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Carbon Monoxide, Reactive Oxygen Signaling, and Oxidative Stress

Claude A. Piantadosi

Duke University Medical Center, Durham, NC 27710 USA

Abstract

The ubiquitous gas, carbon monoxide (CO), is of substantial biological importance, but apart from its affinity for reduced transition metals, particularly heme-iron, it is surprisingly non-reactive—as is the ferrous-carbonyl—in living systems. CO does form strong complexes with heme proteins for which molecular O₂ is the preferred ligand and to which are attributed diverse physiological, adaptive, and toxic effects. Lately, it has become apparent that both exogenous and endogenous CO produced by heme oxygenase engender a pro-oxidant milieu in aerobic mammalian cells which initiates signaling related to reactive oxygen species (ROS) generation. ROS signaling contingent on CO can be segregated by CO concentration-time effects on cellular function, by the location of heme proteins, e.g. mitochondrial or non-mitochondrial sites, or by specific oxidation-reduction (redox) reactions. The fundamental responses to CO involve overt physiological regulatory events, such as activation of redox-sensitive transcription factors or stress-activated kinases, which institute compensatory expression of anti-oxidant enzymes and other adaptations to oxidative stress. In contrast, responses originating from highly elevated or protracted CO exposures tend to be non-specific, produce untoward biological oxidations, and interfere with homeostasis. This brief overview provides a conceptual framework for understanding CO biology in terms of this physiological-pathological hierarchy.

Keywords

heme oxygenase; reactive oxygen species; nitric oxide; mitochondria; heme proteins

Introduction

Carbon monoxide (CO) is a primordial gas that people have long equated with incomplete combustion and silent asphyxiation. However, since the 1950's CO also has been recognized as an endogenous product of heme metabolism¹ and today its importance is appreciated more prevalently in biology than even a decade ago.² It is known, for instance, that CO is produced and metabolized by organisms from prokaryotes to mammals and serves as an important intrinsic signaling molecule predominantly via its propensity to bind to reduced transition-metal centers in heme proteins³⁻⁵.

Correspondence: Dr. Piantadosi, Department of Medicine, Box 3315 CR II Building White Zone, Duke University Medical Center, Trent Drive, Durham, NC 27710 USA, Telephone: 919.684.6143, Fax: 919.684.6002, E-mail: piant001@mc.duke.edu.

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In the bacterial world, CO is a central metabolite in the anaerobic carbon cycle, and some aerobic chemotrophs even utilize it as their sole source of energy.^{6,7} In these microbes, as well as in the endosymbiotic mitochondria of eukaryotes, CO undergoes oxidation to CO₂.⁸ In higher animals, especially vertebrates, CO is recognized as a by-product of the normal enzymatic degradation of heme by the heme oxygenases (HO).⁹ The excess CO is carried by hemoglobin to the lungs for excretion, while that in the cell services physiological processes from platelet aggregation¹⁰ to vasodilation¹¹ to apoptosis—including both pro- and anti-apoptotic effects.¹²⁻¹⁸ Moreover, the HO/CO system is part of the integrated defense against cell stress including heat shock, heavy metals, ROS, lipopolysaccharide (LPS), and other inflammatory processes.¹⁹⁻²³

The gaps in our understanding CO biology exist mainly in the biochemical mechanisms of CO signaling in integrated systems—specifically when and how CO's effects are conveyed as physiological signals based on interactions with iron- or other metallo-proteins that contain active-site transition metals. Perhaps most widely appreciated in this regard is CO's activation of guanylate cyclase (GC), a pivotal heme enzyme whose product, guanosine 3,5-cyclic monophosphate GMP (cGMP), is involved in vascular tone, gene regulation, neurotransmission, and many other cellular processes.²⁴⁻²⁷ CO's capacity to activate GC however is much weaker than is its gaseous alternative, nitric oxide (NO), which dominates cGMP-dependent cell signaling.²⁸

Apart from its high affinity for reduced transition metals such as Fe²⁺, CO is notably un-reactive in biological systems, as is the ferrous-carbonyl, which is dissipated slowly by displacement of CO with molecular O₂ or by oxygenation of CO to CO₂.⁸ Nevertheless, CO does not go unattended physiologically, for its binding to reduced transition metal centers is relatively tight, especially among heme proteins for which molecular O₂ is the preferred ligand.²⁹ The formation of metal-CO complexes generates diverse biological effects ranging from those where a specific enzyme is inhibited and the substrate accumulates to those that redirect electrons and lead to reactive oxygen and nitrogen species (RNS) formation. Indeed, NO does induce CO production through up-regulation of HO-1 mRNA and protein, thereby increasing the potential for the interaction of CO with reduced iron and influencing NO signaling independently of GC.³⁰⁻³⁴ Moreover, it has been proposed that CO and NO may in some cases bind different sides of active heme moieties.³⁵

Although modest in number, each CO reaction is potentially important in both cell signaling and oxidative stress, and thus contributes to the theme of this overview, which also places special emphasis on how CO induces adaptive responses to assist mammalian systems in the tolerance of oxidative stress. A discussion of the basic physiological chemistry of CO, O₂ and transition metal centers however is not intended, and the reader is referred to earlier publications for that information.^{4,36-38}

The Realm of CO— Physiology, Adaptation, and Toxicity

From the outset, it is important to place the responses to CO *in vivo* in line with archetypal physiology. In other words, in order to understand CO biology, core physiological processes that are tightly-regulated spatially and temporally need to be differentiated from secondary or adaptive responses to CO arising from interference with physiological reactions or due to secondary ROS or RNS generation. As CO concentration rises, specificity of effect is dissipated, analogous to the progressive cellular effects of oxidative and nitrosative stress. It is also imperative to identify CO toxicity, and in particular, the cellular and tissue responses that compensate for it. Customarily, the inputs and outputs for these contrasting processes are determined by studies of molecular localization, for instance, by measuring the sensitivity and specificity of an exact reaction or pathway to a particular CO concentration-time integral. An

approach to stratification-of-effect is illustrated schematically in Figure 1, which conceptualizes the presumed relationships among the physiological, adaptive, and toxic effects of CO.

The general concept of effect-stratification is straightforward, but as usual, the devil is in the details. Simply put, it is rarely easy to decide when an effect is homeostatic or constitutes physiological adaptation, whether such effects should be lumped or split, or how they merge into pathological responses. For instance, a CO exposure that causes widespread apoptosis in the hippocampus is clearly pathological³⁹ while an ROS-dependent conditioning effect produced by CO against ischemia or hypoxia⁴⁰ would usually be interpreted as adaptive. On the other hand, an arterial system that fails to dilate in response to CO after heme oxidation associated with large-conductance calcium-activated potassium channels implicates a physiological mechanism^{41,42} that may also have relevance to pre-conditioning, while CO-dependent blockade of the elaboration of pro-inflammatory cytokines after exposure of leukocytes to lipopolysaccharide (LPS)⁴³, could be viewed alternatively as adaptation or along the lines of inhibition of normal chemokine or adherence mechanisms. Loss of an innate inflammatory response, however, would be difficult to interpret as immune counter-regulation because the endogenous CO signal is driven strictly by the degradation of heme substrate, which represents the clearance of pro-oxidant or pro-inflammatory substances with intrinsic pathogenic chemistry.

As CO concentration rises, the gas assumes a decided pleiotropy; hence, diverse, overlapping biological processes confound the interpretation of brute-force experiments. For instance, the multifarious literature on CO is frequently inscrutable with respect to when mimicry by exogenous CO can be taken as proof-of-signaling by the heme oxygenase (HO/CO) system, particularly when experimental manipulations involve massive over-expression of HO-1 or the prolonged or administration of excessive CO or CO-donating molecules to cells, tissues, or animals. Close attention to differences in CO effect due to concentration, timing, molecular specificity, and sub-cellular localization are the proximate steps for unraveling the implications of activating HO/CO in intricate injury-response paradigms.

Tissue and Cellular CO Concentrations

A fundamental factor in understanding the effects of CO from the perspective of oxidation-reduction (redox) chemistry is to know how much CO cells and tissues contain, produce normally, and take up exogenously, as well as the extent to which CO concentration varies under different types of stress. Because endogenous HO activity and heme turnover rates are difficult to quantify, it is useful to measure quasi-steady-state CO concentrations in cells and tissues using sensitive analytical techniques. Although most available techniques are destructive or lack sensitivity at the cellular level, the pioneering work of Vreman and Stephenson cannot be undervalued, as it provides an indication of just how low physiological CO levels are normally.⁴⁴⁻⁴⁶ Even so, the dynamic range can be substantial, although the amount of endogenous CO generated to service physiological or even pathological responses is restricted on the one hand by the aforementioned need for heme substrate, and bounded on the other by the capacity of exogenous CO to exceed physiological stringency and overwhelm the cell. Moreover, *in vivo*, the importance of perfusion cannot be ignored, as blood flow optimizes the cellular PO₂ for heme catalysis, and perfusion eliminates CO from cells and tissues via hemoglobin in the circulation.

A key experimental concern is the use of excessive CO to mimic physiological homeostatic or adaptive mechanisms that instead produce compensatory responses to injury. Although tested guidelines have yet to be developed, cellular standards call for CO exposures that avoid macromolecular damage, and also *in vivo*, CO concentration-times that account for the effects

of secondary cellular hypoxia. Adherence to these principles allows a range of tissue CO concentrations to be explored for physiological effects that are below those produced at 20-25% carboxyhemoglobin, the conventional threshold in mammals for serious poisoning (Figure 2). As a rule, the use of slow CO-releasing agents at concentrations of 50 micromolar or less in cells is advisable, and in rodents, inhaled CO concentrations that acutely do not exceed 500 ppm for one hour or 50 ppm continuously. In humans, the OSHA permissible exposure limit (PEL) of 50 ppm for 8 hours and the NIOSH recommended exposure ceiling of 200 ppm appear safe from overt toxicity while engendering physiological effects.

Heme Protein Interference by CO

The lugubrious biology of CO involves functional interference with various heme proteins, most importantly, binding ferrous heme centers on a competitive basis with molecular oxygen (O_2). The binding is defined by the Warburg partition coefficient—the ratio of CO to O_2 at which half the reactive sites are occupied by CO.⁴ It is widely known that CO avidly binds hemoglobin's ferrous heme moieties with an affinity of some 200 times that of molecular O_2 , thereby interfering with O_2 carriage by hemoglobin. The upshot of this so-called high M value is a decrease in the O_2 content of arterial blood and a change in hemoglobin allostery that hinders O_2 release from the tetramer (the oxyhemoglobin dissociation curve shifts to the left). For decades, these features have been known to account for the asphyxiating actions of CO because they lower the PO_2 in the tissues.³⁷

Once it became apparent that CO binds to a range of intracellular heme proteins, including the ferrous heme a_3 of cytochrome *c* oxidase—the terminal electron acceptor of the mitochondrial respiratory chain—an array of new effects were revealed about the cell's handling of molecular O_2 .⁴⁷⁻⁴⁹ Paramount to living cells, however, is the CO inhibition of mitochondrial electron transport and increased reduction levels of the upstream electron carriers,⁵⁰ most conspicuously *in vivo* in the cytochrome *bc₁* region of the chain.⁵¹ The result of CO binding to cytochrome oxidase is a slower rate of cellular respiration, an increase in the Complex III peroxide leak rate, and more limited turnover of adenosine diphosphate (ADP). On the other hand, in defense of its main energy-producing function, as noted earlier, the oxidase oxygenates CO to CO_2 reflected by the slow burning of CO observed in living tissues.⁵²

Another site of CO binding is the cytoplasmic family of mixed function oxidases, i.e. the P_{450} cytochromes, originally named for the strong absorption peak of the CO complex.⁵³ Indeed, cytochrome P_{450} was first demonstrated to be a monooxygenase by means of the photochemical action spectrum technique to reverse CO inhibition of the 21-hydroxylation of adrenal 17α -OH progesterone.⁵⁴ Other sites of CO-complex formation include myoglobin,⁵⁵ as well as the more recently discovered and related monomeric globins, neuroglobin and cytoglobin.⁵⁶⁻⁶⁰ In each case, CO binds only the ferrous heme, encumbering its capacity to handle O_2 in the usual way. The presence of the CO-bound form, however, does keep iron from participating in redox cycling, a potentially important anti-oxidant mechanism that may limit superoxide-driven Fenton chemistry in the cell. Such a mechanism, for instance, has recently been proposed for the protective effects of CO in cerebral malaria.⁶¹

Endogenous CO Production

As noted earlier, CO is an important endogenous gas synthesized by the heme oxygenases (HO)—enzymes that catalyze the rate-limiting step in heme degradation. Two main isoforms, HO-1 and HO-2, are fairly well understood while the status of a putative third (HO-3) is uncertain.^{2,62} HO breaks the α -methene carbon bond of the porphyrin ring using NADPH and molecular O_2 in a reaction that releases equimolar amounts of biliverdin, iron, and CO. In mammalian cells, biliverdin is rapidly converted to bilirubin, which have anti-oxidant and anti-

inflammatory properties, while the converting enzyme—biliverdin reductase—also has kinase activity.⁶³ The central role of HO in the heme turnover process is illustrated in Figure 3.

Gene transcription for HO-1 (*Hmox1*) is inducible by a range of factors while *HO-2* (*Hmox2*) is expressed constitutively. HO-1 responds to conditions that increase oxidative stress, and *Hmox1* is induced by transcription factors, such as the NF-E2-related factor-2 (Nrf2), which binds anti-oxidant response elements (ARE) in the gene promoter region.^{64,65} The ARE is critical to enzyme induction by LPS, cytokines, heat shock, hyperoxia, oxidants, hypoxia-ischemia, NO, and UV.^{2,62} Also, negative regulators of HO-1 expression, especially ROS scavengers, are well described. HO induction and endogenous CO is protective in numerous experimental models of vascular, cardiac, and pulmonary injury, and against damage from certain inflammatory conditions.^{33,66-70} In some cases, exogenous CO replicates cell protection, which has been interpreted as cell-specific anti-inflammatory, anti-proliferative, or anti-apoptotic effects, although the biochemical mechanisms are mostly unknown.

The normal rate of endogenous CO production results in very low cellular CO concentrations—picomolar to nanomolar—that can increase several fold by heme turnover, whereas exogenous CO can raise cellular CO content by perhaps 20 to 50 fold.^{44,45,71,72} At high CO levels, the physiology is superseded by the emergence of myriad adaptive and oxidative stress-related responses, overlapping hypoxia, and cellular toxicity, which are difficult to organize and categorize. Moreover, CO in the cell modulates not only heme biochemistry, but the metal-carbonyl influences the signaling and noxious activities of NO.^{73,74} It has already been said that CO can induce NO production but highly elevated CO concentrations may interfere with the NO synthases, especially in the absence of adequate arginine or tetrahydrobiopterin.⁷⁵⁻⁸¹

In spite of the requirement of all cells for heme, excess free heme is reactive and causes cellular damage by generating oxidative stress.⁸² Because free heme is lipophilic and naturally interacts with plasma and organelle membranes, its clearance by HO controls untoward membrane oxidations. The prevention of ROS generation by heme is facilitated by the enzyme's requirement for molecular O₂, which reduces PO₂ at the sites of the substrate pool and the free iron release, so the latter can be safely sequestered by ferritin.⁸³

The extent to which endogenous CO protects against iron-mediated biological stress directly through iron-carbonyl formation is not well understood, but it is clear that HO activity does induce the iron defenses.⁵ Moreover, *Hmox1* deficient mice develop iron-overload and a pro-inflammatory state⁸⁴ and HO-1 over-expression in fibroblasts increases iron export and decreases free intracellular iron content, which imparts resistance to apoptosis.⁸⁵ Despite some direct anti-oxidant effects of CO, it has become increasingly clear that exogenous and endogenous CO promulgate a pro-oxidant state in most mammalian systems that engages ROS-mediated signaling and the induction of vital anti-oxidant enzyme systems. These anti-oxidant responses include, among others, the mitochondrial superoxide dismutase (SOD2; MnSOD) as well as HO-1 itself^{16,17}, in part via CO stimulation of heme release from mitochondria.⁷² And protection by HO-1 in PC12 cells in response to peroxynitrite involves induction of the catalytic subunit of glutamate-cysteine ligase (GCLC), the rate-limiting enzyme in GSH biosynthesis, via the Nrf2 transcription factor and activation of PI3-kinase.⁸⁶ This pro/anti-oxidant duality fits the proclivity of CO both to facilitate adaptation and to cause cell injury, illustrated, for instance, by discrete pro- and anti-apoptotic events during inflammation.⁸⁷

CO and signaling through mitochondrial H₂O₂ production

For most of the 80 plus years since CO was first identified as a respiratory inhibitor³⁷ and the nearly 20 years since it was shown to bind to cytochrome *c* oxidase *in vivo*⁸⁸ mitochondrial CO effects in living tissues were considered minor because the *a*₃ heme is primarily in the

oxidized state. However, even at physiological PO_2 , CO does bind cytochrome oxidase, as some a_3 does remain in the reduced state, especially in metabolically-active tissues like the brain and the heart. Moreover, this CO binding produces unique reduction responses in the cytochrome bc_1 region of the chain,⁸⁹ as well as mitochondrial ROS production sufficient to deplete mitochondrial glutathione stores,⁹⁰ which is independent of hypoxia.⁹¹ CO's penchant to generate mitochondrial ROS also has been demonstrated for endogenous CO.⁹²⁻⁹⁴

Today, the area of CO signaling through mitochondrial ROS production is still in its early stages, and the issues of how much CO binding occurs physiologically and what the role of H_2O_2 egress from mitochondria is in cell signaling are not fully understood. Mitochondrial ROS have been implicated in a number of cell regulatory processes including stabilization of hypoxia-inducible factor-1 (HIF-1),⁹⁵ cell proliferation,⁹⁶ angiogenesis factor expression,⁹⁷ metalloproteinase expression in tumor cells,^{98,99} and mitochondrial biogenesis.⁹²

The HIF-1 example is instructive from the standpoint of intricacy and controversy, for instance in macrophages, where CO reportedly increases HIF-1 stability through mitochondrial ROS production.¹⁰⁰ Such stability is clearly conditional¹⁰¹ as any mitochondrial ROS mechanism would require a high peroxide leak rate and therefore substantial CO binding, for instance, as occurs at hundreds of mmHg in the carotid body where photochemical reversibility of HIF-1 effect has actually been demonstrated by the a_3 -CO action spectrum.¹⁰² Frank inhibition of the respiratory chain by CO causes chemical hypoxia, which invokes non-ROS mechanisms by slowing ATP turnover and cell growth.¹⁰³ Inhibition of respiration also increases PO_2 and Krebs' cycle metabolite accumulation while the abrogation of the HIF-1 effect in respiration-deficient cells sidesteps the issue of regulation since a high PO_2 opposes iron-carbonyl formation and suppresses HIF-1 α in the classical way. Therefore, in the interest of clarity, I will use activation of mitochondrial biogenesis, which is sensitive to low CO levels, to illustrate the principles of H_2O_2 -mediated cell signaling by mitochondria.

Mitochondrial biogenesis is a specialized bi-genomic program that maintains the cell's mitochondrial mass and allows it to compensate stresses associated with high energy demand or which involve mitochondrial damage.¹⁰⁴ The regulation of mitochondrial volume density and functional capacity through mitochondrial biogenesis requires close coordination between nucleus and mitochondrion at the level of both genomes. Communication of nucleus with mitochondria—anterograde—and mitochondria with nucleus—retrograde—appear to involve two endogenous gases, NO and CO, and the retrograde CO signal clearly engages mitochondrial H_2O_2 production as it is blocked by mitochondrial targeting of catalase.⁹²

The main source of H_2O_2 production by mitochondria is Complex III, regulated specifically by single electron production of superoxide in the Q cycle.¹⁰⁵ The rate of superoxide production is enhanced by slowing the terminal transfer rate of electrons to molecular O_2 by CO binding at cytochrome c oxidase (and NO in some cases) for two reasons. First, the delay in electron transfer allows the upstream carriers to become more reduced and fill the Q cycle electron pool. Second, the rate of mitochondrial electron transfer from semi-reduced Q to molecular O_2 to form superoxide is proportional to the product of the concentrations of semi-reduced Q and O_2 , and the O_2 concentration increases with inhibition of the oxidase. Another factor is SOD2, which vectors H_2O_2 out of the mitochondrion.⁹⁸ In this respect, SOD2 induction by CO serves a dual purpose: the first is as a classical superoxide scavenger and the second as a ROS-signaling molecule. These concepts are illustrated in Figure 4.

CO-mediated mitochondrial H_2O_2 production serves mitochondrial biogenesis in the following way. Mitochondrial H_2O_2 oxidizes functional thiol groups on counter-regulatory phosphatases, like PTEN and PTP1B, inactivating them.^{96,106,107} This phosphatase inactivation permits unopposed activity of the pro-survival kinase Akt (PKB)—as observed after CO.^{40,92,108} In

the mitochondrial biogenesis program, Akt is regulatory, for example, in the phosphorylation of nuclear respiratory factor-1 (NRF-1), a key transcription factor for some 100 genes involved in oxidative phosphorylation, heme synthesis, and mitochondrial biogenesis.¹⁰⁹ An example of how such CO regulation operates is illustrated in Figure 5 using the nuclear-encoded mitochondrial transcription factor-A (Tfam) as the readout. Tfam is essential for mitochondrial DNA (mtDNA) transcription, replication, and the maintenance of mtDNA copy number.¹¹⁰ CO also stimulates the NRF-2 transcription factor as well as the co-activator PGC-1 α , also required for the expression of mitochondrial transcriptosome proteins needed in mitochondrial biogenesis.¹¹¹

An important factor in the active mitochondrial biogenesis program initiated by CO is HO-1 induction, supported in part by mitochondrial heme release.⁷² This would provide substrate for the continued production of endogenous CO to stabilize the retrograde signal in the on-position.⁹² In this case, endogenous CO, like NO, may also activate GC, which has an important gene regulatory role in mitochondrial biogenesis.¹¹² Although the importance of mitochondrial biogenesis to homeostasis is self-evident, the program is also essential for cells and tissues to survive and recover from mitochondrial damage, for example, during sepsis,¹¹³ as well as from other damage that activates energy-requiring repair processes. The HO/CO system also protects the heart from doxorubicin-induced cardiotoxicity by restoring mitochondrial biogenesis, which opposes necrosis and apoptosis¹¹⁴, and HO/CO has been implicated in the adjustment of mitochondrial phenotype to oxidative stress.¹⁶

CO and non-mitochondrial ROS signaling

ROS-dependent signaling, of course, also occurs via non-mitochondrial sources, perhaps most notably via the family of NADPH oxidase (Nox) and Duox enzymes that generate ROS in various tissues under physiological circumstances.¹¹⁵ The Nox enzymes are di-heme, flavin-adenine dinucleotide-containing membrane proteins that catalyze the NAD(P)H-dependent single electron reduction of O₂ to superoxide anion. CO binding to Nox cytochrome b₅₅₈ moiety has been demonstrated, although the affinity is low and the reaction is slow.^{116,117} This effect of CO would of course decrease ROS production and signaling by Nox, but simultaneously increase ROS production by mitochondria.¹⁰³ In any event, suppression of ROS production by Nox has been invoked to explain inhibition of toll-like receptor (TLR) targeting to lipid rafts in macrophages¹¹⁸ as well as in the anti-apoptotic effects of CO during hyperoxia.¹¹⁹ The theory that CO inhibits pro-inflammatory signaling by “differentially-inhibiting” TLR trafficking to lipid rafts, thereby blocking access to ligands at the plasma membrane, is said to involve caveolin-1, but how ROS inhibition participates is unknown.

The search for the mechanisms of the anti-inflammatory effects of CO through blocking pro-inflammatory cytokine production while augmenting several anti-inflammatory cytokines stems from observations of CO and cGMP-independent, p38 mitogen-activated protein kinase (MAPK) signaling.^{13,66} The topic of p38 MAPK activation by CO is distinguished from mitochondrial ROS production because p38 isoforms in mammalian cells are broadly sensitive to oxidative, environmental, and inflammatory stress, e.g. cytokines.¹²⁰ The p38 MAP kinases (α , β , γ and δ) act in a cascade in which the membrane-proximal components activate upstream kinases—primarily MEK3 and MEK6—in response to physical and chemical stressors including oxidative stress (most notably H₂O₂), UV, hypoxia, ischemia, and early-phase cytokines, especially tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1). Therefore, in considering CO an “anti-oxidant” and “anti-inflammatory” molecule because of p38 MAPK activation one should keep in mind that CO likely activates the kinase by generating oxidants and that p38 activation is a component of an integrated adaptive response to oxidants.

The precise details of p38 MAPK activation by oxidative stress (as well as many other protein kinases) are still in process in most cell types, but as noted earlier, significant data for Akt activation by exogenous and endogenous CO already indicates that mitochondrial H₂O₂ production⁹² serves multiple adaptive signaling and protective functions. The sources of ROS for p38 MAPK activation may be non-mitochondrial, e.g. from the vascular NAD(P)H oxidases, or in tissue or vascular injury, stem from ROS production by the influx of inflammatory cells. This point requires better resolution, but it is foreseeable that the predominant sources of ROS production under the influence of CO or the CO/HO system will depend on cell type and injury model as well as on the CO dose and the duration of dispensation. At the same time, CO binding to multiple NAD(P)H-requiring heme proteins in the cell could expand the cytosolic NAD(P)H pool and produce an assortment of secondary redox effects.

The role of endogenous CO in modulating the innate immune response is also far from settled, and data on interactions with the early-phase cytokine response, i.e. mediated by the NF- κ B transcription factor, are conflicting. One study supports HO-1 transcriptional induction by LPS through NF- κ B and p38 MAPK¹²¹ and another that heme induction of HO-1 opposes neutrophil apoptosis, but requires nuclear translocation of the NF- κ B p65 subunit and ERK2 along with Akt phosphorylation.¹²² On the other hand, heme-driven HO-1 induction inhibits NF- κ B and TNF- α mediated E-selectin and VCAM-1 expression in endothelial cells, but this does not require p38 MAPK and cannot be replicated with CO.¹²³ In other situations, cytokines induce HO-1 and endogenous CO production; for instance IL-10 induces HO-1 expression through activation of STAT-3 (signal transducer and activator of transcription) as well as activation of the PI-3 kinase pathway, yet this does not appear to play a role in the anti-inflammatory activities of the cytokine.¹²⁴ HO-1 gene expression is also induced in hepatocytes by IL-6, and activation of the JAK/STAT pathway by the IL-6 receptor is necessary.¹²⁵

Finally, CO has been implicated in the regulation of heme-based transcription factors stemming from observations some years ago that CO might serve as a gaseous neurotransmitter.¹²⁶ More recently, a role of CO is emerging in the regulation of so-called gas-sensitive transcription factors for which CO binding to the heme moiety clearly enhances DNA promoter binding and activates gene transcription. At least two heme-based transcription factors have been reported to be CO-sensitive, NPAS2-BMAL1 and the insect nuclear receptor, e75.¹²⁷⁻¹²⁹ The regulation of NPAS2, which helps control the hypothalamic circadian clock in the mammalian brain, involves PAS domains that bind heme as a prosthetic group, forming a gas-regulated sensor that exerts heme-control of DNA binding. A model of the pathway involves light-induction of heme- and iron homeostasis-related transcripts including *Hmox2* and cytochrome P₄₅₀ oxidoreductase (*Por*). HO-2 thus generates CO as a signal, and the redox state of the cell is influenced by the NADPH/NADP ratio.¹²⁷ Presently however, the involvement of ROS in this type of CO signaling is unknown.

CO and NO

Earlier it was mentioned that both CO and NO activate the GC system by binding the active heme and enhancing cGMP production. Although NO is decidedly more potent than CO in activating GC, for instance in vasodilation,¹³⁰ CO does operate in the system under specific physiological conditions; for instance, by regulating vascular smooth muscle cell proliferation in hypoxia.¹³¹ This is an example of a GC-mediated physiological process apart from vasodilation that may be activated by CO, particularly if NO production is restricted,¹³² for instance, by limited availability of O₂, tetrahydrobiopterin, or L-arginine. On the other hand, CO does interfere as a function of dose with cell growth by inhibiting respiration¹⁰³ as well as by stimulating the cellular egress of iron.^{85,133} Therefore, similar to NO, the elucidation

of physiological effects of CO on cell proliferation is difficult, and like apoptosis, both pro- and anti-proliferative effects of CO have been reported.^{131,134-137}

NO is the more reactive of the two gases, having the same effective size and polarity as the O₂ molecule, and unlike CO, binds to both Fe²⁺ and Fe³⁺ heme. Moreover, low CO concentrations stimulate NO release and are associated with peroxynitrite generation in vascular cells.^{73,138} The chemistry of CO-mediated NO release depends on the initial distributions of NO and CO in the cell and according to the differing equilibrium constants for transition-metal binding of the two gases.^{4,34,139}

The association constants for NO binding to Fe²⁺ heme proteins such as myoglobin and hemoglobin are greater than those for CO (see 4 for references). For hemoglobin, the affinity of Fe²⁺ for NO is at least a thousand times greater than for CO; however, CO dissociation is much slower than for NO or O₂.¹⁴⁰ Therefore, CO gradually displaces NO from Fe²⁺ heme proteins, requiring minutes or longer for equilibrium even at CO excess. NO displacement from Fe²⁺ by CO may also be enhanced in proximity to reduced thiols that serve as a sink for SNO-protein formation. The kinetics of CO displacement of NO from heme at physiological concentrations of NO and O₂ are not obvious because CO is usually introduced at far above the relevant concentrations.

Although this brief review has focused on exogenous CO and the endogenous HO/CO system, it is also known that tiny amounts of CO are produced by lipid peroxidation during periods of oxidative stress.^{141,142} CO formation also occurs during the P₄₅₀ metabolism of the halogenated methanes,¹⁴³ some of which have proven quite useful as CO donating molecules. The physiological significance of such alternative sources of endogenous CO remains unknown, and their interactions with the mechanisms discussed here remain to be investigated.

Conclusions

The biological significance of and interest in carbon monoxide is mounting, but apart from its high affinity for reduced transition metals, especially heme-iron, CO is surprisingly un-reactive—as is the ferrous-carbonyl moiety. CO does form relatively tight complexes with heme proteins for which molecular O₂ is the preferred ligand, and this chemistry is responsible for the diverse physiological, adaptive, and toxic effects of the gas. Over the past few years, it has become increasingly apparent that exogenous CO exposure and the endogenous production of CO by HO favors a pro-oxidant milieu in aerobic mammalian cells that promotes ROS-dependent signaling, including the activation of redox-sensitive transcription factors and protein kinases, which induce certain anti-oxidant enzyme systems, including HO-1 itself. Early CO-dependent ROS events can be partitioned on the basis of site of origin—mitochondrial or non-mitochondrial—of heme proteins that play unique roles in the cellular adaptation to oxidative stress, while the cell's responses to exposure to high or protracted CO concentrations lack specificity and lead to interference with homeostasis.

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Figure 1.

A plot of the putative concentration-time relationships involved in the physiological, adaptive, and toxic effects of carbon monoxide (CO) in cells and tissues. As intracellular CO concentration increases from the picomolar into the nanomolar range, adaptive responses to the cell stress overlay the physiological homeostatic effects and the period for overt toxicity to supervene becomes progressively shorter.

Figure 2.

Box plot of average cellular CO concentrations in different organs in mice. The mice were either air controls or had received one hour of CO inhalation at 500 ppm to achieve blood carboxyhemoglobin levels of ~25%. One hour later, after euthanasia, tissues were harvested after perfusion of the vascular system with phosphate buffered saline (pH 7.4) to remove the hemoglobin. CO measurements were made in tissue homogenates using reduction gas chromatography as reported in references 44-46. The boxes represent the middle quartiles and the bars are the data ranges for 5 to 10 mice per tissue. Basal physiological CO levels are low, except for the spleen, but demonstrate significant intra- and inter-organ variability. CO exposure at the mouse's injury threshold increases cellular CO concentration by 4 to 10 fold; significant by ANOVA in all organs except the liver and the spleen (asterisks indicate $P < 0.05$).

Figure 3.

Diagram of the heme oxygenase (HO) enzyme system in the context of heme synthesis and heme protein turnover showing endogenous CO production and sites in the pathways of pro-oxidant and anti-oxidant effects.

Figure 4. Schematic diagram of the effects of cytochrome a_3 -CO binding on mitochondrial electron transport and superoxide (O_2^-) and H_2O_2 production by Complex III. The increase in the reduction state of the cytochrome bc_1 region of the respiratory chain has been demonstrated *in vivo*.⁹² The reduced, oxidized, and semi-quinone forms of ubiquinone are indicated by QH₂, Q, and Q^{•-} respectively.

Figure 5.

Role of CO-dependent mitochondrial H_2O_2 signaling in mitochondrial biogenesis. The egress of H_2O_2 from mitochondria allows unopposed Akt kinase activity via inactivation of thiol moieties in counter-regulatory phosphatases. Akt phosphorylates NRF-1, which translocates to the nucleus and transactivates the gene for mitochondrial transcription factor-A (Tfam), required by the bi-genomic mitochondrial biogenesis program for mtDNA transcription and replication.