INVITED REVIEW

Biological signaling by carbon monoxide and carbon monoxide-releasing molecules (CO-RMs)

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List of abbreviations: BKCa, large-conductance Ca2+ and voltage-activated K+ channel; CBS, cystathionine β-synthase; CcOx, cytochrome c oxidase; CO, carbon monoxide; COHb, carbonmonoxy hemoglobin; CO-RMs, carbon monoxide-releasing molecules; Hb, hemoglobin; HO-1, heme oxygenase-1; H2S, hydrogen sulfide; Mb, myoglobin; NO, nitric oxide; PGRMC1, progesterone-receptor membrane component 1.
Abstract

Carbon monoxide (CO) is continuously produced in mammalian cells during degradation of heme. It is a stable gaseous molecule that reacts selectively with transition metals in a specific redox state and these characteristics restrict the interaction of CO with defined biological targets that transduce its signaling activity. Because of the high affinity of CO for ferrous heme, these targets can be grouped into heme-containing proteins, representing a large variety of sensors and enzymes with a series of diverse function in the cell and the organism. Despite this notion, the progress in identifying which of these targets are selective for CO has been slow and even the significance of elevated carbonmonoxy hemoglobin, a classical marker used to diagnose CO poisoning, is not well understood. This is also due to the lack of technologies capable of assessing in a comprehensive fashion the distribution and local levels of CO between the blood circulation, the tissue and the mitochondria, one of the cellular compartments where CO exerts its signaling or detrimental effects. Nevertheless, the use of CO gas and CO-releasing molecules (CO-RMs) as pharmacological approaches in models of disease has provided new important information about the signaling properties of CO. In this review we will analyze the most salient effects of CO in biology and discuss how the binding of CO with key ferrous hemoproteins serves as a post-translational modification that regulates important processes as diverse as aerobic metabolism, oxidative stress and mitochondrial bioenergetics.
Introduction

Carbon monoxide (CO) is a ubiquitous gas produced by heme oxygenases during the degradation of heme (61; 116). Heme oxygenases exist in constitutive (HO-2) and inducible (HO-1) isoforms and are products of two distinct genes, *HMOX2* and *HMOX1*. While HO-2 has distinct tissue localization, being predominantly expressed in testes, brain and the endothelium, HO-1 is up-regulated in all tissues examined following several kinds of stress stimuli, including oxidative stress, which is an underlying factor in different pathological states (61). In addition to CO, heme catabolism generates ferrous iron (Fe^{2+}) and biliverdin, an aqueous tetrapyrrolic pigment that is then reduced to bilirubin by biliverdin reductase (6). The products of this energy-consuming reaction participate to the modulation of cellular function as they are produced by heme oxygenases: in the context of cellular and tissue pathological stress they elicit cytoprotective actions that are mediated by the anti-oxidant properties of biliverdin/bilirubin and by the signaling/anti-inflammatory effects of CO (6; 71; 80). In contrast, iron exerts pro-oxidant effects and following its release cells respond by increasing the expression of the iron storage protein ferritin (8). For the scope of this review we will focus on CO, although biliverdin and bilirubin also possess interesting signaling transduction activities that are being investigated (11; 37; 64) and may prove to be as relevant as those of CO to explain the beneficial role of HO-1. In general, induction of HO-1 is correlated with protection of organs and tissues after injury. The elimination of heme as a pro-oxidant and danger molecule and HO-1-derived products contribute to different aspects of this protection (6; 71; 76).

The variability in tissue expression of HO-2 and HO-1 as well as the levels of expression of the proteins in normal or stressful conditions implies a variation in the amounts of CO generated over time. Fluctuations in CO levels will undoubtedly lead to varied interactions with cellular and extracellular targets, based on the notion that the sensitivity of CO-sensing molecular targets and their affinity for CO is different. Because CO does not interact with aminoacids or other major cellular components apart from having a preferential reactivity
towards transition metals in a specific redox state, we and others have reiterated the notion that only selected metal containing proteins, including ferrous heme-dependent proteins, are the putative targets for the signaling action of CO (26; 31; 32; 48) (see below for further elaboration of this concept in relation to other intracellular gaseous mediators). This hypothesis is only recently being supported by emerging reports on newly identified ferrous heme-containing targets (45; 133), but much remains to be discovered about conditions, selectivity of targets and the specific reactivity of CO within the intracellular environment that might determine the sensitivity of unknown targets to the signaling action of CO. An example that considers guanylate cyclase and myoglobin (Mb), two heme-dependent proteins that bind CO, can help to understand this point. A $K_D = 2.6 \cdot 10^{-4}$ M ($k_{off}/k_{on}$) of CO for soluble guanylate cyclase has been reported, while for whale Mb the parameter is $3.7 \cdot 10^{-8}$ M (118), indicating a higher affinity of CO for Mb compared to guanylate cyclase. Overlooking the limitations that these values are obtained from studies using isolated proteins and that the presence of other gases (O$_2$, nitric oxide (NO), hydrogen sulfide (H$_2$S)) may influence the binding and dissociation of CO to its targets, one may infer that in a cellular environment where CO will find in close proximity Mb and guanylate cyclase, Mb will be predominantly targeted by the gas. This example also serves to illustrate that the distribution of target proteins will greatly affect the specificity of CO-mediated effects in cells and tissues. Additional factors to consider are our limited knowledge of the actual number and identity of heme-binding proteins in the cell, the fact that heme may bind to proteins only in a transient manner rather than being covalently attached, and the lack of tools that can help us to discover a CO ‘signature’ in the cell, that is, some kind of detectable, protein-based marker that signals a change once heme- or metal-proteins are targeted by CO.

CO gas administration and CO-releasing molecules (CO-RMs), substances that liberate controlled amounts of CO to biological systems, have been employed to reproduce the beneficial properties of endogenously generated CO in a variety of studies (9; 71; 73; 80; 99; 119). Both approaches have been useful in addressing certain questions about pathways
activated or affected by CO that ultimately mediate its pharmacological activities (31). The
evidence that exogenous application of CO to cells or tissues recapitulates many of the
effects observed following HO-1 induction, which results in enhanced production of heme
catabolites, led to the interpretation that some of the protective properties of HO-1 originate
from CO signaling. This review will consider the signaling properties of CO, discussing the
limitations of the current methodological approaches used to evaluate the distribution and
accumulation of CO in blood and tissues and to corroborate its role CO in biology. We will
then focus on heme-CO interaction, highlighting the role of heme proteins that bind O₂ and
control the fate of CO signaling as well as the idea that CO is a physiological protector of
heme oxidation.

Investigating the biological signaling of CO: use of CO gas and CO-
releasing molecules

At physiological concentrations, derived from basal production by heme oxygenase, CO has
been shown to mediate signaling processes in the brain (40; 41), liver (109) and endothelium
(136). CO also regulates vascular tone (72; 96) and the production of inflammatory mediators
(25; 79; 132) and has been implicated in a multitude of mechanisms mitigating apoptosis and
ischemic injury (15; 18; 98), cell proliferation (82; 108; 138) and bacterial infection (17; 27;
127). CO administered exogenously via CO gas or CO-RMs should interact with the same
biological targets used by endogenous CO. However, the fact that CO will likely be
generated in cells in proximity to specific targets also suggests some differences compared
to the exogenously applied CO. In addition, the high binding affinity of CO for hemoglobin
(Hb) points to the blood as a potential barrier for the delivery of CO to tissues. Indeed, when
animals inhale CO or are given CO-RMs via diverse routes (i.p., i.v., oral gavage), Hb may
be the first CO-binding protein and only once a certain percentage of carbonmonoxy Hb
(COHb) is formed can some of the CO be re-directed to tissues. This might explain why high
levels of inhaled CO or doses of CO-RMs that liberate supra-physiological CO concentrations are required to mimic the effects of small amounts of endogenous CO.

The delivery of CO to cells and tissues is also most likely different between CO gas and CO-RMs. Among CO-RMs, their diversified chemical structure will also lead to various amounts of CO release to biological systems (29; 71). Data obtained using a selective genetically-encoded fluorescent probe for detection of CO in living cells support convincingly this point (122) (see also the section ‘Assays to detect CO’ for detailed description of this and other recently synthesized CO-sensitive probes). It was observed that small concentrations of CORM-2 (1-5 μM) elicited a higher signal intensity of the probe compared to similar concentrations of CO added to cells in CO-saturated solutions, highlighting a difficulty in delivering or uptake of CO to cells with CO-saturated solutions (122). This does not mean that CO-saturated solutions cannot be useful in cell studies dedicated to elucidate the role of CO, but the above observation indicates that the amount of CO that we apply exogenously using different chemical or delivery approaches does not necessarily result in the same amount of CO accumulated inside the cell. The intracellular levels of CO that are required to exert biological action should be defined in order to better exploit the pharmacological properties of this gas. In relation to CO-RMs and delivery of CO to cells, our laboratory has been employing in the last few years COP-1, another fluorescent probe that detects CO (29; 77; 131). We have observed in various cell types, including endothelial cells, microglia and cardiomyocytes, that application of the same concentration of structurally different CO-RMs does not elicit the same fluorescent signal of COP-1 (see Figure 1). This result is likely due to the distinct rates of CO release by the compounds, the number of CO liberated by the compounds (1 CO/mole for CORM-A1 and CORM-3, 3 CO/mole for CORM-401) and the specific chemical reactivity of each CO-RM towards cellular components (18; 22; 29; 74). In addition, whether the compounds possess the same ability to enter the cell and whether the transition metal present in some of the molecules used (ruthenium in CORM-3 and manganese in CORM-401) affects cellular uptake, delivery and targeting of CO in cells are
factors that remain to be investigated. As a general rule, it should not be assumed that exposure of cells and tissues to certain concentrations or doses of CO gas and CO-RMs will provide always the same levels of endogenous CO. Thus, interpretation of results and comparisons between treatments should take this fact into consideration.

Although this review is not aimed at discussing the biological activity of CO versus that of other gases, it is instructive to compare the chemical reactivity of CO with that of two additional well-established intracellular gaseous mediators, NO and H₂S, in order to have a better understanding of the most plausible molecular target(s) for CO in mammalian systems.

In contrast to NO and H₂S, CO is not a reactive molecule and no significant transformation is expected from its production in mammalian cells to its elimination from the lung through respiration. This may have strong repercussions for the signaling activity of CO, which is not consumed by reacting with other chemical entities, thus potentially being more resistant to conversion into other species within the complex cellular environment and having a more protracted effect over time. NO instead is a free radical and as such can interact with a variety of targets within the cells. It binds either to ferrous (Fe²⁺) or ferric (Fe³⁺) heme although its affinity is much higher for the ferrous form; it reacts with thiols to form S-nitrosothiols, with superoxide to generate the powerful oxidant peroxynitrite and with tyrosine to form nitrosotyrosine; and NO is also converted to nitrate and nitrite by reacting with O₂ (see Figure 2). A very similar and diversified reactivity of H₂S with the intracellular targets mentioned above for NO has been clearly documented as H₂S can also interact with ferrous and ferric heme, thiols, superoxide and molecular O₂ (32; 65). In the case of CO, which is not a free radical, there is only one possible type of chemical interaction within the biological systems, and that is binding to a transition metal. Moreover, and most importantly, the metal has to be in a specific redox state; in the case of iron-heme, for instance, CO can bind only to the ferrous heme. Therefore, it can be appreciated that while NO and H₂S are versatile in their chemical reactivity and have a diversified interaction with biological components, CO is very specific and highly selective (see Figure 2).
Assays to determine CO levels *in vitro* and *in vivo*

The levels of COHb in blood is commonly used as a standard parameter to assess the amount of CO *in vivo* following administration of CO gas (81; 89) or CO-RMs (25; 60) and also to detect increased endogenous CO production in the body in pathological states (62; 66; 101; 102). However, evaluating the levels of CO inside cells and tissues is essential in order to assess its signaling and biological activities and distinguish them from its potential toxic effects. Whether COHb is the most suitable marker for this purpose is debatable. We will first address the advantages and limitations of this approach prior to describing recent technical progresses concerning primarily the development of fluorescent probes able to detect intracellular CO, which represents an important step forward within the field of CO research.

Measurements of blood COHb have been traditionally employed in human subjects that have been accidentally exposed to levels of CO gas that lead to intoxication (36; 91). Notably, there are important disparities in the human response to CO exposure, in the sense that similar symptoms of mild CO poisoning, such as headache and dizziness, are observed in subjects with significantly different COHb levels, while other individuals with relatively high COHb (15-20%) can be asymptomatic (10; 34; 35; 50). Nevertheless, the percentage of COHb in blood is universally accepted as a crucial parameter for assessing the gravity of CO intoxication. As a consequence, it is not surprising that measurements of blood COHb also became a parameter of reference in pre-clinical studies when CO gas inhalation and CO-RMs were introduced as novel pharmacological strategies to investigate the therapeutic effects of CO (70; 73; 81). COHb will give valuable information on the doses of CO gas/CO-RMs, treatment times and modes of administrations that can be safely used for experimental purposes. Pharmacological actions of CO that results in beneficial effects in animals are usually observed in concomitance with COHb levels less than 15%, which are below the
reported toxic threshold \(10\). Oximeters are normally employed in humans and animals and
it should be noted that the sensitivity of these machines to low levels of COHb \(0.2\text{-}1.5\%\) is
not as good as biochemical/spectrophotometric assays that can detect more accurately and
reliably differences in low percentages of COHb \(94; 121\). Apart from this, the greatest
limitation is that these measurements do not provide any information about the content and
the exact distribution of CO in organs and tissues after gas inhalation or CO-RMs treatment.
Furthermore, whether increased local CO concentrations due to an augmented heme
metabolism in tissues are high enough to be detected by differences in COHb levels remains
unknown. COHb levels are also affected by the blood and tissue oxygenation status, which
may vary in individuals and will also be influenced by pathological conditions. An interesting
experiment conducted by Coburn in an anesthetized dog clearly showed that blood COHb
did not change as the animal inspired gas that progressively decreased arterial \(\text{PO}_2\) from 200
to 40 mmHg \(19\). However, below 40 mmHg there was a sharp decline in COHb, which the
author interpreted as a disappearance of CO from blood and redistribution of CO to
extravascular stores that might include muscle and other organs containing high Mb or
heme-bound proteins that could trap CO. Conversely, when the arterial \(\text{PO}_2\) returned to
normal values CO ‘reappeared’ in blood and COHb levels were normalized to initial values.
The implications of these results are very profound and the relevant message that needs to
be emphasized here is that the competition between \(\text{O}_2\) and CO for the same targets is
probably occurring constantly in the body and organs, dynamically affecting the action of CO.

Associating simultaneous assessment of COHb levels with CO content in organs already
offers a better insight on CO pharmacological activity and has been performed by the groups
of Vreman \(121\) and Vanova \(120\) in comprehensive studies. Tissue CO content can be
precisely quantified by gas chromatography after collection of small tissues samples. The
above investigations confirmed that inhalation of CO gas causes an increase in COHb that is
accompanied by augmented levels of CO in tissues. The amount of CO in blood is far
superior to that accumulated in tissue and the distribution of CO is tissue-dependent. As
expected, the dose of inhalation affects the distribution of CO in organs; counter intuitively, the muscle, which contains considerable amounts of Mb, was not the tissue with higher accumulation of CO. The spleen, lungs and liver exhibited increased CO. It is interesting to report that Vreman and colleagues also showed that endogenous CO production, enhanced by a bolus treatment with heme (substrate of heme oxygenase activity), could be detected with their methodology in certain organs (121), indicating how heme metabolism can temporarily and specifically affect CO distribution. This has clear relevance for hemolytic diseases and other stress conditions causing local or systemic increases in heme tissue levels. In general, these measurements suggest that certain organs may be more susceptible to the signaling action of CO due to their high accumulation of the gas.

Concerning the detection of CO endogenously produced by cells or tissues, different methods have been developed. Measurements of CO in exhaled end-tidal breath of patients using an infrared analyzer revealed interesting correlations between the activity of heme oxygenase in tissues and the progression of certain diseases. For instance, increased exhaled CO levels have been reported in patients with sickle cell anemia (113), asthma (38), diabetes (86), cystic fibrosis (5) as well as critically ill patients (68) confirming that tissue HO-1 is activated in hemolytic diseases and disorders characterized by oxidative stress and inflammation. Another infrared laser absorption spectroscopy has been used for the real-time measurements of CO production from vascular cells. With this technique, it was reported that the sensitivity of the sensor for CO was 6.9 pmol and that the basal CO levels increased in vascular smooth muscle treated with HO-1 inducers such as heme and sodium nitroprusside and were markedly reduced in the presence of a heme oxygenase inhibitor (69).

An interesting and more recent progress in the field of CO detection has been the development of selective fluorescent probes that track CO in cells. In 2012 Michel et al (63) described the synthesis of a palladium-based probe which fluoresces upon binding to CO. The group demonstrated that carbonylation of palladium induced by CO generated a signal
that can be detected in aqueous solutions and in cells exposed to CORM-3, a water-soluble compound previously synthesized in our laboratory (18). The probe, **COP-1**, was shown to be selective for CO as reaction with gases such as NO and H₂S or oxidants such as hydrogen peroxide, peroxynitrite or superoxide hardly triggered fluorescence. We have used COP-1 in our own studies on the characterization of new pharmacological molecules that release CO (29; 77; 131) and developed a method in cells using flow cytometry, which allows us to assess several cell samples in a short period of time. Other laboratories have also started to employ this probe with good success (83). The probe is useful when assessing intracellular CO but we have never been able to detect any increase in endogenous CO production after exposure of cells to heme, despite trying different permutations of the experiment in relation to time, concentration of the probe or of heme (unpublished observations). Our interpretation is that COP-1 fluorescence can be turned on only once a certain amount of CO has entered the cell but that endogenously generated CO never reaches concentrations high enough to activate the probe. Taking advantage of the ability of CO to bind transition metals, Wang and colleagues also developed in 2012 a fluorescent probe for detection of CO (122). In this case the group undertook a genetic approach and transfected cells with **COSer**, a biosensor composed of a yellow fluorescent protein attached to CooA, a heme-dependent transcription factor known for its very high sensitivity to CO derived from the bacterium *Rhodospirillum Rubrum* (93). Thus, by inserting this construct into cells and exposing them to CO the authors could visualize an increase in fluorescence that was correlated with the concentration of the given CO. In the article there is no description of experiments where the probe was used to assess increases in endogenous CO. Therefore, the utility of this tool in real time assessments of CO in normal and stress situations, and not when CO levels are artificially elevated with addition of CO gas or CO-RMs, still needs to be proven. In addition, the fact that genetic manipulation is required in order to render cells capable of sensing CO may represent a further limitation compared to chemical probes that can be simply loaded into cells. Interestingly, in the last two months two additional new probes have been added to the series of fluorescent CO detectors available
so far. **ACP-2** is an azobenzene-cyclopalladium-based fluorescent probe that is constructed with azobenzene-cyclopalladium as the CO recognition part combined with borondipyrrromethene as the fluorophore (56). The second compound, **FL-CO-1**, is an allyl chloroformate functionalized fluorescein which signals CO and works in combination with PdCl$_2$ as a trap for CO (30). When CO converts Pd$^{2+}$ to Pd$^0$, the latter stimulates fluorescein to activate the fluorescent signal in FL-CO-1 and thus only when PdCl$_2$ is present can the CO be detected. It does not appear that the concentration of PdCl$_2$ (1 μM) necessary to trap CO is toxic to cells, which may have been an obstacle in its use as PdCl$_2$ is normally considered quite toxic. The advantage and technical advancement of these two latter probes compared to the previous ones is that both apparently can detect endogenous CO generation: ACP-1 was activated when HepG$_2$ were subjected to hypoxia or ischemia/reperfusion events and to prove that the signal was due to CO the authors showed that fluorescence was blocked by zinc protoporphyrin IX, an inhibitor of heme oxygenase activity (56); FL-CO-1 was instead activated in a time-dependent manner (0.5, 4 and 10 h) upon exposure of A549 human lung carcinoma cells to 100 μM heme (30).

It is foreseen that these new tools will expedite our understanding of the signaling action of CO as being able to visualize at the cellular level increases in CO is a real step forward. However, these approaches are still far from addressing the targets of CO in cells. Ideally, probes that are sensitive to CO and are organelle-specific would allow a better identification of cellular ‘hubs’ that are particularly targeted by CO. Heme or metal-recognition probes that fluoresce when CO binds to the metal could be the ultimate aim in the development of CO-sensitive probes. Over the years we have also considered with chemists the possibility of creating CO-RMs possessing radioactive carbonyl groups, which could be excellent to follow the fate of CO in the cell once it is released by the compound. Nevertheless, it appears that synthesizing a radioactive CO-RM is a very difficult and costly task and therefore we have not been able to pursue it so far. Other methods, mostly spectrophotometric and using isolated proteins, have been used to assess potential targets of CO. In the following section
we will analyze more in details heme sensor proteins that have been recognized as targets of CO and that modify their function upon CO binding.

Hemoproteins as sensors and transducers of CO signaling

As anticipated above, it is well established that a large number of proteins containing heme as a prosthetic group in mammals function as receptors, sensors or transporters of gaseous molecules including O₂, NO, H₂S and CO. The most renowned of these proteins are undoubtedly Hb and Mb, which alongside mitochondrial cytochrome c oxidase (CcOx), represent a peculiar triad of heme-based proteins obligatory for O₂ binding, transport and consumption, and thus critically involved in cellular respiration. Their specific role in determining either CO signaling or CO toxicity will be discussed separately in the section below.

It is important to stress that there are two major factors that determine the selectivity and affinity of heme-dependent proteins for gaseous molecules: 1) the intrinsic reactivity of ferrous heme with diatomic gases and 2) the chemical nature and geometry of the ligand on the distal side of the heme pocket (118). These factors, alongside steric constraints and electrostatic interactions of the bound ligand on the distal side of the heme, can greatly affect the selectivity toward gaseous molecules. This is exemplified by the binding of NO and CO to guanylate cyclase, a heme-dependent protein that can be activated to generate the second messenger cGMP, which controls several important physiological processes (51). NO binds to the heme of guanylate cyclase to form a 5-coordinate NO complex leading to more than 100 fold activation of the enzyme. In contrast, CO causes only a minor increase in guanylate cyclase activity as a result of its binding to the heme pocket to form a 6-coordinate complex. Interestingly, O₂ does not bind to guanylate cyclase even in a 100% O₂ atmosphere and with very little oxidation of the heme. On the other hand, there are several sensor hemoproteins that appear to favor the binding of CO and in some cases their activation have been
demonstrated to occur at concentrations of CO that are physiologically relevant (28). For instance, the mammalian heme-based transcription factor neuronal PAS domain protein 2 (NPAS2) and the heme-containing nuclear receptors REV-ERBα and REV-ERBβ, which are all involved in the regulation of circadian rhythm, can bind CO with high specificity even though the structural basis for heme binding and the affinity of gases to these proteins has not been fully characterized yet (28; 85). In the case of NPAS2, resonance Raman spectra indicated that CO coordinated to the ferrous heme histidine on the proximal side, whereas NO is not able to bind to the heme group (117), indicating that this protein may function as a CO sensor in the brain.

The human BK<sub>Ca</sub> channel, a large-conductance Ca<sup>2+</sup> and voltage-activated K+ channel involved in the hypoxic response in the carotid body and controlling vessel relaxation and neuronal excitability, is another interesting example of a CO responsive protein (39). The cytoplasmic segment of the channel is capable of coordinating heme, which in the reduced state may become a target for CO binding and regulation of channel activity (43; 130). It has been shown that low micromolar concentrations of CO gas or a CO-releasing molecule (CORM-2) markedly increased the open probability of BK<sub>Ca</sub> channels in patch clamp experiments using HEK293 cells (130). Interestingly, knockdown of HO-2 expression, which prevents endogenous CO production, reduced channel activity in normoxic or hypoxic conditions and addition of CORM-2 rescued this loss of function. HO-2-dependent regulation of BK<sub>Ca</sub> channels was also demonstrated in carotid body cells suggesting a control of BK<sub>Ca</sub> channels activity during oxygen deprivation. These data support several studies highlighting the importance of BK<sub>Ca</sub> channels in sensing CO and how the transduction of this signal is implicated in a variety of physiological processes such as cerebral and systemic vasodilation (42; 43; 123) with relevant consequences in pathological states including hypoxic pulmonary vasoconstriction (135) and liver cirrhosis (14). Other ion channels have also been reported to be regulated by CO gas or CO-RMs and these include epithelial sodium channels (3; 124), voltage-activated potassium channels (23), ligand-gated P2X receptors (95; 128), L-type
calcium channels (100) and tandem P domain potassium channels (TREK1) (24; 88; 129).

Emerging evidence reinforces the concept that heme may not serve only as inherent prosthetic group in proteins but also as “bridge signaling molecule” that regulates the activity of various ion channels with different layers of biological control, knowing that heme concentration is dependent on heme oxygenase activity and that heme oxygenase-derived CO would ultimately regulate the activity and function of these channels (16).

Another intriguing hemoprotein that has been recently identified to interact with CO is cystathionine β-synthase (CBS), a key enzyme in sulfur metabolism and in regulating substrates for remethylation cycle. CBS catalyzes the condensation of homocysteine and serine to generate cystathionine, which is then hydrolyzed to cysteine and H₂S by cystathionine γ-lyase (114). CO binds to the ferrous heme with relatively high affinity, and in this state, it inhibits the enzyme activity with a Kᵢ in the low micromolar range (5 µM) (90; 105). The physiological relevance of this inhibition is currently unclear but recent evidence suggests that heme oxygenase-derived CO controls the production of H₂S and regulates hypoxia-induced vasorelaxation in the cerebral circulation (67). In addition, activation of protein methylation has been shown to be mediated by CO via inhibition of CBS (134) and this signaling process appears to be crucial in the regulation of key enzymes that shunts glucose towards the pentose phosphate pathway, consequently having important implications in cancer cell survival (133). It is also interesting to point out that an increase in hepatic CO content or administration of a CO-releasing molecule to mice cause a decrease in H₂S in the liver, suggesting that CO can indeed inhibit CBS activity in vivo (104). One of the crucial questions, which can be extended to other heme-containing receptors and sensors of CO, is how the iron heme in the CBS enzyme is reduced and what type of reducing system is engaged in vivo to favor the binding of CO (48). In the context of cancer, a more recent finding reveals that the heme-dependent protein progesterone-receptor membrane component 1 (PGRMC1) receptor, which interacts with epidermal growth factor receptor (EGFR) and cytochromes P450 to regulate cancer proliferation and...
chemoresistance, is sensitive to CO. This gas interferes with PGRMC1 dimerization by binding to the sixth coordination site of the heme molecule and by doing so reduces the proliferation of tumor cells and their resistance to anti-cancer drugs (45). It is possible that the affinity of hemoproteins to CO may continuously vary in response to the levels of tissue O₂, the fluctuation of oxidants affecting the redox state of heme and the expression of HO-2/HO-1 both in physiological and pathological states. Thus, assessing the redox state of hemoproteins in real time in vitro and in vivo in association with their sensitivity to CO is a key aspect to be investigated in the future. Other hemoproteins may function as sensors and transducers of CO. A plausible candidate is BACH-1, a heme-binding factor that serves as a critical physiological repressor of the HO-1 gene. In normal conditions, BACH-1 binds to multiple Maf recognition elements (MAREs) present in two upstream enhancers of the HO-1 gene thus repressing its transcription; however, following an increase in intracellular heme concentration and/or after an oxidative stress event, heme binds with high affinity to BACH-1 and regulates its DNA-binding activity to the HO-1 promoter region (78; 112). To date it is not known whether the heme-BACH-1 complex is also sensitive to CO or other diatomic gaseous molecules. However, it is tempting to speculate that, once heme is bound to BACH-1, the activity of this transcription factor may be subjected to finer regulation by the subsequent interaction with gases. Other heme-dependent proteins have been shown to be a plausible target of CO such as nitric oxide synthase (44), cytochrome P450 (75), NADPH oxidase (115) and fibrinogen (20) but further experiments need to be conducted to confirm their functional role in response to endogenously generated CO.

**Hemoglobin, myoglobin and mitochondrial cytochrome c oxidase: a triad of hemo-proteins determining the fate of CO signaling**

As anticipated above, Hb, Mb and CcOx form an important triad dynamically participating in the binding, transport and consumption of O₂ and thus playing key roles in mammalian
aerobic metabolism. These hemoproteins are the most representative of the three major compartments whereby aerobic respiration takes place: the blood circulation (Hb), the tissue (Mb) and the mitochondria (CcOx). Notably, all these proteins have also the ability to bind CO with a different affinity. The competition between O₂ and CO for the ferrous heme of Hb, Mb and CcOx can be represented by the CO/O₂ affinity ratio, also referred as M partition coefficient value. As indicated in Figure 3, this value is 200-250 for Hb in red blood cells, decreases to 25-50 for tissue Mb and is much lower for mitochondrial CcOx (0.1) (49; 125).

Knowing that the intrinsic affinity of globin-free heme models for CO is >10⁴ times higher than that for O₂ (21), the considerably lower CO/O₂ affinity ratio of heme incorporated into a globin structure clearly indicates that Hb and Mb have evolved to disfavor CO binding and that they are essentially designed by nature to transport and deliver O₂ to the tissues. Moreover, the fact that the intrinsic affinity of CcOx is totally in favor of O₂ binding while the CO/O₂ affinity ratio of Hb and Mb declines in a progressive manner suggests the interesting possibility that Hb and Mb provide different layers of buffering and protection against the possible inhibition of cellular respiration by CO or other ligands. This has been elegantly demonstrated in vivo experiments on dogs which are totally resilient to the potential toxic effects of CO following transfusion with blood-saturated CO but succumb when CO is administered as free gas by inhalation despite in both cases COHb in the blood circulation reached “lethal” levels (HbCO>60%) (33). The study argues that the toxic effects of free CO gas are due to its ability to escape the scavenging properties of blood Hb and diffuse into the cells where it will impair cellular respiration. Thus, this triad of hemoproteins concertedly operates to guarantee that enough O₂ is finally delivered to and captured efficiently by the mitochondria where ultimately it will be consumed and converted to water while coupled with the production of chemical energy (ATP) essential for survival. Within this scenario, it is ultimately the extent of CO bound to CcOx within complex IV of the mitochondrial electron transport chain that would determine the toxic effects of CO. This has important implications on the transduction activities of CO in regulating aerobic metabolism and consequently it may be the cornerstone of one of the most important signaling effects of CO.
Because mitochondria are also the site of heme synthesis, they are intimately linked with CO by controlling the availability of the substrate for CO generation. Thus, what are the effects of non-toxic levels of CO? Is it possible to envision alternative and more refined interactions between CO and mitochondria that do not necessarily result in compromised respiration at the site of CcOx? Even though the mechanisms are far from being elucidated and ultimately they might not involve heme binding by CO, accumulating evidence indicate a beneficial role of CO on mitochondria bioenergetic. We observed in isolated kidney and heart mitochondria that CO-releasing molecules at low concentrations elicit an uncoupling activity that is dependent on CO release (58; 59; 97) and precedes the inhibition of respiration. We have recently confirmed these findings in whole endothelial cells (47), microglia cells (manuscript currently under revision) and adipocytes (unpublished data), where we observed a mild increase in respiration that does not affect ATP production. Interestingly, mitochondrial BK_{Ca} channels appear to mediate this effect in endothelial cells (47) but not in microglia cells, suggesting that multiple mechanisms are involved. In addition, this interaction of low CO levels with mitochondria affects metabolism, since treatment of endothelial cells with CO results in a shift from glycolysis to oxidative phosphorylation (46). Almeida et al have also shown in astrocytes that exposure to CO increased ATP generation and O_{2} consumption with a general improvement in oxidative phosphorylation (2). Despite the fact that the above findings were considered novel when they were recently published, a literature search on this subject made us discover that more than 30 years ago it was already reported that exposure of rats to CO for different times and levels differentially affected ATP production in the brain (107). Specifically, acute exposure for 4 min to 1.3% CO significantly reduced ATP, in support of inhibited respiration, while a 12 h treatment at 0.13-0.15% CO caused an increase in ATP content. In the absence of specific mechanistic studies in this report, we can at least deduce that the effect of CO on mitochondria and energy production are more complex than anticipated and certainly go beyond the classical inhibition of CcOx by CO poisoning. Stimulation of mitochondrial biogenesis may be one of the mechanisms by which treatment with low doses of CO will ultimately result in increased ATP production and a better
bioenergetic profile of the cell (111). Suliman and colleagues were the first to demonstrate that transient increases in CO intracellular levels by 5 to 20 folds led to augmented mitochondrial content over a period of 24 h (111). Remarkably, the group also extended their observations from animal to human subjects exposed to CO gas inhalation (92), indicating the consistency of this response in mammalian systems and the relevance of these studies for human physiology. Importantly, induction of mitochondrial biogenesis is part of the therapeutic effects of CO gas and CO-RMs in physiological and pathological conditions such as sepsis, cardiomyopathy and inflammation (54; 55; 87; 110). From a mechanistic point of view, they have shown that the transcription factors NRF1, Tfam, Nrf2 and others are involved in CO-mediated mitochondrial biogenesis (111). In addition, production of hydrogen peroxide as a redox signaling molecule appears to be necessary in this process, as already demonstrated in inflammatory responses (137). Regulation of reactive oxygen species (ROS) generation is possibly one of the key components in CO signaling and its interaction with mitochondria but also other heme proteins that generate ROS, such as NADPH oxidase (115). Since ROS mediate a variety of adaptations and are common denominators that converge into defined cellular responses, we suggest that some of the transduction activities of CO are dependent on ROS. In fact, although the literature describes other, non-heme containing targets that are modulated by CO (p38 MAPK (79), p21 (1), AP-2α (57)), it is most unlikely that a direct reaction of CO occurs with these proteins. Therefore, the current hypothesis is that these pathways are affected as a consequence of some primary interaction of CO with heme or transition metal-containing proteins that precedes their activation/induction or repression.

CO as a protector of heme oxidation

As discussed above, the high affinity of certain heme-containing proteins for CO supports the signaling action of this gas as the function of these proteins is modulated by the binding of CO. Nevertheless, another critical task carried out by CO appears to be directly related to its
ability to prevent heme oxidation once bound to the ferrous form of the molecule, which has relevance for normal and pathological conditions and may be a unique characteristic of CO due its exclusive binding to the reduced form of iron. In biochemical investigations in which this precise hypothesis was investigated, Sher and colleagues showed how, in the presence of low concentrations of Hb or Mb and peroxides mimicking those found in plasma, the ferric forms of the proteins were converted by CO to the ferrous carboxy forms (103). This reaction depends on peroxides as the source of electrons for the reduction and is expected to occur in cell-free Hb, which is susceptible to oxidation once outside the red blood cell. Importantly, the end result is the simultaneous elimination of two types of damaging molecules, oxidized Hb/Mb and peroxides, that affect vascular and tissue integrity. Reactions of nitrosopersulfide with hemoproteins also require a vacant heme as they cannot take place in the presence of COHb (13), suggesting that once COHb is formed in blood a whole series of reactions with different species will be prevented. In other words, CO could be considered as a preventative agent for heme-catalized redox-reactions.

That blocking heme oxidation has relevance *in vivo* has been corroborated in normal conditions (52) and pathological states (84). Kitagishi et al. have reported the synthesis of very interesting compounds, hemoCDs, which have the capacity to bind CO and O2 with different binding affinities (52; 53). Among the hemoCDs tested, hemoCD1 exhibited a much higher affinity for CO compared to Hb in the R state, providing a new tool for scavenging of CO in biological systems and creating what the authors aptly call ‘a pseudoknockdown state of CO’. When injected into mice, hemoCD1 was capable of removing endogenous CO from blood and COHb levels decreased from 0.2% in control mice to 0.03% in hemoCD1-treated mice. An unexpected and intriguing effect caused by the removal of CO from COHb was the rapid increase in HO-1 expression in the liver, followed by re-establishment of COHb to normal values. Accompanying these events was also a temporary increase in free hemin and circulating bilirubin. Based on these observations, the authors proposed a feedback mechanism in which CO depletion leads to formation of oxy-Hb that quickly becomes methHb,
which then releases hemin that is responsible for HO-1 induction and replenishment of CO in
the circulation. Although actual measurements of metHb formation were not performed in this
work, the central role of CO in this scenario is attributed to its ability to prevent ferrous heme
being oxidized to ferric Hb and maintenance of constant COHb levels in blood. Thus, these
results raise a fundamental question on the basal production of CO that may serve as a
buffer to inhibit the deleterious consequences of oxidized cell-free Hb even in homeostatic
conditions. A similar mechanism was also described previously by Pamplona et al in the
context of malaria (84), which is characterized by extensive hemolysis. By focusing on
cerebral malaria as one of the most critical complications of malaria infection caused by the
Plasmodium parasite, this elegant work demonstrated that HO-1 induction and CO
administration protected against neuroinflammation and the destruction of the blood brain
barrier. However, the authors also identified a biochemical explanation to this protection
according to which exogenously applied CO suppressed the oxidation of Hb and the
formation of metHb in vitro. This effect was supported in vivo by data showing that upon CO
treatment or after HO-1 induction the levels of extracellular free heme in the circulation,
significantly increased by the infection, nearly returned to basal values. Most striking are very
recent data published by Bisse and colleagues supporting this concept in humans (12). The
authors identified a new mutated form of Hb (Hb Kirklareli) in a female patient and her father.
While the daughter suffers from anemia, the father, a smoker with COHb levels of 16%, is
not anemic. A biochemical analysis of Hb Kirklareli shows that the mutant α subunit of the
protein autooxidizes ~8 times more and loses heme ~200 times faster than the native
subunit. Interestingly, the affinity of Hb Kirklareli for CO is ~80,000 fold higher than for O₂
explaining why the father, being a smoker, is protected against heme oxidation and Hb
denaturation (12).

Altogether, this series of studies highlight the versatile significance of CO binding to heme
that includes different layers of regulation, on one side related to modification of protein
function and on the other side to control of heme release from oxidized hemoproteins. In
view of recent hypotheses suggesting that free heme liberated during hemolysis and tissue injury is a danger molecule alerting the organism of a stress situation (80; 106; 126), the induction of HO-1 and consequent increase of CO can be seen as an obligatory counter response to limit heme-mediated oxidation and consequent damage.

Conclusions/Future prospects

The recognition that CO is not only a toxic gas but it is also produced in cells through an energy consuming process has stimulated scientists to explore the function of this molecule in physiological and pathophysiological contexts. The advancements of the last 20 years of research have opened new prospects for CO as its biological and protective actions may be translated into therapeutic applications for diseases with underlying oxidative stress and inflammation. Much of the action of CO is dictated by its chemical reactivity that restricts its targets to specific metal-containing proteins in the organism. As a consequence, hemoproteins have been considered the most likely transducers of CO signaling. Intriguingly, the high affinity of CO for ferrous-heme may well offer much needed pharmacological tools for the treatment of CO intoxication, as recently exemplified with the development of new synthetic molecules that tightly bind CO, such as hemoCD (52) or a mutated neuroglobin with enhanced CO scavenging activity that was proven in mice to effectively protect against CO poisoning (7). Surprisingly, this is a conceptually simple idea that was never investigated before. In relation to this, it is also remarkable how little we know about the ability of heme sensors to protect themselves from the poisoning effect of CO (4) as well as the strategies that these proteins have evolved to discriminate different gas ligands, which would be extremely helpful in elucidating the specificity of CO signaling. Progress in the field of CO signaling is still limited by the lack of technological tools that allow the visualization of CO in cells and tissues. Specific questions, such as the concentrations and levels of intracellular CO that are needed to elicit biological activity, where the CO is bound in the intracellular and
extracellular environment and how CO is disposed by cells, still need to be addressed in order to fully characterize and take advantage of the beneficial features of this ephemeral and dynamic gaseous molecule.

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References


Figure Legends

Figure 1. Intracellular delivery of CO by different CO-releasing molecules (see text for details). Figure modified with permission from Fayad-Kobeissi et al. Biochem. Pharmacol. 102:64-77, 2015.

Figure 2. Molecular targets of NO, H₂S and CO in biological systems (see text for details). While NO and H₂S have a more diversified reactivity towards multiple intracellular targets, CO reacts uniquely with transition metals in a specific redox state, ferrous (Fe²⁺) heme-dependent proteins being the preferential targets.

Figure 3. Competition between CO and O₂ for key hemoproteins involved in respiration (see text for details). * from Weber RE and Vinogradov SN. Physiol. Rev. 81: 569-628, 2001.
**Figure 1**

Intracellular CO (mean fluorescence)

Endothelial cells

<table>
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<tr>
<th></th>
<th>CON</th>
<th>CORM-3</th>
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<td>Intracellular CO (mean fluorescence)</td>
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*C* indicates statistical significance.
Chemical reactivity of NO, $H_2S$ and CO

Figure 2
Hemoglobin

*CO/O₂ Affinity Ratio

200-250
Hemoglobin
Blood stream

20-50
Myoglobin
Tissue/Cell

0.1
Cyt c Oxidase
Mitochondria

O₂ Consumption

Figure 3