ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 12, 2007
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DOI: 10.1089/ars.2007.1803

Forum Review

Mitochondrial and Cellular Heme-Dependent Proteins as Targets for the Bioactive Function of the Heme Oxygenase/Carbon Monoxide System

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ABSTRACT

The toxic effect of high concentrations of CO gas in living organisms is coherently typified at biochemical levels by the high affinity of CO for hemoglobin and cytochromes, heme-dependent proteins that are indispensable for oxygen transport and mitochondrial respiration. However, the basal production of CO during heme degradation and the ability of heme oxygenase-1 (HO-1) to increase CO availability pose the question of how this gaseous molecule interacts with metal centers within the intracellular milieu to serve as one of the most unconventional signaling mediators. Emerging evidence indicates that the diverse and multifaceted beneficial effects exerted by "low concentrations" of CO cannot be explained solely by the activation of classic prototypic targets (*i.e.*, guanylate cyclase/potassium channels) but entails the dynamic and concerted activation/inhibition of a group of CO-responsive proteins. As the complexity of the temporal and spatial action of CO is progressively being appreciated, this review aims to (a) highlight the current knowledge on certain metal-containing proteins that interact directly with CO; (b) analyze the latest notions on their functional role in response to CO; and finally (c) propose a rational view on the mode these CO targets may interrelate with and be regulated by the HO/CO pathway. *Antioxid. Redox Signal.* 9, 2139–2155.

INTRODUCTION

A substantial body of literature points to the prominent role of heme oxygenase-1 (HO-1) as a new tier of control over cellular dysfunction imposed by oxidative and nitrosative stress, which are the common denominators in the progression and development of several disease states. In this respect, the identification of the first human case of (HO-1) deficiency (171) is of particular interest because the pathologic disorders and clinical manifestations associated with this condition reinforce the concept that HO plays an essential physiologic role in the control of heme and iron distribution and suggests that this pathway (and probably its products CO and bilirubin) are crucial for counteracting endothelial cell damage, atherogenesis, and oxidation of low-density lipoprotein. Based on the notion that biliverdin and bilirubin possess intrinsic antioxidant actions and that CO can function as a signaling mediator, an increasing number of studies reported in the last decade on how the action of HO is translated into beneficial effects. We begin to appreciate that the biologic role of HO-1 activity and the function of its products cannot be separated, as each element of the HO pathway takes part in a coordinated and complex set of reactions when cells are under stress conditions. This coordinated response finds its best

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demonstration in the pleiotropic activities that have been attributed so far to the HO-1/CO/biliverdin pathway. The crucial task now is to find a precise mechanism of action for each of the HO products, and this is particularly challenging for CO. This gaseous molecule has been known for more than a century to be toxic when inhaled at relatively high concentrations because of its preferential affinity for the heme-containing proteins hemoglobin and cytochrome oxidase (118). These proteins crucially depend on molecular oxygen to accomplish their natural function; thus, the binding to CO with consequent inhibition of their activities is a process that should be avoided, especially in organisms that rely on oxygen transport and mitochondrial function for energy supply. However, when CO is generated at physiologically relevant levels, are hemoglobin and cytochrome oxidase still the preferential targets of CO? What other heme- and metal-dependent proteins could directly interact with CO? Can the binding of CO to these proteins fully explain some of the physiologic effects observed? Is there more than one target for CO when considering a given physiologic effect attributed to this gaseous molecule? Although we do not have precise answers to these questions, in the next sections of this article, we attempt to illustrate some of the new aspects that are gradually emerging in the field of CO signaling and the important role of CO interaction with intracellular metal centers.

CO-RELEASING MOLECULES (CO-RMS): A THERAPEUTIC TOOL TO IDENTIFY THE INTRACELLULAR TARGET(S) OF CO

A possible biologic function of CO as an important cellular mediator in mammals started to be fully appreciated only decades after the discovery that endothelium-derived relaxing factor (EDRF), a diffusible autocoid responsible for mediating vessel relaxation, inhibition of platelet aggregation, and neuronal signaling, is the gaseous molecule nitric oxide (NO) (50, 56, 58, 115, 137). The proposal that CO could act as a neurotransmitter and activate the heme-dependent enzyme guanylate cyclase in a similar fashion to NO (86, 158) encouraged scientists to explore in a more persuasive way how endogenous CO could regulate and mediate physiologic processes. Consequently, evidence corroborating a prominent role for endogenously HO-derived CO in controlling vascular and hepatic sinusoidal tone, as well as acute hypertensive responses under stress conditions (61, 96, 127, 147, 161), instigated more assiduous experimental approaches to understand the functional role of endogenous CO in biology. In the last decade, progress has been made in this area, demonstrating that CO gas produced by HO contributes not only to alleviating vascular and inflammatory disorders in animal disease models, but also its intrinsic bioactivity could be exploited for therapeutic needs. Initial studies on rodents subjected to inhalation of defined doses of CO gas over a restricted period (250–500 ppm for 1–24 h) revealed unprecedented and unexpected antiinflammatory actions of this HO product (92, 111, 112). These "paradoxical" beneficial effects of CO have been confirmed and extended to other pathophysiologic conditions whereby administration of this gas appears to reduce postoperative ileus (92), organ rejection (71, 107, 132), posttransplant arteriosclerosis associated with intimal hyperplasia (113), and pulmonary hypertension (174). More recently, it has been reported that bubbling CO gas into cold preservation solutions improves the function of organs and grafts after transplantation (104). The mechanism(s) of action exerted by CO in these situations have been attributed to the ability of the gas to activate selective intracellular pathways based on the notion that CO binds almost exclusively to heme-dependent proteins (14). For instance, activation of guanylate cyclase and large-conductance potassium channels containing heme moieties in their functional subunits have been implicated in both cardiovascular and cerebral effects mediated by CO on vasodilatation (59, 161, 163, 168). Despite these advances, still a lack of consensus exists on whether the interactions of CO with guanylate cyclase or potassium channels or both can fully explain the diverse beneficial actions of CO gas or whether other metal-containing targets that are more sensitive to CO remain to be identified in the various cell types present in mammals (14).

A technical restraint in the use of CO gas as both a therapeutic agent and a tool for searching for its intrinsic intracellular target(s) is represented by its chemical nature; indeed, gaseous compounds are difficult to manipulate and to deliver directly into living cells or organisms in an accurate, safe, and measurable fashion. Moreover, a foreseeable administration of gaseous CO as a pharmaceutical in the clinic will have to face a strict approval standards by regulatory authorities on the safety and efficacy of CO gas delivery to human patients. The advent of CO-releasing molecules (CO-RMs), a novel class of compounds capable of carrying and liberating controlled amounts of CO in cellular systems (93), appears a plausible step forward in the attempt to overcome the limitations of CO gas. The chemical structures of CO-RMs that are biologically active are illustrated in Fig. 1. The first class of CO-RMs identified were transition metal carbonyls, chemicals that have been used for a century as catalysts in organic synthesis but clearly possess the inherent ability to carry CO groups. Despite their poor solubility in water, transition metal carbonyls such as $Mn_2(CO)_{10}$ (CORM-1) and $Ru(CO)_3Cl_2$-dimer (CORM-2) were initially discovered to liberate CO in aqueous biologic environments and at the same time proved to mimic the physiologic activities exerted by CO gas (93). Subsequently, major progress to improve the pharmacologic profile of metal carbonyls and related compounds enabled our group to synthesize Ru(CO)3Cl-glycinato (CORM-3) and sodium boranocarbonate (CORM-A1), two water-soluble CO releasers having interesting and diverse chemical features (26, 101). CORM-3 is a ruthenium carbonyl complex that liberates CO very rapidly ($t_{1/2} \approx 1$–2 min) when exposed to cells or tissues (26), whereas CORM-A1 contains a carboxylic group that is converted to CO in the presence of hydrogen ions (101). Specifically, the more acidic the environment, the faster the generation of CO from CORM-A1, which has a $t_{1/2} \approx 21$ min under physiologic conditions (pH, 7.4; T = 37°C). The chemical structure of lipid- and water-soluble CO-RMs that have been shown to be pharmacologically active is reported in Fig. 1.

The introduction of water-solubility into metal carbonyl complexes and the ability to generate "fast" and "slow" CO releasers have been achieved with the initial intention of implementing the development of CO-RMs as pharmaceuticals (62, 100).

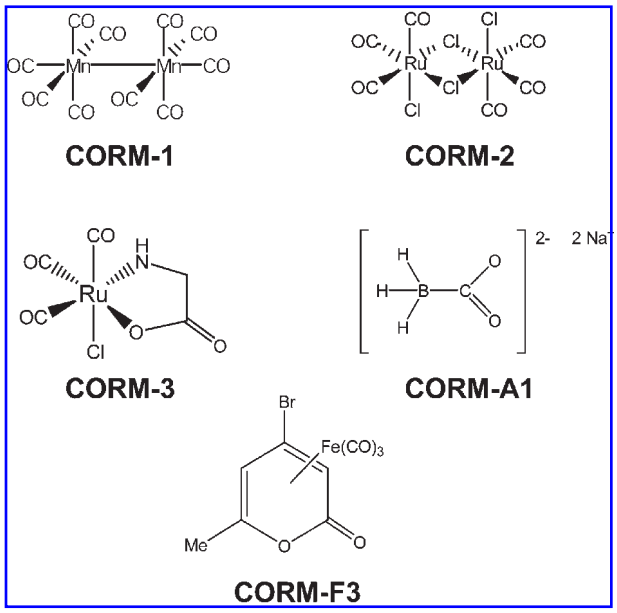

FIG. 1. Chemical structure of bioactive CO-RMs. The chemical structures of lipid-soluble (CORM-1, CORM-2, and CORM-F3) and water-soluble (CORM-3 and CORM-A1) compounds that have been shown to be pharmacologically active through the liberation of CO are represented. See text for more details.

Consequently, water-soluble CO-RMs have been used in a variety of experimental models and proved successful in eliciting pharmacologic effects. CO-RMs–mediated relaxation of isolated vessels and mitigation of acute hypertension via activation of both guanylate cyclase and potassium channels were the first effects to be recognized (42, 101) (see example in Fig. 2). However, other beneficial actions by CO-RMs were recently identified, and these include cardioprotection against ischemia and myocardial infarction (26, 145); prevention of cardiac graft rejection and positive inotropic effects on the heart (26); suppression of the inflammatory response (10, 133); inhibition of platelet aggregation (23); attenuation of endotoxin-mediated vascular dysfunction (46); and improved kidney function after cold-ischemia preservation and protection against cisplatin-induced nephrotoxicity (129, 154). The renoprotective effects of CORM-A1, which are summarized in Fig. 3, are of particular relevance, as other reports have demonstrated an enhanced renal function and protection against renal failure after treatment with water-soluble CO-RMs (125, 157). In addition to these multiple bioactivities that could be refined and optimized for therapeutic applications (99), both water-soluble and lipid-soluble CO-RMs are used progressively more as suitable tools the better to understand some of the mechanism(s) by which CO is liberated (108) and how it acts as a signaling molecule (15, 33, 39, 63, 81, 122, 167, 169). Furthermore, CO-RMs are very useful in assessing to what extent CO contributes to antiinflammatory (3, 47, 77, 88, 109, 139, 156), antiapoptotic (11, 18, 25, 66, 80, 116), antiproliferative (24, 114, 144, 151), and antioxidant/cytoprotective (7, 11, 103, 116, 123, 139) effects.

The versatility and practicality of the CO-RMs technology is also indicated by the increasing number of published articles quoting the original reports on CO-RMs (26, 93) (Fig. 4). Conceivably, as in the case of chemicals releasing NO (NO donors), a great advantage of CO-RMs is that they can be used at precise concentrations in cells, organs, and whole animals, and could ultimately reach the intracellular targets more effectively than CO gas. Although an ideal molecule able to simulate more closely the activities of endogenous CO has not been formulated, the great flexibility of CO-RMs make this class of compounds promising pharmaceuticals for the future, as their synthesis is very reliable, and the release of CO can be finely controlled by a given stimulus. This is the case with metal carbonyls such as CORM-1, CORM-2, CORM-3, and CORM-F3 (see chemical structure in Fig. 1), in which the rate of CO liberation from these metal complexes can be triggered by light or modulated by ligands with a higher or lower affinity to the metal center (26, 36, 93, 99, 134); similarly, in the case of boranocarbonate (CORM-A1), the rate of CO release is strictly pH dependent (101). These few examples emphasize the potential of expanding CO-RMs to a large portfolio of compounds from which the amount, kinetics, and specificity of CO released can be tuned according to a specific need. It is then not surprising that the accessibility of CO-RMs coincided with the ability of scientists to explore new intracellular targets for CO, different from those typified by guanylate cyclase and potassium channels, which were identified some time ago when only CO gas was available (49, 162). In the last few years, emerging evidence points to a wider range of metal-containing enzymatic and protein systems that appear to be responsive to CO-RMs and CO gas; these systems could ultimately work in synchrony to orchestrate the transduction of different chemical signal(s) by CO into specific cellular functions. Furthermore, the way CO interacts with and controls the activity of some "classic" targets in mammalian cells (*i.e.*, cytochrome oxidase) are being reexamined in the search for the precise mechanism(s) underlying its protective effects (148). These targets of CO in-

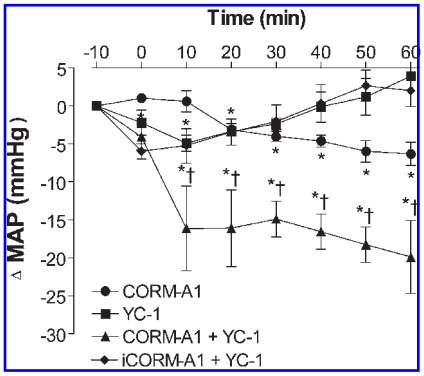

FIG. 2. The hypotensive effect of CORM-A1 *in vivo* is mediated by guanylate cyclase. Mean arterial pressure (MAP) was measured in anesthetized and chronically catheterized Sprague–Dawley rats. CORM-A1 (30 μmol/kg, i.v.) or the inactive compound that does not liberate CO (iCORM-A1) was injected alone or in combination with YC-1 (1.2 μmol/kg, i.v.), a guanylate cyclase sensitizer. $^{*}p < 0.05$ *versus* baseline values (time, 10 min); $^{\dagger}p < 0.05$ *versus* YC-1, CORM-A1, or iCORM-A1 plus YC-1. Modified with permission from ref. 101.

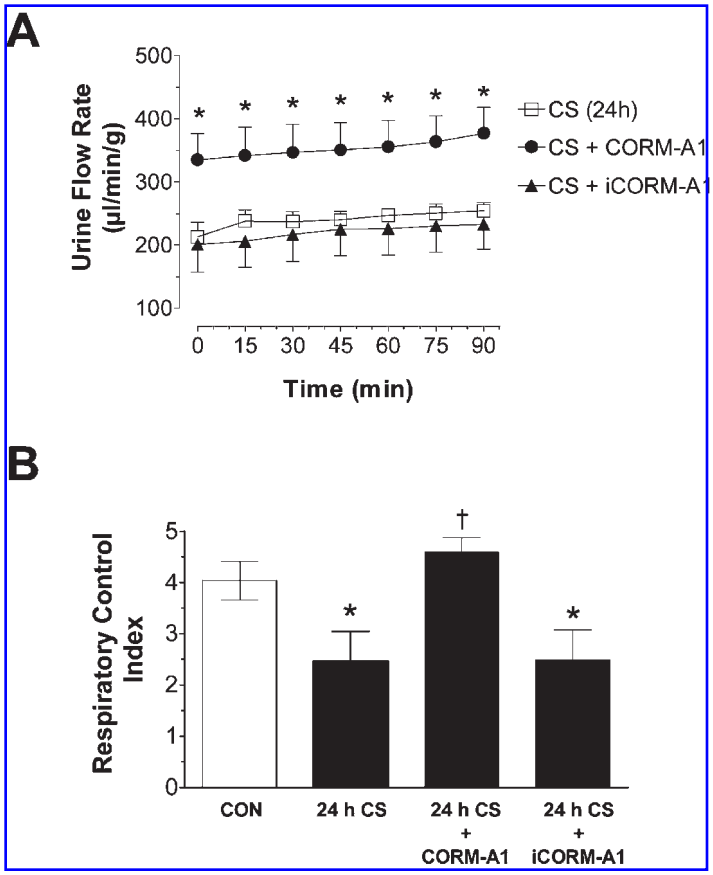

FIG. 3. Renoprotective effects of CORM-A1. (**A**) Rabbit kidneys were isolated and initially flushed with Celsior solution alone or supplemented with 50 μM CORM-A1 and then subjected to 24-h cold storage (CS). Urine flow rate was then measured in isolated kidneys perfused with physiologic buffer. iCORM-A1, which does not release CO, was used as negative control. $^{*}p < 0.05$ *versus* CS and CS + iCORM-A1. (**B**) Kidneys were freshly isolated (CON) or subjected to a 24-h cold storage (CS) period in the presence or absence of CORM-A1 (50 μM). Mitochondria were then isolated, and the respiratory control index (RCI) assessed in the presence of malate and glutamate as substrates and ADP. $^{*}p < 0.05$ *versus* CON; $^{\dagger}p < 0.05$ *versus* 24 h CS and 24 h CS + CORM-A1. Modified with permission from ref. 129.

clude but are not restricted to (a) NADPH oxidase (151); (b) cytochrome oxidase and mitochondrial complexes (128, 129, 148, 151); and (c) nitric oxide synthase (133, 134, 139). Circumstantial evidence also indicates that other hemoproteins, such as cytochrome P_{450} (27, 28), and proteins containing transition metals could be possible targets of CO (33). In the following sections, we analyze and elaborate more closely on how this class of proteins interrelates with and is regulated by the HO/CO pathway.

NADPH OXIDASE AS TARGET OF THE HO/CO SYSTEM

NADPH oxidase is a ubiquitous protein system and is the major source of superoxide anion ($O2_2^{\bullet -}$) production in phagocytes. This enzyme is one representative of the Nox family of oxidases (Nox1-Nox5, Duox1, and Duox2) that exhibit diverse expression patterns and appear to serve a variety of functions related to the generation of reactive oxygen species (ROS). It is made of an assembly of *gp91^{phox}* (otherwise known as Nox2), *p22^{phox}*, *p67^{phox}*, *p47^{phox}*, and Rac1 or Rac2 subunits. The catalytic moiety of the oxidase (*Gp91^{phox}*) is a flavohemoprotein, containing one flavin-adenin dinucleotide and two hemes that catalyze the NADPH-dependent reduction of oxygen (O_2) to form $O_2^{\bullet -}$ (136). This is accomplished by transferring one electron from NADPH to O_2 via oxidation of ferrous heme to its ferric form. Production of ROS by NADPH oxidase and other members of the Nox family have been increasingly recognized as important components of various biologic events, including hormone biosynthesis, cell signalling, and host defense (73).

Heme oxygenase-1 (HO-1) exerts antioxidant properties, and recent evidence suggests that, in addition to the scavenging effects of bilirubin on ROS production, these are linked to control of NADPH expression or activity or both. HO-1 induction has been shown to decrease *gp91^{phox}* expression and whole NADPH oxidase activity by limiting the cellular heme content (152) (Fig. 5). The obligatory role of heme and the requirement of this protoporphyrin molecule for the assembly and activity of NADPH has been well demonstrated (32). Heme incorporation into *gp91^{phox}* is required for ensuring protein stability and forming the functional component of the oxidase. Heme oxygenase-1 induction does not modify *gp91^{phox}* mRNA expression but results in its protein degradation by the proteasome and an impaired translocation to the membrane (152). However, decreasing cellular heme content is not the only mechanism through which HO-1 can interact with NADPH oxidase. Previous reports have shown that endogenous HO-1–derived bilirubin or micromolar concentrations of bilirubin applied exogenously to airway smooth muscle cells can decrease $O_2^{\bullet -}$ production (126, 150). The precise mechanism of this inhibition remains to be investigated, but this effect could be related to the oxidant-scavenging properties of bilirubin (90) or a di-

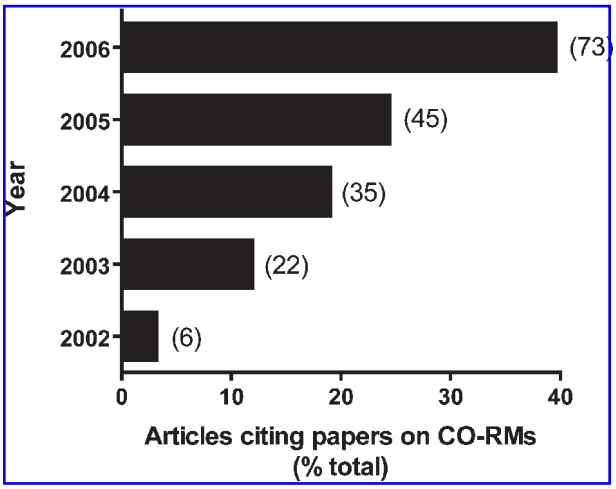

FIG. 4. Analysis of articles citing CO-RMs. This graph illustrates the percentage of articles citing CO-RMs each year since the first publication on CO-releasing molecules was published in 2002 (93). The percentage is calculated based on the total number of citations, and in brackets is the number of articles quoting CO-RMs per year, as analyzed by ISI Web of Science (http://wos.mimas.ac.uk/).

rect or indirect inhibition of NADPH oxidase activity by this bile pigment (74, 76) or both.

As outlined earlier, the other product of heme degradation by HO-1 (CO) interacts preferentially with heme- and transition metal–containing proteins. Although a substantial number of reports corroborated that CO in cells and tissues binds to and inhibits cytochrome oxidase activity, consequently affecting mitochondrial redox signaling and function (see later in more details), recent studies have shown a "nonmitochondrial" mechanism that involves a direct binding of CO to the heme of NADPH oxidase, leading to downstream changes of redox signaling events. Experimental evidence by Taille and co-workers (151) showed that in human airway smooth muscle cells and neutrophils, exogenous CO liberated at low micromolar concentrations from CORM-2 can inhibit NADPH oxidase activity, as evidenced by a decrease in $O_2^{\bullet -}$ production (assessed by the superoxide dismutase–inhibitable cytochrome c reduction assay). This inhibition was obtained with "noncytotoxic" concentrations of CORM-2 (1–50 μM) (93) and was associated with a change in the spectrum of the heme-containing membrane-bound component of the NADPH oxidase, confirming a direct interaction of CO with the heme moiety. These results are in agreement with a previous publication showing that after addition of CO gas to either intact cells or membrane fractions, the

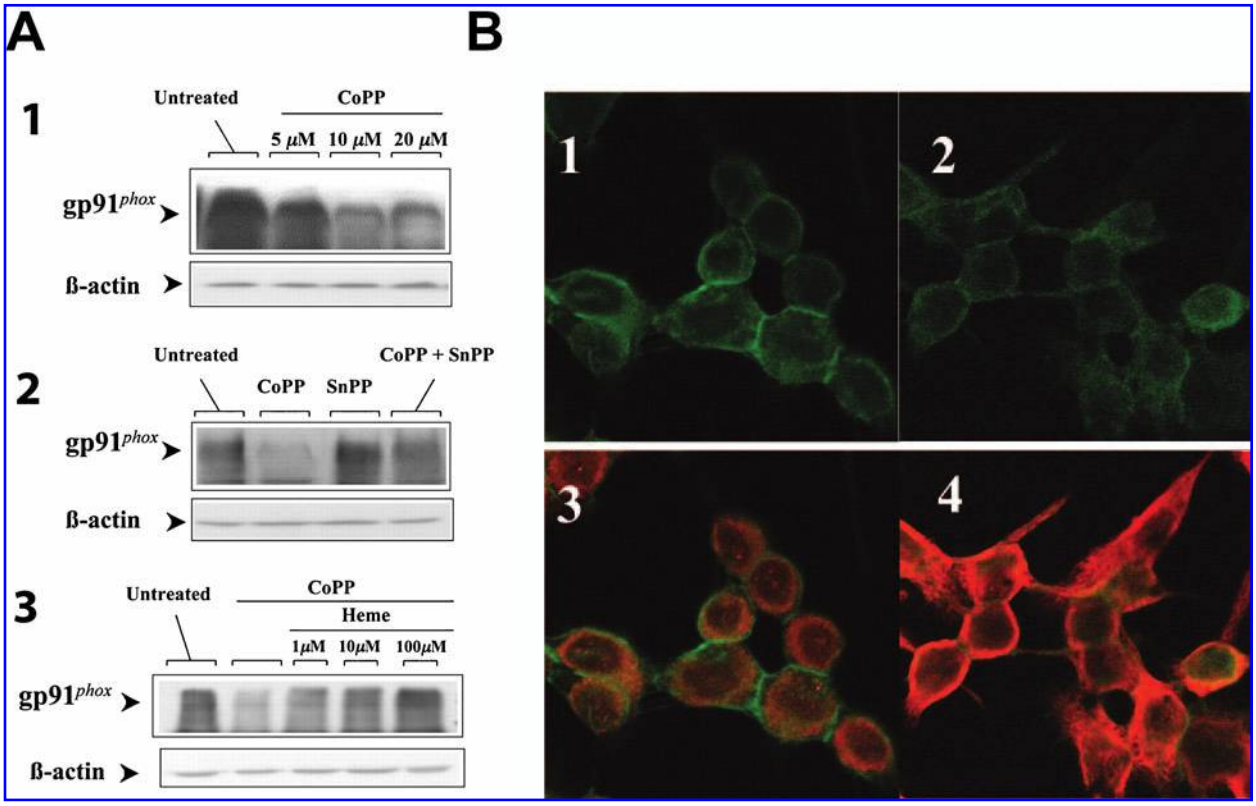

FIG. 5. Effect of HO-1 on NADPH oxidase expression. (**A**) Western blot analysis of gp91^{phox} and β-actin expression after murine macrophages were treated with (a) the HO-1 inducer cobalt protoporphyrin (CoPP, 5–20 μM); (b) the HO inhibitor tin protoporphyrin IX (SnPP, 20 μM) alone or in combination with CoPP; and (c) CoPP plus increasing concentrations of hemin (1–100 μM). (**B**) Laser confocal microscopy analysis of gp91^{phox} expression (*green*) and HO-1 (*red*) in nontreated cells (1 and 3, respectively) or cells treated for 24 h with 20 μM CoPP (2 and 4). Modified with permission ref. 150. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

spectrum of reduced cytochrome changed to that typical of a heme–CO complex (30). This NADPH oxidase inhibition by CO seems to be associated with antiapoptotic effects (164). Another recent study showed that CO from enhanced HO activity or liberated by CORM-2 inhibits $O_2^{\bullet-}$ production in LPS-stimulated murine macrophages (139). Notably, redox signaling events occurring in mitochondria can modulate intracellular ROS production by the Nox system. Desouki and co-workers (34) have shown that mitochondrial ROS induce Nox1 expression and activity in breast and ovarian tumor cells (34). Therefore, the amount at which CO modulates redox signaling could depend on the interplay between the different oxidants-producing systems. The decrease in cell viability observed in rat cardiomyocytes challenged with either paraquat-induced ROS production or hypoxia–reoxygenation can be markedly attenuated by CORM-3 (1–50 μM); the contribution of CO in inhibiting NADPH oxidase in this cell type has not been investigated. However, the role of CO in controlling redox reactions from systems generating ROS could have a major impact in conditions of hypoxia or ischemia, as ROS production is enhanced under these conditions (53, 82). It is plausible to suggest that CO could better compete with O_2 and other gaseous molecules (*e.g.*, NO) for heme-dependent targets when intracellular O_2 concentration is decreased or NO bioavailability is impaired or both. This is particularly relevant to NADPH oxidase, as the activity of this enzyme is profoundly affected in conditions of reduced O_2 tension, leading to endothelial dysfunction and several vascular disorders (48, 79). Future studies in this direction will provide important information on whether controlled amounts of CO delivered to specific cells or tissues under limited oxygen availability will result in major antioxidant effects.

INTERACTION BETWEEN THE HO/CO SYSTEM AND NO SYNTHASE

Nitric oxide synthase (NOS, EC 1.14.13.39) generates NO in mammalian cells. Although none of these isoenzymes has a tissue-specific expression, the family of NOS is categorized into three thoroughly characterized isozymes (55, 165): the neuronal (nNOS, type 1), the endothelial (eNOS, type 3), and the inducible (iNOS, type 2) isoforms. Neuronal NOS and eNOS are constitutively expressed, and their activity is Ca^{2+} dependent, whereas the iNOS enzyme is Ca^{2+} independent. The subcellular localizations of NOS proteins vary within the cell. eNOS can be inserted in plasmalemmal caveolae, whereas nNOS is bound to the sarcolemma of skeletal muscle fibers via its interaction with syntrophin, and iNOS is most likely cytosolic. Interestingly, NOS isoforms can be also localized in the mitochondria; this localization varies depending on the conditions and the type of tissue being considered (20, 51). All NOS isoforms are homodimeric heme-containing proteins with a monomeric molecular mass of ~126–160 kDa, use L-arginine and molecular O_2 to produce NO and L-citrulline, and are inhibited by L-arginine analogues.

NO is a free radical that possesses a highly selective chemical reactivity, which is somehow restricted to (a) high-affinity binding with transition metal centers in proteins (12), (b) nitrosylation of cysteine residues of target proteins (140), and (c) rapid reactions with $O_2^{\bullet-}$ and other free radicals (12). The broader chemistry of NO is, however, extremely complex, as it involves an array of interrelated redox forms, such as nitrosonium cation (NO^+) and nitroxyl anion (NO^-) (143). Binding with transition metal centers is involved in the reaction of NO with different enzymes present in the mitochondrial respiratory chain (see later) and with soluble guanylate cyclase, a heme-containing protein (6) responsible for the formation of cyclic guanosine monophosphate (cGMP). As anticipated earlier, this small cyclic nucleotide is involved in different transduction pathways and physiologic effects such as smooth muscle relaxation, inhibition of platelet aggregation, and decrease in cell proliferation (57, 102). Nitros(yl)ation of cysteine residues is also an important pathway in NO signaling. S-nitrosylation appears to serve as a functional switch in the control of gene expression and regulation of protein activity. For example, poly-S-nitrosation of several free thiols contained in the cardiac ryanodine receptor induces reversible activation of this protein (170). In contrast, S-nitrosylation, can inhibit the activity of caspases, a family of cysteine proteases that execute programmed cell death (85). Furthermore, thiols and cysteine-containing systems seem to be crucial for NO binding, stabilization, and transport in biologic systems (142). Conversely, excessive production of NO in combination with increased ROS leads to the formation of reactive nitrogen species (RNS), resulting in a well-characterized impairment of the cellular redox balance known as "nitrosative stress" (141). This is exemplified by the key reaction of NO, which, under conditions of increased oxidant stress, leads to the formation of the highly damaging species peroxynitrite. Peroxynitrite is produced by the near diffusion–limited reaction of $O_2^{\bullet-}$ and NO with a very rapid reaction rate of 2×10^{10} M/sec (68). Although SOD concentrations in the micromolar range can prevent the formation of peroxynitrite *in vivo* (13), the second-order rate constant of the disproportionation of $O_2^{\bullet-}$ by copper-zinc and manganese-superoxide dismutase is one order of magnitude slower than the reaction between NO and $O_2^{\bullet-}$. Thus, formation of peroxynitrite appears to constitute a real threat to the redox equilibrium, suggesting that overproduction of RNS must be precisely controlled by protective antinitrosative systems to maintain cellular homeostasis (97).

Many similarities and interrelations are found between HO and NOS enzymes. Both families have constitutively expressed and inducible isoforms, and both generate diatomic gases as final products. As outlined earlier, the gases CO and NO produced by these two systems exert similar biologic effects contributing to regulation of vascular and bronchial tone, cell proliferation, apoptosis, inflammation, and platelet aggregation. CO, like NO, binds to the heme of the prototypic target guanylate cyclase, although with a lower degree of activation compared with NO (121, 146), and this partially explains why additional targets for CO are being investigated. The similarity of the two families of enzymes and the emerging roles of gaseous molecules in physiology and medicine points to possible cross-talk between these systems and raises the question of how NO and CO dynamically coordinate different responses in cells and tissues. The fact that inducible forms of HO-1 and

iNOS respond to and can be overexpressed by an array of similar stimuli and conditions, including ROS, oxidative stress, hypoxia, and inflammatory cytokines, is another indication of a possible direct interaction between these two pathways (44). Intriguingly, and this is perhaps the most striking link between NO and the HO system, mammalian cells are highly susceptible to HO-1 induction by nitric oxide (NO) and its redox-activated forms (NO^+ and NO^-) (97). This aspect initially originated from the observation that NO^+-donating compounds, such as sodium nitroprusside (SNP) and S-nitroso-*N*-acetylpenicillamine (SNAP), can highly increase HO activity in endothelial cells (95). Subsequently, it was demonstrated that activation of endothelial HO by NO^+ releasers is strictly associated with overexpression of the HO-1 protein, and this effect can be extended to NO, NO^-, and peroxynitrite (41, 45, 97, 98, 105).

The finding that enhanced HO-1 expression by NO redox forms has been confirmed in different cell types *in vitro* (2, 16, 31, 35, 69, 105, 159), in tissues *ex vivo* (127), as well as in *in vivo* systems (89, 98) points to HO-1 being a crucial inducible system in the cellular adaptation not only to oxidative but also to nitrosative reactions (97). It has also been demonstrated that increased endogenous NO levels as a result of increased iNOS expression lead to upregulation of HO-1 (35, 94, 95, 172). In addition, bilirubin has been reported to possess "antinitrosative" properties (65, 84, 90), and recent *in vivo* studies reveal that continuous administration of organic nitrates and sodium nitroprusside is associated with both hepatic HO-1 expression and augmented carboxyhemoglobin levels (83, 89). More recently, our group showed that heme or methemoglobin (metHb), which is highly damaging to endothelial cells and is known to upregulate HO-1 (8, 9, 60), act synergistically with NO redox forms to amplify HO-1 expression and activity and maximize heme uptake (Fig. 6). These data corroborate a strong association between increased heme degradation–derived products and attenuation of oxidative/nitrosative stress (40, 43, 83, 89). Collectively, these results suggest that the products of heme degradation may be crucial to counteract effectively the cytotoxic actions of excessive amounts of NO (44, 97). A primary contribution to this effect may be found in the ability of CO to bind to the heme of iNOS protein (166). For instance, pharmacologic inhibition of HO activity in isolated smooth muscle cells (22) and macrophages (155) results in increased NO production, suggesting that HO may exert an inhibitory effect on NOS. Turcanu and co-workers (139) demonstrated that, in macrophages, inhibition of NOS activity by HO is not associated with a decrease in NOS mRNA expression, suggesting a post-transcriptional mechanism of regulation. This mechanism seems in part related to CO production, because inhibition of endotoxin-mediated increase in NO by enhanced HO activity can be reproduced by a CO-RM. Similarly, in endotoxin-stimulated macrophages, the increased iNOS protein expression is unaffected by both CORM-2 and CORM-3 despite nitrite levels being significantly reduced by these compounds in a dose-dependent manner (133). Nevertheless, the antiinflammatory activity of CO seems to be more complex than a simple direct inhibition of the NO-generating enzyme, as other inflammatory mediators (*i.e.*, TNF-α) are also inhibited by CO gas and CO-RMs (Fig. 7). In a rat model of lipopolysaccharide-induced multiorgan failure, Sarady and co-workers (131) showed that exposure to CO had opposite effects in lung and liver, with prevention of NO overproduction in lung and an increase in NO production in liver. In the same study, CO exposure led to a reduction in lung alveolitis, a diminution of serum alanine aminotransferase, and an increase in rat survival. CO overproduction from enhanced HO-1 activity has been demonstrated to provide cytoprotective effects against the toxicity caused by overproduction of NO in animal experimental models of endotoxemia (130), hemorrhagic shock (153), ischemia/reperfusion (4), and xenograft rejection (138). Thus, the increase in HO-1 expression by NO can act as negative feedback on NO production to limit its cytotoxic effects; it is important to emphasize that the effect of CO on NO production may differ in each organ, but the data so far suggest that the HO/CO might serve as a system to control iNOS activity and its detrimental effects on tissues.

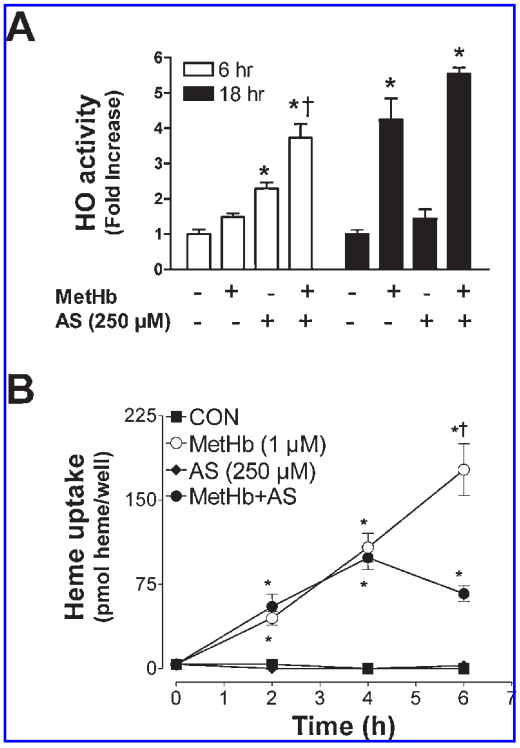

FIG. 6. Heme interacts with NO redox forms to amplify endothelial HO activity and heme uptake. (**A**) Endothelial cells were exposed to 1 μM methemoglobin (MetHb) in the presence or absence of 250 μM Angeli's salt (AS, a generator of NO^-), and heme oxygenase activity was measured after 6 or 18 h. Cells were also exposed to AS or medium alone (CON). (**B**) For the heme-uptake experiments, cells were exposed to medium alone (CON), Angeli's salt (AS), or MetHb in the presence or absence of AS. The intracellular heme levels were assessed after 0-, 2-, 4-, or 6-h incubation. $^*p < 0.05$ *versus* CON; $^\dagger p < 0.05$ *versus* each different hemoglobin alone. Modified with permission from ref. 40.

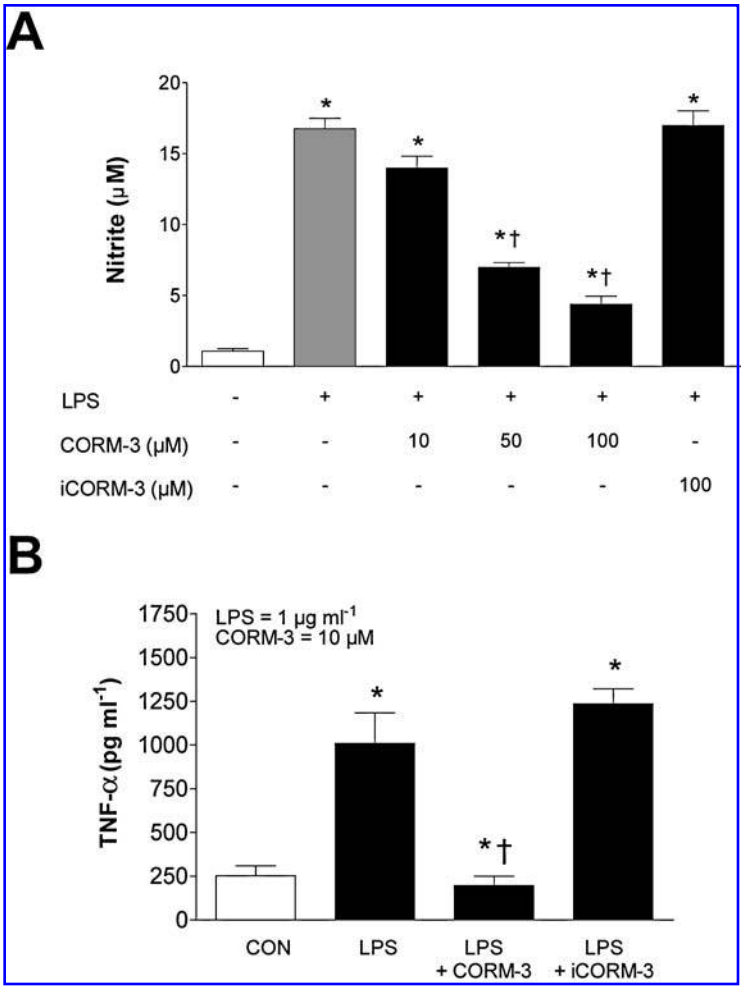

FIG. 7. Antiinflammatory actions of CORM-3. (**A**) RAW264.7 macrophages were exposed to 1 μg/ml lipopolysaccharide (LPS) in the presence or absence of CORM-3 (10–100 μM), and nitrite production was assessed at 24 h. The inactive compound iCORM-3 (100 μM) was also used to determine the contribution of CO released by CORM-3 to the observed effect. *$p < 0.05$ *versus* control (white bar); †$p < 0.05$ *versus* LRS alone (gray bar). (**B**) Macrophages were exposed to 1 μg/ml LPS in the presence or absence of 10 μM CORM-3 or iCORM-3, and the levels of TNF-α in the culture medium were measured after 24 h. *$p < 0.05$ *versus* CON; †$p < 0.05$ *versus* LPS alone. Modified with permission from ref. 134.

HO/CO INTERACTION WITH MITOCHONDRIAL CYTOCHROME OXIDASE

High concentrations of CO in mammalian cells have deleterious effects by different mechanisms, including binding to the heme moiety of mitochondrial enzymes, such as in cytochrome c oxidase. However, interaction of physiologic and nontoxic concentrations of CO with the respiratory components of the mitochondria may have important metabolic effects. Redox respiratory components are thermodynamically organized to provide electrons to cytochrome oxidase (cyt *a*-*a*3). This terminal enzyme catalyzes electron transfer from cytochrome c to reduce molecular O_2 to H_2O; although CO_2 and H_2O are equally produced during biologic or chemical combustions, in living organisms, energy is obtained mainly from water synthesis, whereas in the case of chemicals, the energy comes from carbon oxidation to CO_2. Cytochrome oxidase belongs to the heme-copper superfamily, having common features with phylogenetically distant organisms, like plants, archaea, and mammals (117); the inertness of O_2 requires that its degradation proceed through metal active sites of the respiratory oxidases (135). To accomplish electron transfer, cytochrome oxidase has four redox-active sites: two hemes, low-spin *a* and a_3, and two Cu^{2+} sites (CuA and CuB). In recent years, structural information emerged from crystallographic analysis and confirmed that cyt α-α3 is in general composed of several polypeptides, which integrate up to VIII subunits in the beef-heart oxidase. Three subunits, I, II, and III, that are encoded by mitochondrial DNA contain the metals and participate directly in O_2 reduction. It is noteworthy that fast internal electron transfer occurs among these subunits. Likewise, electrons associate at a diffusion-controlled rate to Cu_A in subunit II as initial acceptor, and afterward, they are derived to heme a in an intramolecular reaction with an apparent rate constant of $\sim 2 \times 10^4$/sec, and finally, to binuclear center a_3-Cu_B in subunit I, which binds O_2 as terminal acceptor (37). The other 10 subunits, IV, Va and b, VIa-c, VIIa-c, and VIII, are nuclear encoded, surround and stabilize the central structure, and contribute to dimerization. Finally, the 13 subunits dimerize with themselves (26 subunits), and the functional protein is ~400 kDa.

This particular structure allows cytochrome oxidase to be finely regulated. The kinetics of respiratory chain has been extensively studied to establish where the weight of the control

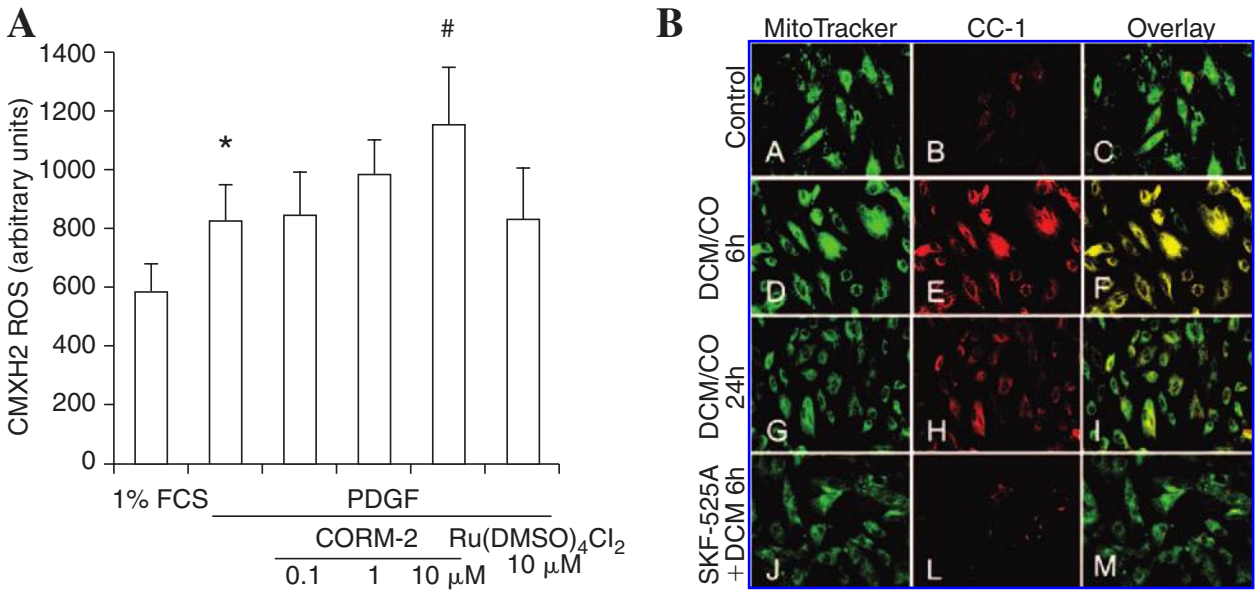

FIG. 8. Effect of CO on mitochondrial ROS production. (**A**) The effect of CORM-2 on PDGF-induced mitochondrial ROS production in airway smooth muscle cells was assessed by measurement of the specific MitoTracker CM-H_2XRos oxidation. Modified with permission from ref. 151. $^{*}p < 0.05$ *versus* 1% fetal calf serum (FCS); $^{\#}p < 0.05$ *versus* PDGF alone. (**B**) ROS localization in the cardiomyocyte cell line H9c2 after exposure to CO. Cells were treated with dichloromethane (DCM), which is metabolized intracellularly by cytochrome P_{450} enzymes to release CO. Mitochondrial localization and ROS production were assessed by a mitochondrial-selective dye MitoTracker (*green*) and a redox sensor CC-1 (*red*), respectively. (**A–C**) Control cells. (**D–F**) For 6 h. (**G–I**) Cells exposed to CO (100 μM DCM) for 24 h. (**J–M**) Preincubation with cytochrome P_{450} inhibitor (SKF-525A) followed by CO exposure (100 μM DCM). Merged images for MitoTracker and CC-1 (**C, F, I, M**), indicate production of ROS inside the mitochondria. Modified with permission from ref. 148. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

of electron flow, and therefore, of oxidative phosphorylation resides. Most authors think that ATPase, succinate-cytochrome c reductase, NADH-ubiquinol reductase, and cytochrome oxidase are main steps of regulation; other experimental data and predictive computational studies indicate cyt a-a_3 as the rate-limiting step for the electron-transfer chain (72), whereas the other steps are close to thermodynamic equilibrium. On the basis of very low K_m for O_2 ($\sim 10^{-8}$ M), the *in vivo* rate reaction for cytochrome oxidase depends on O_2 at only very low concentrations. In addition, rapid transition of electron flow by increased demands is based on the ATP/ADP ratio. In recent years, NO and CO gained significance with respect to cytochrome oxidase activity (67). Almost a decade ago, several investigators observed a rapid and reversible inhibition of cytochrome oxidase and of O_2 uptake by NO (119). Although the effects were considered toxic in initial work, we postulated a physiologic resource to adapt O_2 utilization to availability, to environmental changes, or to endocrine effects (21). The effect of NO relies on the formation of Cu^{+}_{B}-a_3-NO, which proceeds independent of substrate and with high affinity (10^9 per M; k_{on}, 1×10^8 M/sec) (17); it is apparent that NO can react with fully oxidized or reduced cytochrome oxidase. Interestingly, we reported that NO utilization produces O_2 active species via ubiquinol, and depletion of endogenous mitochondrial ubiquinol leads to enormously prolonged O_2-uptake inhibition (120); inhibition of terminal oxidase increases reduction on the substrate side and favors monovalent electron transfer directly from semiubiquinone radical to O_2 and forms $O_2^{\bullet -}$. From our perspective, little NO is converted to nitrite, and most reacts with $O_2^{\bullet -}$ to generate peroxynitrite ($ONOO^-$). Therefore, formation of $O_2^{\bullet -}$ and $ONOO^-$ is required to clear NO, and the formation of $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) participates in cell signaling inside and outside mitochondria (19).

Both NO and CO are produced in cytosol and mitochondria (29). It is still difficult to understand a scheme of combined physiologic effects of both small gases in the organelles. Differently, NO inhibits electron transfer at sites other than cytochrome oxidase, whereas CO reacts only with reduced cytochrome oxidase, depends on substrate, and the reaction occurs with a lower reaction constant rate ($k_{on} = 0.8 \times 10^5$ M/sec). As with NO, CO increases mitochondrial H_2O_2 generation. We have shown in human airway smooth muscle in culture that exogenous CO delivered by CORM-2 (10 μM) inhibits muscle proliferation by stimulating ROS production in the mitochondria (151) (Fig. 8A). This was the first demonstration that effects of nontoxic concentrations of CO could be secondary to an increase in mitochondrial ROS production. Similar results were shown later by Suliman and co-workers (148), who demonstrated an increased H_2O_2 production in H9c2 cardiomyocytes exposed to dichloromethane, which generates CO after being metabolized by cytochrome P450 enzymes (see Fig. 8B). In contrast to NO, CO upregulates SOD-2 expression, which scavenges $O_2^{\bullet -}$ and consequently augments mitochondrial H_2O_2 release (173). Increased mitochondrial ROS production

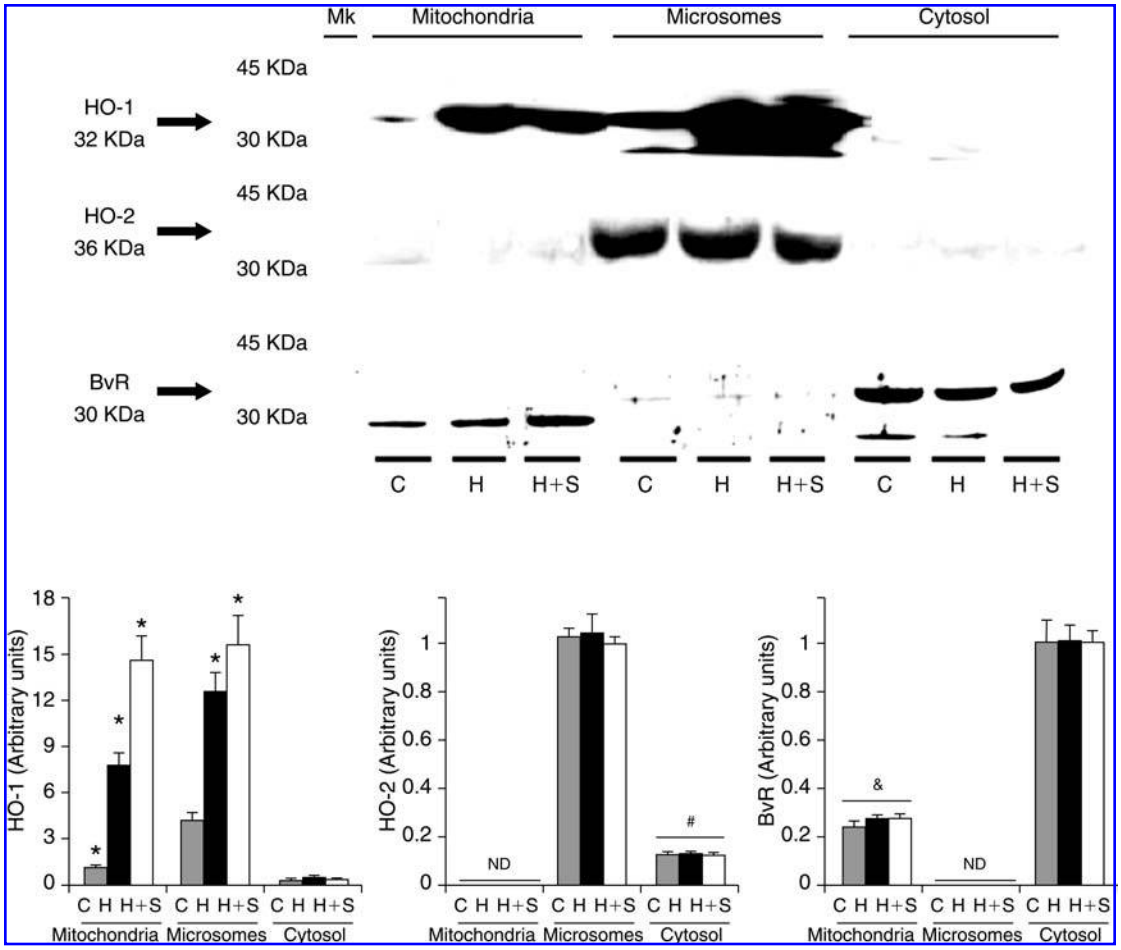

FIG. 9. HO and biliverdin reductase (BvR) expression in microsomes and mitochondria. Typical Western-blot analysis of HO-1, HO-2, and BvR expression in liver mitochondria, microsomes, and cytosol of untreated rats (C) and rats treated with hemin (H) or hemin plus tin-protoporphyrin-IX (H+S). Densitometric analysis of protein expression is shown in the lower part of the panel. $^{*}p < 0.05$ *versus* the respective control organelle; $^{\#}p < 0.05$ *versus* the microsomes; $^{\&}p < 0.05$ *versus* cytosol. Modified with permission from ref. 29.

by CO is likely to have signaling properties (54), as it can (a) decrease smooth muscle cyclin D1 expression, resulting in inhibition of muscle proliferation (151); and (b) increase Akt activation through an established oxidant mechanism involving NRF1, which, in concert with NRF2, activates *Tfam* phosphorylation and its binding to *Tfam* promoter in cardiomyocytes, leading to mitochondrial biogenesis (148). The effect of modest noncytotoxic increases in cellular CO on mitochondrial biogenesis is of both physiologic and pathophysiologic importance. Interestingly, in parallel with these effects on redox signaling, CO can protect mitochondria against oxidative stress. Compounds that liberate small amounts CO (CO-RMs) are capable of modulating respiration in isolated mitochondria and can improve the respiratory control index of mitochondria from kidneys exposed to cold ischemia and reperfusion, showing protective effects of CO against oxidative stress (129).

CO produced endogenously by heme degradation by HO-1 should have some different effects from those of exogenous CO, because HO-1 can degrade heme necessary for NOS activity located in mitochondria (29). In this case, we can speculate that CO decreases NO-dependent inhibition of O_2 uptake and contributes to clearing NO without eliciting excessive $O_2^{\bullet -}$ and $ONOO^-$ formation. In line with this concept, it has been reported that upregulation of HO-1 in the mitochondria (Fig. 9) decreases mitochondrial NOS expression and activity as well as NO-dependent H_2O_2 production, without affecting maximal mitochondrial H_2O_2 levels (29).

Figure 10 represents a schematic summary of the effects of the HO/CO pathway on the mitochondrial electron-transport chain and ROS production. Future studies in this direction will help to define the exact physiologic significance of low doses of CO in mitochondria.

CYTOCHROME P_{450} ENZYMES ACTIVITY: MODULATION BY THE HO/CO PATHWAY

Cytochrome P_{450} proteins are a very large family of enzymes and proteins named because their CO-bound complex forms reveal an absorption band at 450 nm (70). Cytochrome super-

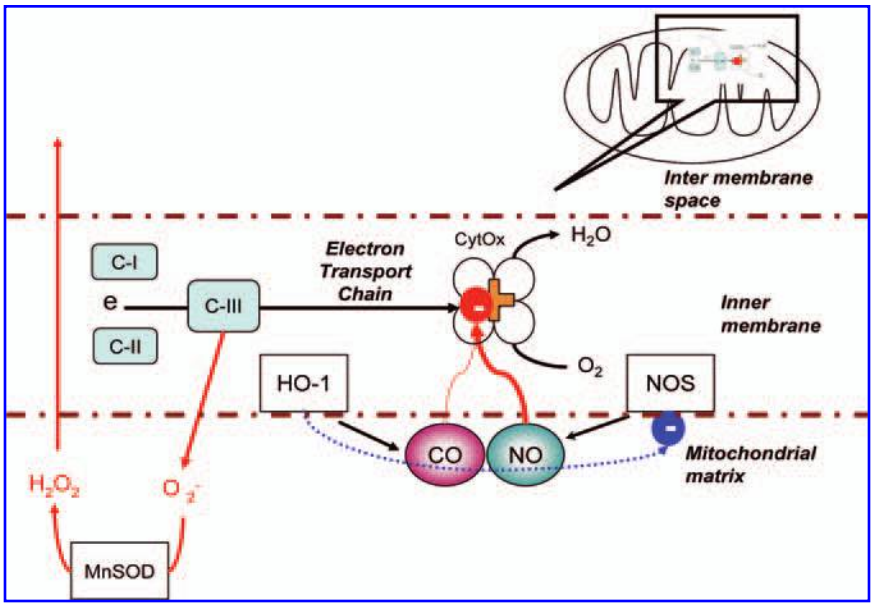

FIG. 10. HO/CO interaction with mitochondrial electron-transport chain. Schematic illustration representing the way CO interacts with mitochondrial components, thereby regulating mitochondrial function. CO binds to heme-bound Fe^{2+} in cytochrome oxidase (CytOx) with less affinity than NO. This inhibition modulates, in turn, electron transport, leading to production of small amounts of $O_2^{\cdot -}$ that is converted to H_2O_2 by a manganese-dependent superoxide dismutase (MnSOD). H_2O_2 then diffuses from mitochondria to cytosol, where it has signaling properties. This pathway may explain some of the signaling effects of exogenous CO. In the case of endogenous CO produced by mitochondrial HO, the picture is probably more complex. If, on one side, endogenous CO acts similar to exogenous CO, on the other side, mitochondrial HO may decrease mitochondrial NOS expression and activity by degrading heme. As a consequence of this effect, an attenuation of the electron chain transport inhibition elicited by NO occurs. Moreover, endogenous CO could directly inhibit mitochondrial NOS by binding to its heme moiety. The overall effect of mitochondrial HO on reactive oxygen species production should be the results of these two antagonistic mechanisms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

family genes (CYPs) are classified by following the recommendations of a nomenclature committee (106) on the basis of amino acid identity, phylogenetic criteria, and gene organization. The symbol CYP is followed by a number for families, a letter for subfamilies, and a number for the gene. The cytochrome P_{450} genes are found in the genome of almost all organisms from prokaryotes to eukaryotes (plants and animals), being soluble in prokaryote, and usually bound to the endoplasmic reticulum or inner mitochondrial membranes in eukaryotes. These proteins are heme-thiolate proteins, and their most-conserved structural features are related to heme binding (110). They catalyze hydroxylation reactions involving the uptake of a pair of electrons from NADPH with reduction of one atom of O_2 to H_2O and incorporation of the other atom in the substrate. Among the extremely diverse functions, these enzymes are known particularly for their role in steroid hormone and xenobiotic metabolism.

Carbon monoxide (CO) can bind to the heme moiety of cytochrome P_{450} and prevent O_2 binding and activation. Although the partial pressure of CO is physiologically too low to inhibit this class of enzymes, it seems that at toxic levels, CO could significantly bind to it and cause biologic effects such as changes in xenobiotic metabolism and vascular tone. The effect of CO binding to cytochrome P_{450} has been hypothesized to explain inhibition of drug metabolism after exposure to CO gas (91), but further studies attributed this effect to cellular hypoxia induced by the high affinity of CO with hemoglobin (124). Nevertheless, it has been shown in fetal rats that CO can inhibit the transformation of the proteratogenic chemical 2-acetylaminofluorene to its teratogenic form by cytochrome P_{450} (38). The CO/O_2 ratio needed to inhibit cytochrome P_{450} is different for each isozyme. This differential inhibition by CO may be a useful tool to identify specific human cytochrome P_{450} isozymes in the early screening of drug-biotransformation catalysts (78).

If CO can bind cytochrome P_{450}, it can also be a product by this enzyme. Lipid peroxidation in rabbit liver microsomes produces CO, and this production is not inhibited by Zn-protoporphyrin IX (an inhibitor of HO activity), suggesting an HO-independent pathway. Conversely, addition of cytochrome P_{450} typical inhibitors SKF 525A and metyrapone had an inhibitory effect on the CO formation rate (5). Some cytochrome P_{450} isozymes may induce HO-1 expression, as has been demonstrated for CYP2E1. CYP2E1 is involved in oxidative injury, and HO-1 induction was observed in the livers of alcohol-fed mice, a condition known to elevate CYP2E1 levels (52). Furthermore, increased levels of HO-1 were observed in HepG2 cells overexpressing CYP2E1 (E47 cells) compared with control HepG2 cells. Catalase and PD98059, a specific inhibitor of ERK MAPK, blocked the HO-1 induction (52). These results suggest that HO-1 expression induced by CYP2E1 is mediated by ERK and oxidants.

From the limited data available in the literature, it is clear that a fundamental and direct role for HO-derived CO in the regulation of cytochrome P_{450} by binding to the heme domain remains to be thoroughly investigated. However, starting from the assumption that a particular signaling outcome comprises an integrated network of signaling pathways that function in a precisely coordinated manner, we cannot rule out *a priori* a plausible important contribution of CO in controlling cytochrome P_{450} activity. For instance, circumstantial evidence indicates that CO can participate in the regulation of vessel tone, not only as an effector of vasodilatory mediators, but also as an antagonist of vasoconstrictor agents. A cytochrome P_{450} appears to be involved in the onset of pulmonary vasoconstriction during hypoxia in pigs, an effect that can be attenuated by

inhalation of 11.5% CO gas (149); these findings were reproduced with similar results in dogs (87). In the ductus arteriosus, CO elicits complete relaxation, and its action is reversed by illumination with monochromatic light, whose photoactivation peaks at 450 nm, suggesting the involvement of a cytochrome P_{450} enzyme (1). Reduction in vascular resistance in perfused rat livers by CO overproduced by HO-1 is also linked to a change in total cytochrome P_{450} activity (160). Although in that study, a direct link with a specific cytochrome P_{450} enzyme was not determined, CO-mediated hepatic vasorelaxation is known to be simulated by inhibitors of cytochrome P_{450} monoxygenases (75). A possible contribution of CO in inhibiting a cytochrome P_{450} isoform (CYP4A), thereby limiting the production of the vasoconstrictor 20-HETE, has also been reported in response to the vasoreactivity of renal arteries to phenylephrine and vasopressin (64). These examples are another demonstration that the ability of CO to promote a specific pharmacologic effect (*i.e.*, vasorelaxation) cannot be confined to a single cellular target (*i.e.*, guanylate cyclase) but is likely to involve the simultaneous regulation of a group of targeted enzymes that act in synergy to amplify a specific effect of CO. Although major criticisms have been made of the fact that, at equal concentrations, CO is a much weaker vasodilator compared with NO, much more attention should be given to the spatial and temporal distribution of CO in a given condition or microenvironment rather than arguing about its potency and amounts being produced. Experiments designed specifically to address these paradigms should enable scientists to define the key elements that determine signaling responses to CO.

SUMMARY AND FUTURE PERSPECTIVES

We are gradually learning that heme-containing proteins involved in redox signaling and inflammation are targets of heme oxygenase and CO. Therefore, the coordinated control of the function of these heme proteins can explain the diverse and ubiquitous protective properties of the HO/CO pathway. However, without sophisticated biochemical means that could clearly distinguish the specific effect of HO products *in vivo*, the relative importance of heme degradation, CO, and biliverdin in physiology and disease remain in question. For instance, is there a hierarchy among the metal-containing targets of HO/CO in a given cell? In other words, is the binding of CO to intracellular targets dependent on their intrinsic affinity for this gas, or do other mechanisms explain this process? What is the interplay between CO, NO, and O_2 in their interaction with heme- and metal-center proteins? What are the differences between exogenously and endogenously produced CO? We envision that in the near future, a multidisciplinary collaboration between chemists and biologists in the HO/CO field will enable scientists to address and possibly answer these and other important questions.

ACKNOWLEDGMENTS

Jorge Boczkowski is a recipient of a Contrat d'Interface INSERM-Assistance Publique–Hôpitaux de Paris, France. Mathieu Desmard is recipient of a Poste d'Accueil INSERM. The works done in Dr. Poderoso's Laboratory had financial support from UBACYT, CONICET, FONCYT, and the Fundación Perez Companc, Buenos Aires, Argentina. Dr. Roberto Motterlini is funded by the Kidney Research UK and the Henry Smith Charity.

ABBREVIATIONS

cGMP, cyclic guanosine monophosphate; CO, carbon monoxide; CO-RMs, carbon monoxide-releasing molecules; CORM-A1, sodium boranocarbonate; CORM-1, dimanganese decacarbonyl (Mn_2CO_{10}); CORM-2, tricarbonyldichloro ruthenium dimer ($Ru(CO)_3Cl_2$-dimer); CORM-3, tricarbonylchloro ruthenium glycine ($Ru(CO)_3Cl$-glycinato); CYP, cytochrome P_{450}; cyt a-a^3, cytochrome oxidase; EDRF, endothelium-derived relaxing factor; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; NO, nitric oxide; NOS, nitric oxide synthase; NO^+, nitrosonium cation; NO^-, nitroxyl anion; $O_2^{\bullet -}$, superoxide anion; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNAP, S-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

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Date of first submission to ARS Central, June 25, 2007; date of acceptance, July 1, 2007.

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