews.org.)

inhibition of twitch response of guinea pig ileum than either agent alone, suggesting some synergy between the two agents. In our laboratory, we have shown that H_2S relaxes pregnant rat uterus in vitro (24). However, methylene blue (an inhibitor of guanylate cyclase) did not significantly affect this tocolytic action (unpublished data), suggesting that H_2S does not cause relaxation by the activation of guanylate cyclase and increased production of cGMP in this tissue. The tocolytic action of H_2S was also not inhibited by glibenclamide (a K_{ATP} channel blocker) or tetraethylammonium (a nonspecific K^+ channel blocker) (unpublished data).

It is becoming increasingly clear that H_2S exerts its effects via different mechanisms in different tissues. In vascular smooth muscle, there seems to be involvement of NO production, because of the neighboring endothelium. However, in other smooth muscle NO production does not seem to be involved, yet there is still synergy between H_2S and NO. Hosoki et al. (25) were the first to suggest synergy between H_2S and NO in relaxing

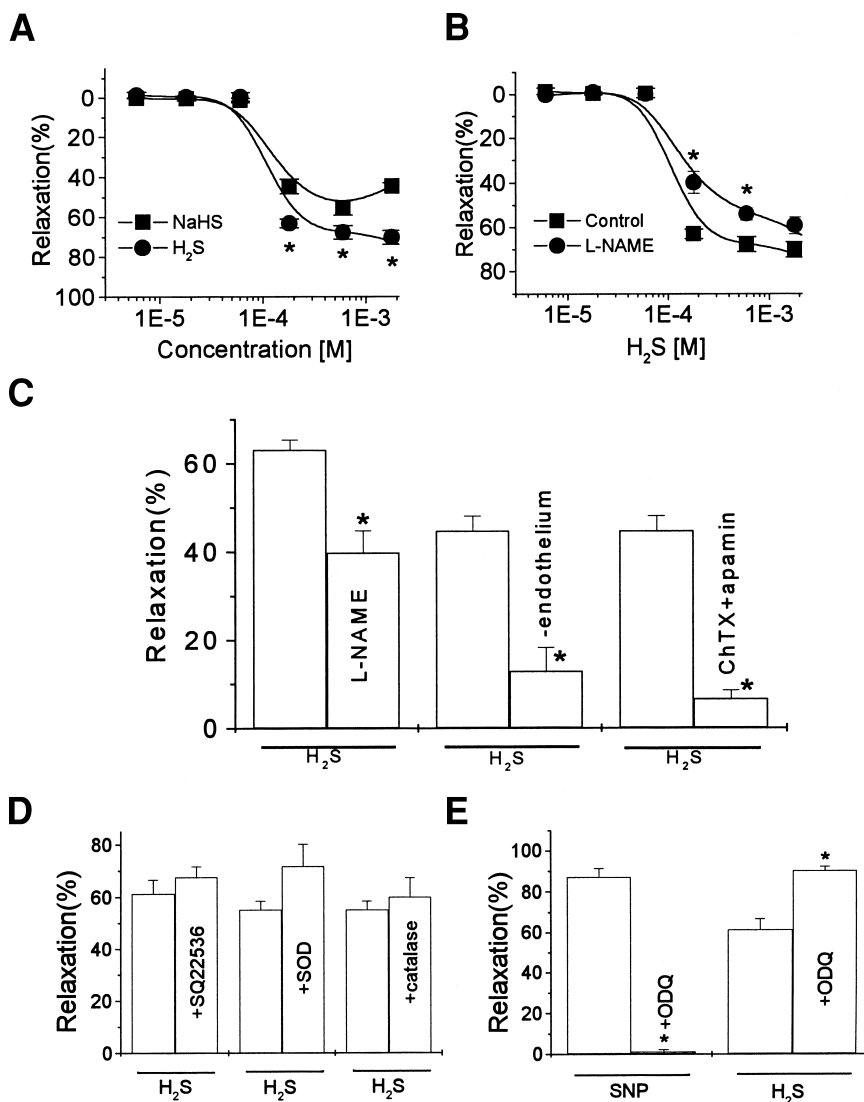


Fig. 3. Dose-response curves to H₂S and NaHS and underlying mechanisms. **(A)** Relaxation of phenylephrine-precontracted tissues by H₂S in form of either standard NaHS solution (■) or H₂S gas-saturated solution (●); **(B)** inhibitory effect of L-NAME (100 μM, 20 min) (●) on H₂S-induced relaxation (control) (■); **(C)** effects of H₂S (180 μM) on endothelium-free or endothelium-intact aortic tissues pretreated with L-NAME or charybdotoxin (ChTX)/apamin; **(D)** the relaxant effect of H₂S was not affected by pretreating the tissues with SQ22536, SOD, or catalase, respectively; **(E)** effect of 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) treatment (10 μM for 10 min) on relaxant effects of SNP (0.1 μM) or H₂S (600 μM). **p* < 0.05 compared to control. (Reproduced from ref. 22.)

vascular smooth muscle. They demonstrated a leftward shift in the dose-response curve for relaxation of rat thoracic aorta by NaHS in the presence of two different NO donors, sodium nitroprusside and morpholinosydnonimine (**Fig. 4**). They reported that a low concentration of H₂S enhanced the smooth muscle relaxant effect of NO by up to 13-fold. However, Zhao and Wang (26) found that low doses of NaHS shifted the dose-response

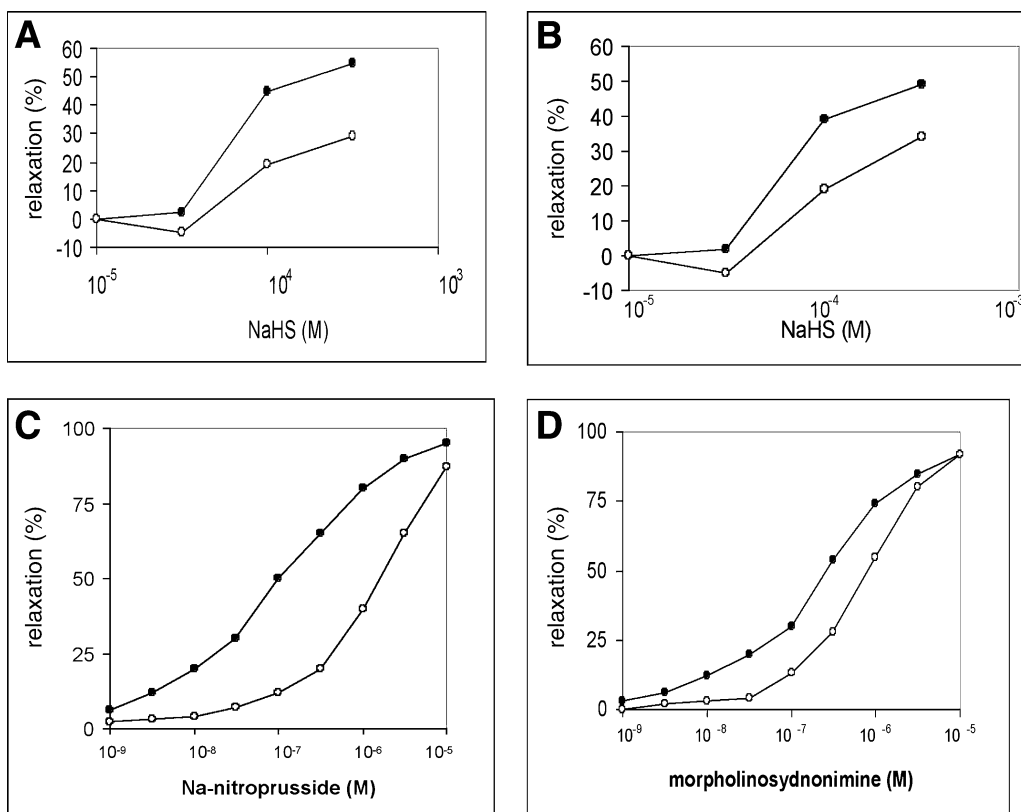


Fig. 4. Dose–response curves for relaxation of rat thoracic aorta helical strips in vitro because of NaHS and NO donors. (A,B) Potentiation of relaxant effects of NaHS by NO donors: (A) 10 nM sodium nitroprusside and (B) 30 nM morpholinosydnonimine; (○) control, (●) with NO donor. (C,D) Potentiation of relaxant effects of sodium nitroprusside (C) and morpholinosydnonimine (D) by NaHS, (○) control, (●) with 30 μ M NaHS. (Reprinted from *Biochem. Biophys. Res. Commun.*, 237, Hosoki et al., The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide, 527–531, copyright 1997, with permission from Elsevier.)

relaxation curve for sodium nitroprusside to the right in rat aortic rings, suggesting that H_2S inhibited the vasorelaxant effect of NO (Fig. 5). The contradiction of these results is not easy to explain. Different preparations, helical strips of Wistar rat aorta were used vs Sprague-Dawley rat aortic rings and different methods of precontraction were used, 1 μ M norepinephrine vs 0.3 μ M phenylephrine. If H_2S does decrease the production of cGMP, then this could explain how it decreases the response to NO, which works via this pathway. By contrast, if H_2S causes relaxation via a totally independent pathway to NO, then it could augment the relaxant effect of NO in an additive manner. It is not yet clear which mechanism is correct.

Li et al. (27) demonstrated that L-cysteine, an H_2S donor, inhibited NO-induced relaxation of rabbit aortic rings. L-Cysteine inhibited an increase in cGMP induced by NO, and superoxide dismutase (SOD) decreased the inhibitory effect of L-cysteine. These investigators concluded that the inhibitory effect of L-cysteine on NO was partly because of superoxide generation by the autooxidation of L-cysteine and partly via a direct interaction of SH groups with NO (27).

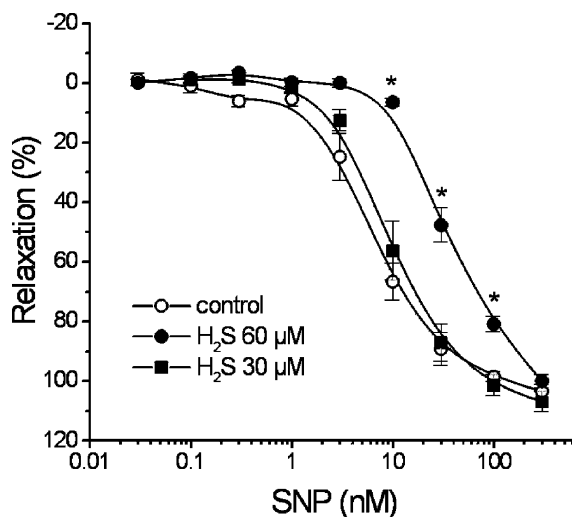


Fig. 5. Dose-response of precontracted rat aortic tissues to sodium nitroprusside. The tissues were pretreated with either 30 or 60 μM H_2S . * $p < 0.05$ vs control. (Reproduced from ref. 26.)

It has been found that the endogenous production of H_2S by homogenized rat vascular tissue was increased by sodium nitroprusside in a dose-dependent manner (22), suggesting a direct stimulatory effect of NO on the enzymes that produce H_2S , cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE). The mechanism of action could be downstream of cGMP, involving the stimulation of cGMP-dependent kinases, which could phosphorylate and activate the enzymes or could involve a direct effect on the enzyme protein, perhaps via nitrosylation. It was also shown that incubating rat VSMCs in culture with a dose range of sodium nitroprusside for 6 h significantly increased mRNA levels for CSE (22); however, the mechanism of action of NO here is not yet clear, but it could involve nuclear factor- κB (NF- κB).

H_2S is produced endogenously from L-cysteine, but there is some debate as to whether L-cysteine is transported into cells as it is or as L-cystine, which consists of two molecules of L-cysteine joined by a disulfide bond (24,28). Zerangue and Kavanaugh (29) have reported that L-cysteine was transported into cells by the neuronal EAAT3 excitatory amino acid transporter, which is known to be expressed in tissues other than neurons. Li et al. (30) showed that a NO donor increased cystine uptake into bovine vascular endothelial cells in a dose-dependent manner. This increase was found to require both RNA and protein synthesis and appears to be because of the induction of expression of a cystine transport system (30). In theory, then, NO could increase the production of H_2S cells by increasing the availability of substrate.

Further interactions among NO, CO, and H_2S may become apparent because it has been reported that the activity of human CBS may be regulated by heme-mediated redox-linked mechanisms (31).

Care must be taken in interpreting and comparing the results of different studies because there are differences in preparations and methods, such as the use of precontracted or spontaneously contracting smooth muscle preparations.

Wang (2) has previously summarized interactions between NO and H_2S (Fig. 6). Known interactions between the NO and H_2S systems are illustrated in Fig. 7.

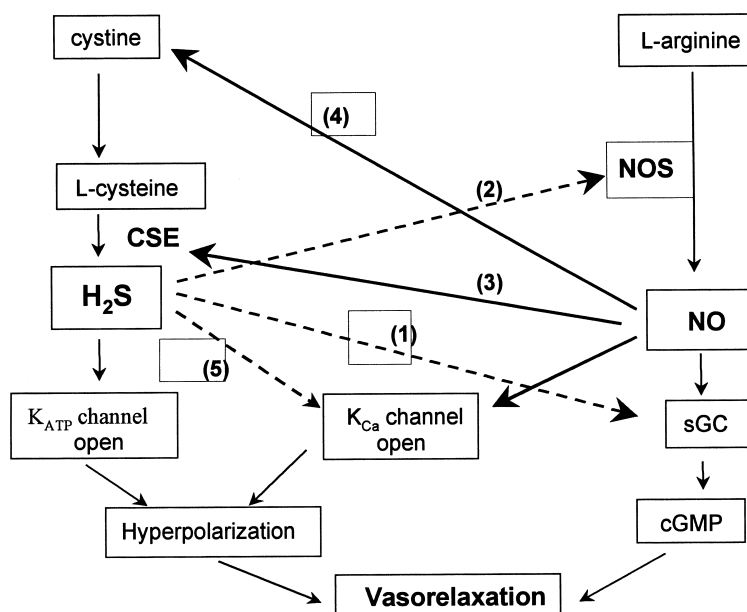


Fig. 6. Hypothesized scheme of interaction of H_2S and NO in vascular tissues as proposed by Wang (2). The solid lines indicate the stimulatory inputs and the dashed lines, inhibitory inputs. (1) H_2S may decrease the sensitivity of the cGMP pathway to NO. (2) H_2S may reduce the expression level of NOS. (3) NO may increase the expression of CSE. (4) NO may increase the cellular uptake of cystine. (5) H_2S may modify K_{Ca} channels to decrease their sensitivity to NO. (Reproduced from ref. 2.)

At the time of this writing, there are no published reports of interactions between the CO and H_2S systems; however, further research is required because interactions are likely.

5. INVOLVEMENT OF FREE RADICALS

The role of NO as a free radical itself and a generator of other free radicals has been well documented. A role for CO as protection against free radicals, in contrast to NO, is becoming increasingly apparent. Increased production of CO via HO is thought to be involved in the protection of tissue against oxidative stress (32). Indeed, HO may be an endogenous protection mechanism against free radicals in acute inflammation (33). Upregulation of HO-1 and consequent overproduction of intracellular bilirubin are associated with protection against ONOO⁻-mediated apoptosis (34), suppression of oxidant-induced microvascular leukocyte adhesion (35), and amelioration of postischemic myocardial function (36). After treatment with hemin, there is resultant HO-1 expression and bilirubin production, and it has been discovered that cells display high resistance to oxidant damage only when actively producing the bile pigment, strongly implicating the HO-1 pathway in cytoprotection against oxidative stress (37,38). Cell injury caused by oxidative stress appears to contribute extensively to the pathogenesis of vascular disease, and HO-1 is widely considered to be valuable in the restoration of vascular function under conditions of increased generation of reactive oxygen species (ROS). It has been recently hypothesized that the HO-1 system may act in a similar fashion to counteract the excessive production of NO and reactive nitrogen species (RNS) (39). The ability of HO-1 to

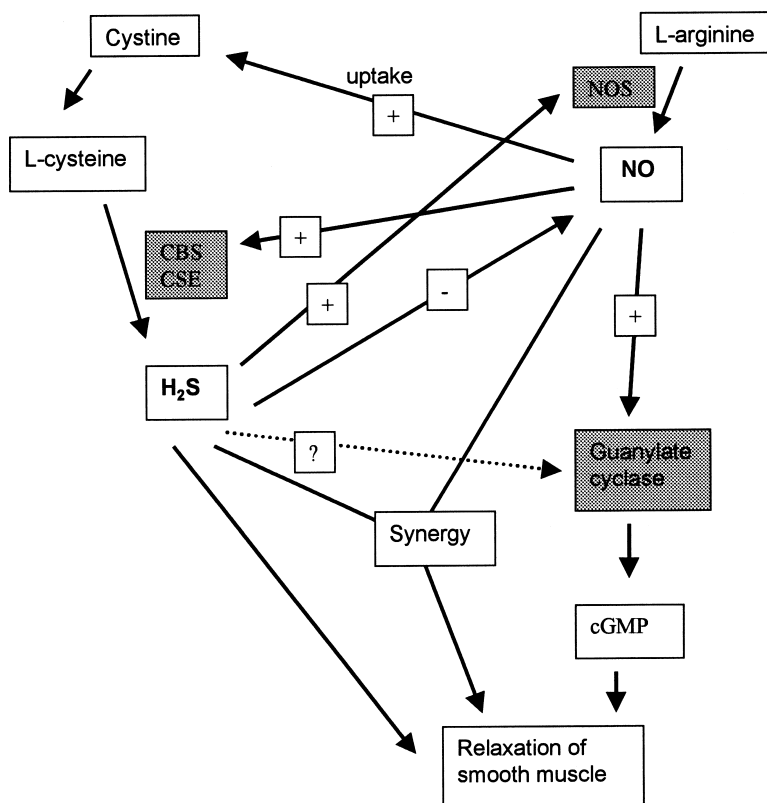


Fig. 7. Summary of interactions between NO and H₂S systems.

be highly induced in eukaryotes in response to NO and NO-related species makes this stress protein a likely contender to participate in NO detoxification (40).

It is now known that the antioxidant protein HO can somehow detect NO and act successfully as a key player in cytoprotection against insults from ROS and RNS. For example, NO and NO-related species induce HO-1 expression and increase HO activity in aortic vascular cells (4,18,39,41,42). Furthermore, cells pretreated with various NO-releasing agents acquire increased resistance to H₂O₂-mediated cytotoxicity at the time HO is maximally activated (18). In addition, bilirubin, one of the end products of heme degradation by HO, has been shown to protect against the cytotoxic effects caused by the strong oxidants H₂O₂ and ONOO⁻ (18,34,37). Given that further investigations have revealed that NO-mediated activation of the HO-1 pathway is a stress response that can be extended to various mammalian systems (39), several important issues on the possible signal transduction mechanism(s) that leads to HO-1 induction by NO and RNS remain to be carefully examined (40).

The physiological significance and the mode of regulation of the HO-1 system by NO have not yet been fully elucidated (39,40). In endothelial and smooth muscle cells, isolated aortic tissue, and cardiac myocytes, certain NO-releasing agents have been shown to induce HO-1 and augment HO activity (18,34,38,42), suggesting that the effect mediated by the NO group is independent of its redox state. It has been concluded that reaction of NO with O₂^{•-} and the extent of the conversion of NO to NO⁺ or NO⁻ by intracellular components could be critical to determine the modulation of HO-1 gene expression (40).

Although H_2S is not a free radical, like $\cdot\text{NO}$, in aqueous solution it is in fact a reducing agent and should be protective against oxygen free radicals. However, it has been shown that under certain conditions, in the presence of peroxidase and hydrogen peroxide, H_2S produced thiyl free radicals, $\text{SH}\cdot$ and $\text{S}\cdot$ (43). Perhaps H_2S could be produced locally in relatively high concentrations to have a cytotoxic role, as is the case for NO. Because H_2S is cytotoxic, we propose that it could also be an inflammatory mediator and be involved in host defense, although there is currently no published evidence to support this. The fact that H_2S can affect xenobiotic metabolic enzymes (44) in the liver and affect the generation of ROS supports the notion that the immune system could be modulated. This may be particularly true in times of stress when there is an increase in the production of ROS (45,46).

6. GAS SIGNALING MOLECULES IN THE CARDIOVASCULAR SYSTEM

6.1. Action of H_2S Within the Cardiovascular System

To date, the cardiovascular effects of both endogenous and exogenous H_2S have not been elucidated fully. The opinion previously has been that H_2S interfered with the cardiovascular function as a result of secondary anoxia rather than a direct action on cardiac myocytes or VSMCs (47). However, more recent evidence has revealed that H_2S may have more of an endogenous physiological role to play within the cardiovascular system.

CBS, one of the enzymes responsible for generating H_2S , has been shown to have no activity or expression in human cardiovascular-related tissues (48,49). On the other hand, CSE expression and endogenous production of H_2S have been shown in rat portal vein and thoracic aorta (25). The expression of H_2S -generating enzyme has been identified in VSMCs, but not in the endothelium (22).

Recently, the physiological function of H_2S in the cardiovascular system has been studied. When H_2S is injected intravenously, a transient decrease in blood pressure is observed in rats, which is antagonized by glibenclamide (a K_{ATP} channel blocker). This concurs with preliminary results from our laboratory showing that H_2S causes a dose-dependent decrease in left ventricular developed pressure (LVDP) and heart rate while increasing coronary flow rate in an isolated rat Langendorff rat heart model (unpublished data). Preliminary results would also indicate a role for K_{ATP} channel, because glibenclamide blocks both H_2S -induced changes in LVDP and heart rate.

6.2. Interactions Between CO and NO in the Cardiovascular System

6.2.1. CO AND THE CARDIOVASCULAR SYSTEM

First, we discuss briefly the actions of CO and NO on the cardiovascular system, and then the interaction between the two gas signaling molecules.

HO is the rate-limiting step in heme degradation; it catalyzes the oxidation of the α -meso carbon of the protoporphyrin ring leading to the formation of CO, free iron, and biliverdin (50). Three isoforms of HO are known: HO-1, HO-2, and HO-3; for the purpose of this section, we concentrate on HO-1 (also termed hsp32), a stress-inducible enzyme. HO-1 is induced in response to oxidant stress, has been shown itself to be cytoprotective, and plays an important role in the regulation of cardiovascular function (51).

The dangers of CO within the cardiovascular system have been well defined—at high concentrations, CO is unquestionably lethal. However, recent studies (for a review, see

ref. 52) consistently support the emerging idea that CO at low concentrations exerts distinctly different effects on physiological and cellular functions, with this revelation motivating the need to re-evaluate its role.

CO, which is the byproduct of HO activity, has recently been attributed to being an important modulator of many physiological processes. It particularly plays a role in the homeostatic control of cardiovascular function (for reviews, *see* refs. 39 and 53). The possible beneficial effects of HO-1 induction in stress are mainly attributed to the vaso-active CO. When the heart is stressed, there is an impressive increase in HO-1 mRNA expression in the heart (for a review, *see* ref. 15). In the stressed heart, HO-1 protein is expressed in particularly high levels in the atrioventricular node and in the myocytes (15). Experimental evidence suggests that in the blood vessels and the heart, CO, generated by HO activity, is a regulator of cGMP production (this is discussed in greater detail later in this chapter). It is highly relevant to the pathophysiology of the cardiovascular system and is integral to the heart's response to oxidative stress (15).

Endogenously released CO is known to cause vasodilation and have antiproliferative actions. CO also has indirect actions producing vasoconstrictors and vascular growth factors, such as ET-1 and platelet-derived growth factors, which may be involved in combating chronic hypoxic stress (54). Motterlini et al. (55) have recently shown that an increased CO production by HO-1 in vascular tissue contributes to the suppression of acute hypertensive responses under stress conditions *in vivo*. Sammut et al. (42) have also reported that HO-1-derived CO significantly suppresses phenylephrine-mediated contraction of isolated aortic rings.

It has been demonstrated in an *in vivo* vascular injury model of xenotransplantation that CO not only can confer protection as effectively as HO-1 but can also confer cytoprotection in the absence of HO-1 (56,57).

Reports have also demonstrated that the HO-1/CO pathway is markedly upregulated by hypoxia in VSMC, cardiomyocytes, and heart tissue (54,58,59). It has been suggested that aortic vasoconstriction following chronic hypoxia in rats involves the induction of endothelial HO-1 and the enhanced production of CO (60).

Recent observations suggest that CO may impart potent antiinflammatory and antiapoptotic effects via the mitogen kinase pathway in macrophages and endothelial cells, respectively (52,61). It has been shown that the cytoprotection via CO requires the activation of NF- κ B transcription factor and is dependent on p38 kinase activity (61). It is well documented that activation of p38 kinase and other mitogen kinase pathways within the cardiovascular system can transduce signals to provide downstream cytoprotection against cellular stresses such as myocardial ischemia reperfusion injury. Therefore, future studies are required to unify the possible importance of myocardial CO production during myocardial ischemia and its influence on the activity of several cytoprotective transcription factors and kinases.

In summary, HO-1 has been shown to have antiinflammatory, antiapoptotic, and antiproliferative effects, and it is now known to have salutary effects in diseases as diverse as atherosclerosis and sepsis. The mechanism by which HO-1 confers its protective effect is still poorly understood, although recently direct links have been postulated concerning stress-induced production of CO (62).

6.2.2. NO AND THE CARDIOVASCULAR SYSTEM

NO plays an important role in the homeostatic regulation of the cardiovascular system (40,63). NO is produced by vascular endothelium and smooth muscle, cardiac muscle,

and many other cell types (64,65). NO serves many important functions in the cardiovascular system, including vasodilation, inhibition of vasoconstrictor influences (e.g., inhibits angiotensin II and sympathetic vasoconstriction), inhibition of leukocyte adhesion to vascular endothelium (anti-inflammatory), antiproliferative (e.g., inhibits smooth muscle hyperplasia following vascular injury), as well as inhibition of platelet adhesion to the vascular endothelium (antithrombotic) and scavenging superoxide anion (anti-inflammatory) (66,67).

The mechanism of many of these actions of NO involves the formation of cGMP. The antiplatelet aggregatory effects of NO are also related to the increase in cGMP. When NO production is impaired, as occurs when the vascular endothelium becomes damaged or dysfunctional, the following can result: vasoconstriction (e.g., coronary vasospasm, elevated systemic vascular resistance, hypertension); platelet aggregation and adhesion leading to thrombosis, vascular stenosis, or restenosis, as occurs following balloon angioplasty and stent placement; increased inflammation and tissue damage mediated by ROS such as superoxide anion and hydroxyl radical.

There is considerable evidence that cardiovascular-related diseases/conditions such as hypertension, dyslipidemia, diabetes, heart failure, atherosclerosis, cigarette smoking, aging, and vascular injury are associated with endothelial dysfunction and reduced NO production and/or bioavailability.

NO is necessary for normal cardiac physiology, but it is potentially toxic in excess concentrations. The role that NO plays in apoptosis is not known because NO has been shown to exert both proapoptotic and antiapoptotic effects in the myocardium (68,69). NO also spontaneously interacts with molecular oxygen and reactive oxygen metabolites to yield potentially injurious oxidizing and nitrosating agents (70), as discussed elsewhere.

6.3. COEXPRESSION OF HOs AND NOSs IN THE CARDIOVASCULAR SYSTEM

HO and NOS are the enzymes responsible for generating CO and NO, respectively. They have intriguing similarities in their isoforms, requirements for activity, and regulation (71). For example, both the CO- and NO-generating systems have constitutive (HO-2, HO-3, endothelial NOS [eNOS], and NOS) and inducible (HO-1 and iNOS) isoforms. Production of CO and NO arises from different substrates (heme for HO and L-arginine for NOS); however, both enzymes require molecular oxygen and the reducing agent NADPH for activity. The differences are that NO synthesis requires additional cofactors (tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide) and that the constitutive isoforms of NOS are calcium/calmodulin dependent (63).

Zakhary et al. (72) have reported marked similarities in the localization of HO and NOS in endothelial cells and adventitial nerves of blood vessels, suggesting a possible coordinated function for CO and NO. Indeed, *in vitro* studies show that under certain pathophysiological conditions, such as hypoxia, downregulation of constitutive eNOS is concurrent with transient increases in inducible HO-1 protein, indicating a potential compensatory regulation between the two systems (54).

It is well established that NO donors can activate HO-1 gene expression and activity in various tissues (40,71), although extensive studies of the cardiovascular system have not concurred this finding.

This section concentrates on literature concerning coexpression of HOs and NOSs solely within the cardiovascular system. It appears that HO and NOS require molecular oxygen for activation, although modulation of these enzymes by hypoxia remains unclear.

Experiments conducted using the isolated heart model confirmed the vasoactive properties of CO-releasing molecules within the cardiovascular system. The metal carbonyl

markedly attenuated an L-NAME-mediated (NO inhibitor) increase in coronary perfusion pressure. Hearts expressing high HO-1 in the vasculature following treatment of animals with hemin also displayed reduced contractility when challenged with L-NAME, and inhibition of HO activity abolished the effect; this confirms the important role of endogenously produced CO in vascular control (42,55). Thus, augmented HO-1-derived CO can profoundly modulate cardiac vessel functions, and this effect can be mimicked by exogenously applied CO-releasing molecules. Motterlini et al. (55) found that induction of the HO-1 system by hemin pretreatment considerably suppressed the increase in mean arterial pressure elicited by intravenous administration of L-NAME, a finding consistent with a previous report by this group; as observed for the isolated aortic ring and heart preparations, SnPPiX (HO inhibitor) restored the vasoconstrictor responses to L-NAME.

In the carotid body, basal levels of CO and NO act together to suppress sensory discharge. However, during acute hypoxia decreased synthesis of CO and NO has been implicated in contributing to the augmentation of sensory discharge (73). Other studies have shown that, in contrast to the apparent decrease in synthesis of CO and NO in the carotid body, hypoxic conditions induce the gene expression of both HO-1 and iNOS, although the mechanisms involved remain unclear (59,74). This apparent discrepancy is most likely attributable to the difference in regulation between constitutive and inducible isoforms. Interestingly, CO and NO themselves have been shown to suppress the hypoxic induction of vascular endothelial growth factor (75) and to inhibit hypoxia-inducible factor-1 (HIF-1) DNA-binding activity by abrogating hypoxia-induced accumulation of HIF-1 α protein (76).

Maulik et al. (77) demonstrated that in isolated working rat hearts made ischemic for 30 min followed by 30 min of reperfusion NO activates HO, which further stimulates the production of cGMP presumably by CO signaling. This study revealed that NO not only potentiates cGMP-mediated intracellular signaling but it also functions as a retrograde messenger for CO signaling in heart.

Studies performed on smooth muscle cells revealed that increases in HO-1 transcript by the NO donor, spermine NONOate, is associated with enhanced activator protein-1 (AP-1) DNA-binding activity (41). By contrast, recent work using HeLa cells suggests that mitogen-activated protein kinase (MAPK) extracellular signal-related kinase (ERK) and p38 pathways, but not the AP-1 transcription factor, are involved in NO-mediated induction of HO-1 (78); in this case, the mechanism of activation would be unrelated to cyclic GMP and also appears to be independent of redox signaling events.

6.4. INTERACTION BETWEEN NO AND CO SIGNALING PATHWAYS AND ATHEROSCLEROSIS

Increased expression of the stress response protein HO-1 in human atherosclerotic lesions (79) and vascular endothelial and smooth muscle cells exposed to oxidized low-density lipoprotein (LDL) (80,81) may serve a multipurpose role, via metabolism to the antioxidant biliverdin and the vasodilator CO (15,82). These adaptive responses may contribute to the maintenance of vascular tone and patency in atherosclerotic vessels and compensate for diminished NO generation and activity (83,84).

Endothelium-derived CO or NO diffuses to subjacent smooth muscle cells where activation of SGC results in elevated intracellular cGMP levels, leading to smooth muscle relaxation (85). As shown in **Fig. 8** and pointed out by Siow et al. (81), CO and NO can also be generated in smooth muscle cells in response to atherogenic stimuli. The metabolic functional links between CO and NO suggest that vasodilator actions of

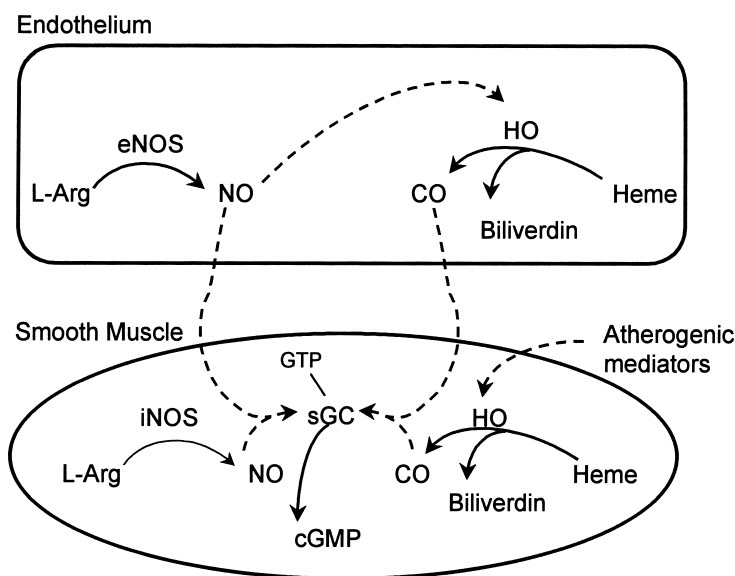


Fig. 8. Importance of the HO-CO and L-arginine-NO signaling pathways in vascular endothelial and smooth muscle cells in atherogenesis. HO₁ metabolizes heme to generate the antioxidant biliverdin and CO, which, like NO, stimulates sGC, resulting in increased intracellular cGMP levels. Atherogenic and proinflammatory mediators such as oxidized LDL and cytokines decrease the expression and activity of eNOS while inducing HO-1 and iNOS in smooth muscle cells. Diminished production or activity of NO by the endothelium in atherogenesis could be compensated for by induction of HO-1. Increased cGMP levels in VSMCs would sustain blood flow, whereas catabolism of heme and generation of biliverdin would attenuate cellular oxidative stress in atherogenesis. (Adapted from ref. 81.)

CO may become important in atherogenesis, where endothelium-derived NO production is inhibited.

As mentioned earlier, there is accumulating evidence demonstrating that NO donors and endogenously generated NO induce expression of HO-1 in vascular endothelial and smooth muscle cells (4,34,39,41). This provides an endogenous adaptive defense mechanism against the oxidative stress associated with sustained production of NO (18,86). It has been shown that the heme moiety of NOS and sGC can serve as alternative substrates for HO; their activity may, under certain conditions, be downregulated. In addition, CO is able to bind to the heme moiety of NOS and thereby inhibit L-arginine turnover and NO production (15). NOS and NADPH-cytochrome P450 reductase are extremely similar, and, therefore, electron transfer from NOS to HO can also occur, fuelling heme catabolism by HO (15). By reducing intracellular heme levels in vascular cells, HO₁ may limit *de novo* synthesis of iNOS, whereas the iron generated by heme catabolism would further limit synthesis of iNOS through inhibition of nuclear transcription (87). It has been shown that iNOS is expressed and has been detected in animal and human atherosclerotic lesions and contributes to the formation of ONOO⁻ (88–90). Induction of HO-1 in atherogenesis in response to ONOO⁻ (34) may attenuate vascular injury.

In the setting of atherosclerosis, impairment of the vascular NO signaling pathway could tip the balance in favor of HO as a salvage mechanism required to maintain vascular tone and function (81).

7. NO, CO, H₂S, AND THE IMMUNE SYSTEM

7.1. *NO and the Immune System*

The notion that NO has an important role to play in the functioning of the immune system has taken time to be accepted, which is interesting because in evolutionary terms, more than 500 million years ago, the horseshoe crab was using the NO pathway to prevent blood coagulation. It is now well established that NO produced via iNOS is an important inflammatory mediator in the body; thus, NO has a proinflammatory role.

7.1.1. IMMUNE CELLS

NO is produced by macrophages and will eradicate many parasites and bacteria that are otherwise difficult to kill. Studies performed on mice infected with *Leishmania major*, a pathogenic protozoan, demonstrated that host defense against this infection depends on the macrophages releasing NO (91). Studies have clearly demonstrated that immunological activation of mouse macrophages induces the activity of NOS, producing NO (92). Much of the antimicrobial activity of mouse macrophages against some fungal, helminthic, protozoal, and bacterial pathogens has been attributed to alterations in the activity of NO. Production of large amounts of NO by activated macrophages contributes to their ability to suppress lymphocyte proliferation. However, no compelling evidence yet exists however that NO synthesis can occur directly in lymphocytes. However, cytokines secreted by activated lymphocytes can certainly regulate NO synthesis by macrophages. Constitutive NOS is activated in neutrophils in response to inflammatory stimuli, and NO has diverse, often biphasic, effects on neutrophil functions (93).

NO acts as an inter- and intracellular messenger molecule, coordinating cross talk between immune cells and endothelial cells; thus, NO plays a vital role in the inflammatory processes. This involves NO derived from constitutive NOS, which appears important in the early stages of an inflammatory response through to high-output production of NO by iNOS, which is fundamental to chronic inflammatory disease (94). Constitutively produced NO released by endothelial cells has been shown to act as an endogenous agent that inhibits the rolling and adhesion of leukocytes in the microcirculation, and the importance of iNOS in modulating leukocyte recruitment can vary according to the type of inflammatory response (95). The significance of NO in this capacity has been reported as almost an incidental observation. The rolling and adhesion of leukocytes within the microcirculation is a significant step in determining endothelial-leukocyte crosstalk (96) and could well play a role in subsequent leukocyte activation and formation of edema.

7.1.2. THERAPEUTIC POTENTIAL

Increased knowledge of the role of gasotransmitters in host defense may lead to novel therapeutic interventions. Researchers from the University of North Carolina led by Schoenfish have developed a gel that can release NO when in contact with biological fluids, such as blood, resulting in a technique that mimics the body's own NO-producing capabilities. Medical implants such as catheters and artificial organs have been coated with such gel-based materials. This targeted release of NO extends the normal, extremely short duration half-life of NO from a few seconds to minutes. The overall effect is that of selectively mimicking phagocytosis, the process by which immune cells release a host of antibacterial agents including NO. The NO released also serves the dual function of reducing bacterial adhesion (97). The genes coding for eNOS, nNOS, and iNOS enzymes are on chromosomes 12, 7, and 17, respectively, and will no doubt offer scope for future therapeutic targeting.

7.2. CO and the Immune System

CO mediates potent anti-inflammatory effects and has been shown to suppress the inflammatory response (32). Both in vivo and in vitro, CO at low concentrations differentially and selectively inhibited the expression of the lipopolysaccharide-induced proinflammatory cytokines tumor necrosis factor- β , interleukin (IL)-1 β , and macrophage inflammatory protein-1 α and increased the lipopolysaccharide-induced expression of the anti-inflammatory cytokine IL-10, involving the MAPK pathway (98). All of these cytokines have significant modulating effects on immune cells. There have been few studies on the effect of CO on the actual functioning of immune cells, primarily because CO has been previously associated with poisoning. Studies examining the effects of CO on the immune system have focused largely on the effects of CO toxicity, with a paucity of research considering its potential as a signaling molecule.

CO poisoning has been reported to temporarily inhibit B2 integrin adherence molecules on leukocytes (99). This has the potential of modifying leukocyte-endothelial cell interactions and could be of tremendous benefit in modifying stress-induced leukocyte activation, which has been in part attributed to B2 integrins (45).

The fact that CO can upgrade the production of free radicals and modulate leukocyte adherence has yet to persuade researchers that it has remarkable therapeutic potential.

7.3. H₂S and the Immune System

H₂S dissociates into free sulfide in the circulation and sulfide binds to many macromolecules, among them cytochrome oxidase (100). Exposure to toxic levels of H₂S resulted in inhibition of complement activity along with the bacteriocidal activity of blood serum (101), both indirect indicators that immune cell function has been modified.

7.4. A New Look at Phagocytic Killing: Therapeutic Possibilities

Phagocytic leukocytes play a pivotal role in the innate immune response against bacteria, fungi, foreign particles, and stress-induced immunosuppression (45,46,102). On the surface of phagocytic leukocytes is NADPH oxidase, a multi-subunit enzyme that can assemble and “shoot” pathogens with highly ROS. These NADPH oxidases are highly controlled and thus prevented from blasting highly reactive superoxide anions into healthy tissues. Recently, it has been shown that once “superoxide shooting” commences, the leukocyte initiates a highly coordinated sequence of events that includes fusion and release of several types of granules and activation of antimicrobial enzymes (103). Therefore, the role of ROS is not just that of a reactive oxygen free radical but may be a signal for subsequent alteration of electrons, movement of ions, and ultimately release of granular contents (103). Thus, an alteration of pH, undoubtedly possible by any one of the intracellular gas signaling molecules, in particular H₂S and CO, could result in selective leukocyte activation. Clearly this offers a novel therapeutic approach to modulating leukocyte activation (**Fig. 9**).

8. CONCLUSION

There are some similarities among NO, CO, and H₂S, in terms of their production and effects, but also some important differences. There is now clear evidence of interactions among NO, CO, and H₂S. In particular, the negative interactions among NO, CO, and their generating systems allows a degree of control in the cardiovascular system, via

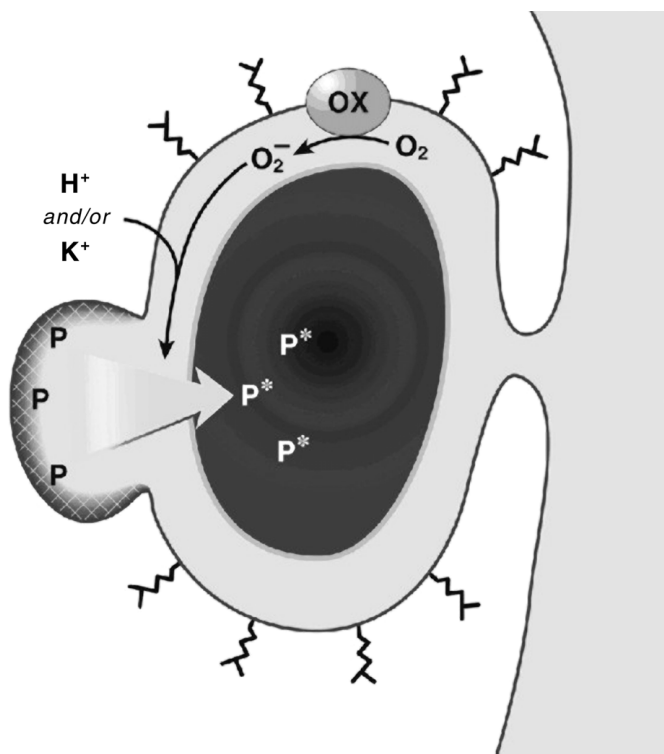


Fig. 9. Fresh look at phagocytic killing. The single electron reduction of molecular oxygen to form superoxide anion by the phagocyte NADPH oxidase (OX), stimulated by bacterial uptake, results in the transfer of electrons into the enclosed phagocytic vesicle. Dismutation of the superoxide generates OH^- , and the accumulating negative charge must be compensated by the influx of H^+ and/or K^+ . The hypertonicity resulting from K^+ transport promotes the release of inactive cationic granule proteases (P) bound to an anionic sulfated proteoglycan matrix (crosshatching). The released and active proteases (P^*) encounter the bacterium under optimal pH conditions within the phagocytic vesicle and degrade it. Cytoskeletal elements associated with the phagocytic vesicle (wavy lines) indirectly affect the killing process by modulating vesicular volume. The pH and movement of ions may well be affected by gas signaling molecules. (From ref. 103 with permission.)

opposing effects. Initial reports of synergy between NO and H_2S in their actions are interesting but require further investigation. Interactions among the three gasotransmitters in the immune system and free-radical production require further research but could open up the possibility of novel therapies.

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