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Forum Review

Cross Talk Between Carbon Monoxide and Nitric Oxide

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ABSTRACT

Carbon monoxide and nitric oxide are two endogenously produced gases that can act as second messenger molecules. Heme oxygenase and nitric oxide synthase are the enzyme systems responsible for generating carbon monoxide and nitric oxide, respectively. Both carbon monoxide and nitric oxide share similar properties, such as the ability to activate soluble guanylate cyclase to increase cyclic GMP. It is becoming increasingly clear that these two gases do not always work independently, but rather can modulate each other's activity. Although much is known about the heme oxygenase/carbon monoxide and nitric oxide synthase/ nitric oxide pathways, how these two important systems interact is less well understood. This review attempts to define the current known relationship between carbon monoxide and nitric oxide as it relates to their production and physiological function. Antioxid. Redox Signal. 4, 301–307.

INTRODUCTION

OUR CURRENT KNOWLEDGE of the heme oxygenase (HO)/carbon monoxide (CO) and nitric oxide synthase (NOS)/nitric oxide (NO) pathways and their contribution(s) to various biological functions has been formed through multiple investigations. A majority of these studies have focused on the physiological and cellular functions mediated by one or the other system without consideration of the potential relationship between the two systems. This review attempts to consolidate our current understanding of the known interactions between CO and NO.

CO, an odorless, colorless, tasteless, and nonirritating gas, is produced from incomplete combustion of organic matter and traditionally viewed as a life-threatening toxic gas. CO, a component of exhaust fumes and cigarette smoke, is the most abundant pollutant found in the lower atmosphere. For decades, CO has been known to induce relaxation of vascular tissues (11), but it was not until the scientific community recognized that NO, another toxic gas, was endothelium-derived relaxing factor that it became appreciated these noxious gases possess important physiological vasoactive roles (33, 35).

This revelation led to the concept of a new class of second messenger gases to which NO and CO apparently belong. It is noteworthy that these two diatomic gases share similar molecular mass (30.01 and 28.01), solubilities in water (4.7 ml/100 ml and 2.6 ml/100 ml), and basal rates of production (850 μmol/day and 500 μmol/day), respectively (13). Numerous studies have characterized the role of NO in multiple biological processes, including vasodilation, inhibition of platelet aggregation, neurotransmission, antimicrobial properties, and modulation of gene expression (34). Similar to NO, CO increases cyclic GMP (cGMP), acts as a neurotransmitter in the brain, decreases vascular tone, and inhibits platelet aggregation (18, 52).

Despite these similarities, there are important differences that suggest these two gases do not represent redundant messenger molecules. NO is a free radical that possesses an unpaired electron that is readily lost to give the nitrosonium ion, which is subsequently involved in the formation of many NO–metal complexes. CO is a stable gas, not a free radical, and therefore does not undergo the various oxidative and reductive reactions characteristic of NO (12). These two gases also have distinct binding properties. CO binds only ferrous heme, whereas NO binds both ferrous and ferric hemoproteins. A comparison of the interaction of these two gases with hemoglobin gives a sense of their unique reactivity. The combination rate of NO with hemoglobin is fast compared with

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that of CO, whereas the dissociation rate of NO is slower than that of CO (43), giving NO an affinity for hemoglobin ~1,500 times that of CO (13). Interestingly, the binding properties of CO to heme appear to be enhanced in the presence of NO. Studies using dioxyhemoglobin showed that the formation rate of carboxyhemoglobin was 10–15 times greater when equal amounts of NO and CO were mixed versus CO alone (32).

HEME OXYGENASES AND NITRIC OXIDE SYNTHASES

HO and NOS, the enzymes responsible for generating CO and NO, respectively, have surprising similarities in their isoforms, requirements for activity, and regulation. For example, both the CO- and NO-generating systems have constitutive [HO-2, HO-3, endothelial NOS (eNOS), and neuronal NOS (nNOS)] and inducible [HO-1 and inducible NOS (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. 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 (35). Another striking difference between HO and NOS involves the free radical superoxide anion. Whereas studies have established that NOS is capable of generating both NO and superoxide anion (51), HO has recently been linked to the induction of manganese superoxide dismutase, an enzyme that scavenges superoxide anion (15).

Regulation of CO or NO activity is not directly dependent on plasma membrane transporters and/or channels. This makes the localization and control of their synthesis via HO and NOS critical in controlling their activity. The constitutive isoforms, HO-2, nNOS, and eNOS, are subject to regulation by the adrenal glucocorticoids. Interestingly, glucocorticoids have opposing effects on the expression of these two proteins in the brain, with HO being up-regulated and NOS being down-regulated (32). Similar findings were reported by Willis *et al.* who demonstrated that NOS and HO activities are inversely related in both the brain and spleen in the presence of various NOS donors (55). Conversely, inducible HO-1 and iNOS are both up-regulated by numerous common stimuli, such as reactive oxygen species, cytokines, and endotoxin (8, 32), but not all inducers of HO-1 regulate iNOS protein or activity. For example, Wagner *et al.* demonstrated that sheer stress and cyclic stretch up-regulate HO-1 gene transcription in vascular smooth muscle cells without affecting iNOS expression (53). Interestingly, Zakhary *et al.* 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 (59). Indeed, *in vitro* studies show that under certain pathophysiological conditions, such as hypoxia, down-regulation of constitutive eNOS is concurrent with transient increases in inducible HO-1 protein, indicating a potential compensatory regulation between the two systems (36). However, the production of NO and CO and their regulation of pulmonary vascular tone during acute and/or chronic hypoxia vary between animal models, making their relationship *in vivo* less clear.

ACTIVATION OF GUANYLATE CYCLASE

Several studies have established that both CO and NO exert many of their biological functions via the activation of soluble guanylate cyclase (sGC). Both CO and NO bind to the iron atom of the heme moiety of sGC. sGC is a heterodimer consisting of an α subunit of 73–88 kDa and a heme-containing β subunit of 70 kDa (46). The ferrous b-type heme is bound to the protein through the imidazole (ImH) side chain of His^{105}. Activation of sGC by NO is initiated by binding to the heme iron, which causes the weak Fe-ImH bond to break, yielding a five-coordinate low-spin ferrous nitrosyl heme (16). It is this conformational reorganization that is thought to be responsible for the 100–400-fold increase in sGC activity converting GTP to the intracellular second messenger, cGMP. CO binds to the sGC heme group with a lower affinity, and binding only results in a four- to sixfold activation of the enzyme. Unlike NO, when CO binds to the heme of sGC it leaves the Fe-ImH bond intact, forming a six-coordinate complex that can only weakly increase cyclase activity (16).

Although CO is a relatively weak stimulator of sGC, increasing evidence suggests a direct physiological role for CO in the regulation of cGMP levels. Morita *et al.* reported that in smooth muscle cells hypoxia up-regulated HO-1 gene expression, leading to increased CO production and intracellular cGMP accumulation (36). This suggests that CO may regulate vascular tone under pathophysiological conditions. In addition, Wagner *et al.* demonstrated that CO generated from smooth muscle cells by exposure to sheer stress inhibited platelet aggregation (53). Elevated CO and cGMP levels have also been shown to decrease blood pressure under *in vivo* stress conditions, such as post surgery (37), and increase renal blood flow in rats exposed to chronic hypoxia (38). Many investigators have shown that HO-2 is prominently expressed in several cell types in the brain where NOS expression is minimal or undetectable (52), and the HO-2 protein or transcripts are colocalized with sGC. This supports the idea that activation of sGC is by CO. Indeed, a direct relationship between CO production and endogenous cGMP concentrations was demonstrated by Ingi *et al.* using elegant metabolic labeling in a neuron culture system (26).

Given that *in vitro* studies show that NO has a higher affinity for sGC compared with CO and that CO does not cause the critical conformational shift to a five-coordinate complex, it remains controversial whether CO is a physiological activator of sGC *in vivo*. As stated above, there is substantial evidence for a direct role of endogenously produced CO to stimulate sGC and increase cGMP levels. So how does one reconcile these differences? One possible explanation could be the existence of an endogenous molecule that sensitizes sGC to CO. Friebe *et al.* reported that the benzyl indazole derivative, YC-1, could independently activate purified sGC (17). More importantly, they demonstrated in the presence of YC-1 that the stimulatory effect of CO was intensified, resulting in enzyme activation similar to that produced by NO (17). The abil-

ity of YC-1 to increase the maximal activity obtained with CO by >4,000% appears to be mediated through the stabilization of sGC in the active conformation. Although the potentiation by YC-1 of cGMP production was more pronounced with CO, the action of YC-1 was not specific as it also enhanced cGMP levels generated in response to NO by >40%. Indeed the stimulatory effects of YC-1 lead to complete inhibition of platelet aggregation at concentrations of NO and CO that are ineffective alone (17). The discovery of a naturally occurring YC-1-like molecule would clearly solidify the role of CO as a modulator of biological function via its ability to stimulate sGC.

NO REGULATION OF HO ACTIVITY

It is well established that NO donors can activate HO-1 gene expression and activity in a variety of tissues (14, 58). We and other investigators have demonstrated that NO-mediated HO-1 induction is dependent on *de novo* synthesis of both RNA and protein and occurs via a cGMP-independent signaling pathway (14, 20), although a recent study suggests that NO may act via cGMP to up-regulate HO-1 (39). We further demonstrated that NO, unlike other stimuli, also augments HO-1 gene expression by increasing the half-life of HO-1 mRNA (20). More recently, Bouton *et al.* reported that NO-mediated stabilization of the HO-1 message does not require *de novo* protein synthesis (3). In addition, we reported that the antioxidant *N*-acetylcysteine significantly reduces HO-1 induction during NO exposure, suggesting that HO-1 gene expression might be up-regulated by oxidative stress (20). Further work by Foresti *et al.* demonstrated that formation of peroxynitrite could independently increase HO-1 gene expression, leading to postulation of a dynamic equilibrium among NO, glutathione, and superoxide anion and their ability to modulate the expression of HO (14). More recently, hypoxia-induced HO-1 gene expression and the up-regulation of HO-1 by lipopolysaccharide have been linked, in part, to iNOS-dependent generation of NO (25).

Conversely, Chen and Maines reported that *N*-acetylcysteine did not inhibit the induction of HO-1 by SNP in HeLa cells, suggesting that oxidative stress is not involved, but rather showed a connection to the mitogen-activated protein kinases ERK and p38 (7). In addition, there are conflicting reports on whether DNA binding activity of the transcriptional factor activator protein-1 is increased during NO exposure (7, 20), and further experiments are necessary to fully define the cis and trans elements mediating NO-induced HO-1 gene transcription. Not all studies confirm the idea that NO up-regulates HO activity to increase CO production. Willis *et al.* demonstrated that the NO donor SNP reduced HO activity in both brain and spleen (55). More recently, Hoetzel *et al.* reported that the NO donor molsidomine attenuated the accumulation of HO-1 activity in a hemorrhagic shock and resuscitation model (22). To date, the mechanism underlying the modulation of HO-1 by NO remains unclear, and the discrepancies between studies may depend on which specific HO isoforms are present and the cellular concentration of HO versus NOS. Another potential variant is the distinct characteristics (*i.e.,* rate of decomposition, capability to generate NO and other molecules, and stability) of the various NO donors (19).

HO REGULATION OF NOS ACTIVITY

There are several different mechanisms by which HO could decrease NO production by NOS. NOS is a cytochrome P450 type hemoprotein requiring two heme molecules in the active site. Therefore, increased HO activity could directly degrade the heme located in the active site of NOS, or could reduce the amount of available heme for the *de novo* synthesis of NOS (32). This is reflected in the reciprocal relationship of cytochrome P450 and HO activities, where stimuli that increase HO-1 activity cause a concomitant decrease in cytochrome P450 (32). Iron released in the degradation of heme by HO could further inhibit production of NOS by inhibiting its nuclear transcription. In addition, both HO and NOS require NADPH as a cofactor, and the subsequent reduction of biliverdin to bilirubin by biliverdin reductase also utilizes NADPH, which could shift competition for electrons in favor of the HO pathway (32). Indeed, induction of HO-1 by hemin negatively modulates iNOS expression and activity in an animal model of glomerulonephritis, demonstrating the potential of regulatory interactions between the two enzymes *in vivo* (9). More recently, Ding *et al.* hypothesized a complex interaction where HO-2 inhibits NO activity by acting as an intracellular "sink" for NO (10). In turn, NO binding would inhibit HO-2 catalytic activity (10).

NOS activity is also susceptible to direct inhibition by CO, as both neural and macrophage forms of NOS have been reported to bind CO and studies have demonstrated that exogenous CO inhibits both macrophage and rat cerebellar NOS activity (54). Interestingly, a variety of HO-1 inducers, including NO donors, attenuated the activation of iNOS by cytokines at the transcriptional level (5). Further support comes from studies showing that inhibition of HO activity increases NO production in internal anal sphincter smooth muscle (6) and mouse macrophages exposed to endotoxin (49), suggesting that HO activity may exert counterregulatory effects on NOS activity. These effects may be largely dependent on the amount of CO present in the system, as Thorup *et al.* reported that whereas high levels of CO inhibit NOS activity and NO generation, lower concentrations induce release of NO from intracellular pools (48). These data support the work of Thom *et al.,* who demonstrated that CO exposure, at concentrations that do not alter mitochondrial function, did not alter NOS activity, but rather increased NO steady-state levels presumably by competitively dissociating NO from hemoproteins (47). These various possibilities are summarized in Fig. 1.

INTERACTIONS OF CO AND NO

Increasing evidence in the literature supports coexpression of HO and NOS in a variety of cells and tissues, suggesting possible interaction between the CO- and NO-generating systems. In cells, Alm *et al.* demonstrated that all four types of islet cells contain the constitutive forms of HO and NOS (1), and other groups have reported dual expression of HO-2 with nNOS in >74% of cell bodies and nerves of human urethral sphincter (21). In addition, Kusner *et al.* showed the presence of both HO and NOS at the neuromuscular junction, implying that both CO and NO can serve as pre- and

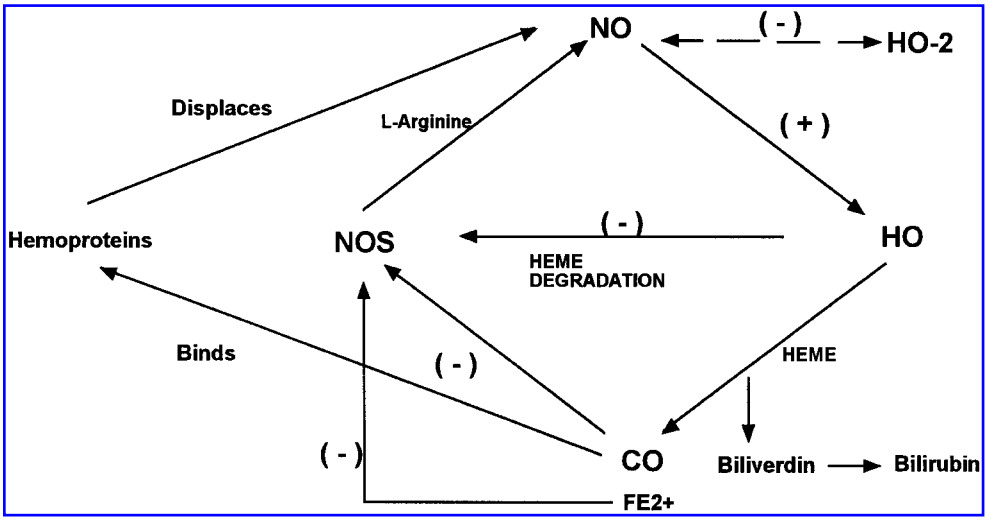

FIG. 1. Schematic of the potential regulatory interactions between HO/CO and NOS/NO systems.

postsynaptic messengers (29). Localization of HO and NOS in discrete cell populations within organ tissue has also been defined in various animal models. For example, in a rat liver injury model of hemorrhage and resuscitation, HO-1 was increased primarily in parenchymal cells, whereas a faint induction of NOS was restricted to nonparenchymal cells (41). Similar studies using a rat model of portal hypertension demonstrated both iNOS and HO-1 mRNA and protein were significantly increased in lung tissue and further localized iNOS expression to the vascular endothelium and HO-1 expression to the bronchiolar epithelium and macrophages (42).

Given that CO is a weak stimulator of sGC compared with NO, it is possible that CO can function as a partial agonist to either facilitate or inhibit NO-mediated activation of sGC. Studies, such as that by Cao *et al.,* support this idea as they report that CO amplifies NO-stimulated augmentation of cGMP in the retina over increases in cGMP levels seen with either CO or NO alone (4). In addition, experiments using HO-2 and nNOS knockout mice show that in the enteric nervous system, NO and CO are neurotransmitters that interact as cotransmitters, resulting in an NO system that does not function in the absence of CO generation (56). Conversely, Ingi *et al.* reported that low concentrations (1.5–5 μM) of CO inhibited increases of cGMP by NO, whereas high concentrations (150–500 μM) of CO potentiated the elevation of NO-mediated cGMP (26). They propose that this observation could be related to a conformational change of sGC that affects the mechanism of NO-mediated activation, or that the mechanism involves allosteric effects (26).

Theoretically, CO and NO could function in a synergistic, compensatory, and/or counterregulatory way, and emerging evidence suggests that any given response depends on the microenvironment. For example, Turnbull *et al.* demonstrated that both NO and CO exert stimulatory effects on the hypothalamic–pituitary–adrenal axis and that they must be present together to account fully for shock-induced adrenocorticotropin release (50). In contrast, Kostoglou-Athanassiou *et al.* reported that although endotoxin stimulated both NO and CO generation, the two gases had counterregulatory effects on the activation of the hypothalamic–pituitary–adrenal axis: NO inhibited corticotropin-releasing hormone release, whereas CO inhibited the release of vasopressin (28).

As stated above, both HO and NOS require molecular oxygen for activation, yet the modulation of these enzymes by hypoxia remains unclear. 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 (40). 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 (27, 30). 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 (31) and to inhibit hypoxia-inducible factor-1 (HIF-1) DNA binding activity by abrogating hypoxia-induced accumulation of HIF-1α protein (24).

CO and NO can be measured in exhaled breath and are being proposed as a new noninvasive way to predict airway inflammation in asymptomatic atopic patients, and as a monitor for both asthma and cystic fibrosis (2, 23, 57). More than just markers for airway disease, clinical studies are linking the production of both CO and NO in the pathogenesis of various newborn diseases. Elevated plasma CO levels have been significantly related to increased NO production in newborn infants with sepsis (44), and both CO and NO plasma levels have been linked to the severity of neonatal hypoxic–ischemic encephalopathy (45), suggesting that they may be important mediators in the disease process. Given the potential of NO to increase CO production by inducing HO-1 activity, the increased clinical use of inhaled NO to treat pulmonary complications makes it imperative to continue investigation into the interactions of CO and NO in human disease.

This review has tried to define the existing known relationships between CO and NO as they relate to physiological

function. However, further studies are necessary to form a clearer picture of how the reported changes in HO and NOS expression and activity in response to CO and NO production affect cellular and biological outcomes. Initially, investigators theorized that CO, being the more stable gas, was responsible for basal activity, therefore providing tonic background stimulation, whereas surges of NO transiently amplified or delivered phasic signaling. To date, no results have fully supported this hypothesis but rather suggest that, based on any given system, CO and NO act together in a complex, dynamic, and adaptable association.

ABBREVIATIONS

cGMP, cyclic GMP; CO, carbon monoxide; eNOS, endothelial nitric oxide synthase; HIF-1, hypoxia-inducible factor-1; HO, heme oxygenase; ImH, imidazole; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase.

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