BioFactors

Review Article

CO metabolism, sensing, and signaling

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Abstract.

CO is a colorless and odorless gas produced by the incomplete combustion of hydrocarbons, both of natural and anthropogenic origin. Several microorganisms, including aerobic and anaerobic bacteria and anaerobic archaea, use exogenous CO as a source of carbon and energy for growth. On the other hand, eukaryotic organisms use endogenous CO, produced during heme degradation, as a neurotransmitter and as a signal molecule. CO sensors act as signal transducers by coupling a "regulatory" heme-binding domain to a "functional" signal transmitter. Although high CO concentrations inhibit generally heme-protein actions, low CO levels can influence several signaling pathways, including those regulated by soluble guanylate cyclase and/or mitogen-activated protein kinases. This review summarizes recent insights into CO metabolism, sensing, and signaling.

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1. Introduction

CO is a colorless and odorless toxic gas produced by the incomplete combustion of hydrocarbons, both of natural and anthropogenic origin. Most of the CO is produced in the atmosphere by reactions of: (*i*) hydroxyl radicals with methane and other hydrocarbons, (*ii*) alkenes with ozone, and (*iii*) isoprene and terpenes with hydroxyl radicals and ozone. Other natural sources of CO include plants and oceans, volcanic activity, and forest fires. The most noticeable human activities responsible for CO production are transportation, stationary source fuel combustion, industrial processes, solid waste disposal, and burning of forest and agricultural materials [1].

Abbreviations: ACS, acetyl-CoA synthase; Akt/PKB, Akt/protein kinase B; bHLH, basic helix-loop-helix; BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1; BV, biliverdin; cav-1, caveolin-1; CBB, Calvin–Benson–Bassham; CBS, cystathionine β-synthase; CC, coiled-coil; cGMP, guanosine 3,5-cyclic monophosphate; CoA, coenzyme A; CODH, carbon monoxide dehydrogenase; CooA, carbon monoxide oxidation activator; CORM, CO-releasing molecule; COX, mitochondrial cytochrome *c* oxidase; CPR, cytochrome p450 reductase; EC, endothelial cell; Egr-1, early growth response transcription factor 1; EPC, endothelial precursor cell; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ETC, electron transport chain; FADD, Fas-Associated protein with Death Domain; GCS, globin-coupled sensor; GTP, guanosine-5′-triphoshate; Hb, hemoglobin; HbCO, carbonylated Hb; HKa, cleaved high molecular weight kininogen; HIF1α, hypoxia-inducible factor 1; HMOX2, heme oxygenase 2; HNOB, heme-NO-binding; H-NOX, heme-NO/oxygen; HO, heme oxygenase; Hsp-70, heat shock protein 70; IL, interleukin; IRF, interferon regulatory factor; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; Mb, myoglobin; MRS, mitochondrial redox signaling; NADP, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NPAS2, neuronal period circadian protein-aryl hydrocarbon receptor nuclear translocator protein-single-minded protein 2; NRF, nuclear respiratory factor; Nrf2, nuclear factor-erythroid 2-p45-related factor-2; NPAS, neuronal (N) period circadian protein (Per)-aryl hydrocarbon receptor nuclear translocator protein (Arnt)-single-minded protein (Sim); OXPHOS, oxidative phosphorylation; PAS, period circadian protein - aryl hydrocarbon receptor nuclear translocator protein - single-minded protein; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PI3K, phosphatidylinositol-3-kinase; PKG, cGMP-dependent protein kinase; PLP, pyridoxal 5′-phosphate; PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SDF-1, stromal cell derived factor-1; sEng, soluble endoglin; soluble guanylate cyclase; STAT, signal transducers and activators of transcription; Tfam, transcription factor A mitochondrial; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor; VILI, ventilator-induced lung injury; VSMC, vascular smooth muscle cell.

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Endogenous CO production depends mainly on heme degradation catalyzed by heme oxygenase (HO), both in prokaryotic and eukaryotic organisms [2–6]. Therefore, despite its toxicity [1,7,8], CO plays a key role in several metabolic processes and in diverse signal transduction pathways in bacteria, archaea, and eukaryotes [9–11].

In both prokaryotes and eukaryotes, the major molecular targets of CO are heme-proteins [12,13], which display diverse biological functions including sensing and transportation of diatomic gases, catalysis, and electron transfer [11,13–15]. A number of signaling effects of CO depend on the modulation of soluble guanylate cyclase (sGC) and/or activation of mitogen-activated protein kinases (MAPKs). In fact, CO is critical in both physiological (*e.g.*, memory and neurotransmission) and pathophysiological (*e.g.*, stress response to pathogens and ischemia) conditions [12,16–21].

Here, recent insights into CO metabolism, sensing, and signaling are reviewed.

2. CO metabolism

2.1. CO and prokaryotic organisms

Several microorganisms, including aerobic and anaerobic bacteria and anaerobic archaea, use CO as a source of carbon and energy for growth [10,11,22]. CO-metabolizing bacteria, spanning multiple phylogenetic lineages, include aerobic carboxydotrophic and carboxydovores bacteria (the latter being unable to grow in the presence of elevated CO concentrations) [9], and obligate anaerobic CO oxidizers (*e.g.*, acetogenic bacteria, hydrogenogenic bacteria, phototrophic purple nonsulfur bacteria, and sulfate-reducing bacteria) [11]. These bacteria are globally distributed in soils and oceans, and include pathogens, plant symbionts, and biogeochemically important lineages [9]. In addition, hydrogenogenic, methanogenic, and both sulfate and sulfur reducing anaerobic archaea are able to metabolize CO [10,11]. Archaeal aerobic CO oxidizers have not been identified so far [9]. Remarkably, microbial CO metabolism helps to maintain environmental CO below toxic levels, by removing about 10^8 tons of CO from the lower atmosphere of the earth every year [23].

CO oxidation by aerobes and anaerobes differs mechanistically [9–11]. Any known CO-dependent energy metabolism depends on the CO-dehydrogenase (CODH) [9,10], an enzyme containing iron and either molybdenum (in aerobes) or nickel (in anaerobes) in its active site [10]. CODH, widely distributed among physiologically and phylogenetically diverse lineages of bacteria and archaea [24], is classified either according to its metal/cofactor content (*i.e.*, Mo- and Ni-containing CODH) or according to its metabolic role (reflected by its subunit composition) and catalytic activity (*i.e.*, monofunctional and bifunctional CODHs) [10,11].

Monofunctional CODHs are characterized by a catalytic subunit (CooS), which encodes the CODH activity. These enzymes produce electrons that can be transferred to a variety of electron shuttles, enabling the coupling of CO oxidation to the reduction of H_2O, metals, nicotinamide adenine dinucleotide (NADP), sulfate, and so on. [11]. Thus, CODHs catalyze the oxidation of CO to CO_2 (and the reverse reaction); CO_2 is then fixed into cellular carbon by one of the reductive CO_2 fixation pathways (*e.g.*, the Calvin–Benson–Bassham (CBB) cycle, the reverse tricarboxylic acid cycle, the 3-hydoxypropionate cycle, and the Wood–Ljungdahl pathway) [23]. When coupled to acetyl-coenzyme A (-CoA) synthase (ACS), CODH forms the bifunctional CODH (CODH/ACS). In CODH/ACS, CODH catalyzes the reduction of CO_2 to CO, while ACS condenses CoA, a methyl group, and CO to form acetyl-CoA [23]. The CODH/ACS complex is pivotal for aceticlastic methanogenesis and acetogenesis in bacteria and archaea to form acetyl-CoA from either CO_2 or CO [23].

2.2. CO and eukaryotic organisms

Although nonenzymatic heme metabolism occurs *in vivo*, about 80% of the CO present in the human body is produced during hemoglobin (Hb) degradation [2]. The heme oxidative degradation is catalyzed by HO in concert with both the microsomal-reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome p450 reductase (CPR; NADPH-CPR) and the NAD(P)H biliverdin reductase (BVR). In mammals, HO, NADPH-CPR, and NAD(P)H-BVR form a complex on the endoplasmic reticulum (ER) [2]. HO breaks the α-methene carbon bond of the porphyrin ring of the heme using NADPH and O_2 in a reaction that releases equimolar amounts of biliverdin (BV), CO, and iron [2,25–27]. BV is rapidly reduced by the BVR to bilirubin, a powerful endogenous antioxidant [25], which undergoes further modification(s) before eventual secretion in urine and feces [28,29], iron being recycled for the synthesis of metal centers [30].

Endogenous CO is removed from the body mainly by expiration and oxidation. Indeed, CO binds to Hb forming carbonylated Hb (HbCO) and is subsequently transported and exhaled through the lung, by rapid diffusion across the alveolar-capillary membrane. This process is influenced by the alveolar gas volume, ventilation, and Hb concentration [12]. Although CO displays a very high affinity for Hb, O_2 can compete with CO for the heme-Fe-atom binding [31]. Moreover, CO can be slowly oxidized to CO_2 by mitochondrial cytochrome *c* oxidase (COX) [32].

The major site of heme breakdown, and therefore the major organ for the production of endogenous CO, is the liver; remarkably, also the spleen, the brain, and the erythropoietic system are additional important catabolic generators of CO [1,33]. Although most heme is derived from senescing red blood cells and ineffective erythropoiesis, about 20% of the total amount of endogenous CO comes from the degradation of non-Hb heme-proteins, such as catalase, cytochromes, myoglobin (Mb), and peroxidases [32]. All of these sources result in a normal blood HbCO saturation of 0.3–1% [34].

HOs from different organisms display essentially the same catalytic mechanism [4], even if there are considerable variations in the number of HO isoforms [6]. Although plants can express multiple HO isoforms, most bacteria, fungi, and animals contain only one isoform [5]. However, in most mammalian cells, three HO isoforms have been identified [2,3].

Although HO-1 and HO-2 isoforms are mostly recognized for their role in the heme oxidation and in the production of CO and BV, the biological function of HO-3 is still unclear [35–37]. The distribution of HO isoforms is largely linked to the specific biological actions of CO in different organs and tissues [2,3,12].

HO-1, also known as the heat shock protein 32 [38], represents the inducible isoform expressed under physiological conditions and at high concentrations in the spleen and in the liver, where it contributes to heme metabolism following erythrocyte breakdown [17]. Besides its role in heme degradation, HO-1 can exert cytoprotective functions (*i.e.*, anti-apoptotic, anti-inflammatory, and anti-proliferative) [20,26,39]. Indeed, by itself or by its enzymatic products, HO-1 acts in tissue homeostasis suppressing oxidative stress and maintaining cellular integrity [20,26]. When expressed under various pathological conditions, HO-1 metabolizes large amounts of heme, thus producing high CO levels that in turn can influence various biological events [40]. HO-1 is primarily expressed by high levels of free heme, by a variety of stressful stimuli (*e.g.*, heat shock, heavy metals, hypoxia, hyperoxia, gaseous nitrogen monoxide (NO), NO-donors, oxidative stress, sodium arsenite, and various cytokines), as well as by growth factors, hormones, disease states, dietary antioxidants, (including various classes of natural products), and drugs [12,20,26,30,41,42]. Furthermore, HO-1 expression is upregulated by exogenous CO [43–45]. The induction of HO-1 is regulated predominantly at the transcriptional level by several cellular transcription factors, such as the nuclear factor-erythroid 2-p45-related factor-2 (Nrf2)) [12,26,39,41,42], which is activated by multiple mechanisms and various signaling pathways, including MAPKs [12,26,41,46]. Remarkably, negative regulators of HO-1 expression, including scavengers of reactive oxygen species (ROS), have been reported [27].

HO-2 is constitutively expressed in most tissues and organs, activated by Ca^{2+}-calmodulin and phosphorylated by casein kinase 2 [47]. HO-2 is a member of the glucocorticoid-regulated proteins family, which is specifically upregulated by adrenal glucocorticoids, opioids, and estrogens [41]. HO-2 is localized in the endothelial layer of blood vessels [48], in discrete population of neurons in the brain [49,50], in many neurons of the myenteric plexus of the intestines [51–53], and in the testes [17,54]. HO-2 appears to be involved in neuronal communication through the action of CO [6,21,55].

Recently, the critical physiological role of the HO/CO system has been highlighted by several lines of evidence: (*i*) the description of the first and unique case of human HO-1 deficiency; (*ii*) the identification of human functional HO-1 polymorphisms; (*iii*) the production of knockout animal models by targeted deletion of *HO-1* and *HO-2* genes; (*iv*) the production of transgenic animals overexpressing HO in a tissue-specific manner; (*v*) the *HO-1* and *HO-2* genes knockdown by RNA interference; and (*vi*) the modulation of HO activity by pharmacological approaches (*i.e.*, HO activators and mimetics, porphyrin and nonporphyrin-based HO inhibitors) [21,41,42,56]. Both the human case of HO-1 deficiency and the HO-1 knockout mouse model provide clear evidence of a role of HO-1 in the heme degradation, in the maintenance of vascular and tissue iron homeostasis, and in the systemic responses to stress [21,41,56]. Remarkably, the protective role of HO-1 has been demonstrated by both preclinical studies using HO-1 knockout and by analysis of transgenic mice models [42]. Even if few studies investigated the specific HO-2 physiological roles, gene deletion in knockout mouse models resulted to be associated with several pathophysiological conditions [41]. Remarkably, all these studies suggest that HO-1 and HO-2 play important physiological functions independently from each other [41].

The HO-catalyzed CO production *in vivo* is modulated by several amino acids [6]. Arginine regulates CO production by upregulating HO-1 expression, likely through a NO-dependent mechanism and by increasing HO-3 expression. Glutamine induces HO-1 expression, revealing a cytoprotective effect. Glutamate and alanine increase CO synthesis in multiple cell types, stimulating the expression of both HO-1 and HO-2 in endothelial cells and brain. Taurine chloramine and taurine bromamine show anti-inflammatory properties by enhancing HO-1 levels in macrophages, thus leading to the inhibition of cyclooxygenase-2 expression and prostaglandin-E_2 production. Taurine chloramine also promotes HO-1 expression in human fibroblasts-like synoviocytes and suppresses the interleukin (IL)-1β-induced production of proinflammatory cytokines. Methionine increases HO-1 expression and CO production in epithelial cells. *N*-acetyl-cysteine reduces HO-1 expression at both mRNA and protein levels in vascular smooth muscle cells, and injured brain protecting cells from oxidative stress. Either cysteine or glutathione may mediate a stimulatory effect of *N*-acetyl-cysteine on CO production, via alteration of the cellular redox state. Some peptides, produced by protein degradation in the intestinal lumen (*e.g.*, Met-Tyr and D-Arg-D-m-Tyr-Lys-Phe), are also potent regulators of CO production, promoting the increase of HO-1 levels [6].

CO can be also obtained by HO-independent heme degradation. In fact, hydrogen peroxide and ascorbic acid catalyze the cleavage of the heme methylene bridges followed by the elimination of a methene-bridge carbon atom as CO. Moreover, the self-inactivation of the cytochrome p450 leads to the breakage of the bond between the heme and the apo-enzyme leading to heme degradation and CO biosynthesis [12].

Remarkably, heme-independent sources for CO production have been reported [12,57,58]. Indeed, minor sources of CO include the auto- and enzymatic-oxidation of flavonoids, halomethanes, and phenols, the photo-oxidation of organic compounds, and the peroxidation of membrane lipids [12,58]. The reduction of cytochrome b_5 is also accompanied by CO production [57].

Endogenous CO levels can be influenced by either pathophysiological conditions or chemicals inducing red blood cells destruction, hemoprotein breakdown, and HO-1 activity [58]. In women, the endogenous CO production doubles during the progesterone phase (0.62 mL/h *vs.* 0.32 mL/h in estrogen phase), and increases during pregnancy (0.92 mL/h)

due to contributions from fetal endogenous CO production (0.036 mL/h) and altered Hb metabolism [58]. Enhanced endogenous CO production is also caused by the increase in HbCO concentration with altitude, and by a number of different pathological conditions, including hemolytic, inflammatory, and oxidative diseases [58]. The administration of drugs, such as allyl-containing compounds (acetamides and barbiturates), contraceptives, diphenylhydantoin, nicotinic acid, phenobarbital, progesterone, and statins induce CO formation. Carbon disulfide and sulfur-containing chemicals (*e.g.*, parathion and phenylthiourea) also induce CO production, facilitating the heme degradation of cytochrome p450 [58]. The metabolic degradation of dihalomethanes by cytochrome p450 also increases the CO synthesis, leading to very high levels of HbCO (>10%), which can be further enhanced by prior exposure to hydrocarbons or ethanol [58]. Finally, additional sources of nonheme CO are thought to be intestinal bacteria, under pathophysiological conditions [15].

3. CO sensing

Heme-based sensor proteins are key regulators of cellular responses to changes in CO, H_2S, NO, and O_2 levels. Heme-proteins act as signal transducers by coupling a "regulatory" heme-binding site to a "functional" signal transmitter region. Four different types of heme-binding modules are known: (*i*) the heme-binding period circadian protein (P)-aryl hydrocarbon receptor nuclear translocator protein (A)-single-minded protein (S) (PAS) domain, (*ii*) the globin-coupled sensor (GCS), (*iii*) the bacterial transcription factor CO oxidation activator (CooA), and (*iv*) the heme-NO-binding (HNOB) domain. The transmitters coupled to such heme-binding domains include cyclase, histidine protein kinases, cyclic nucleotide phosphodiesterases, chemotaxis methyl-carrier protein receptors, and transcription factors of the basic helix-loop-helix (bHLH) and the helix-turn-helix classes [14,15].

The best known CO-sensors is CooA, a homodimeric heme-containing protein, which regulates the CO oxidation system in the photosynthetic bacterium *Rodospirillum rubrum* [59–62], and the mammalian neuronal (N) period circadian protein (Per)-aryl hydrocarbon receptor nuclear translocator protein (Arnt) -single-minded protein (Sim) protein 2 (NPAS2), a member of the bHLH family of transcription factors expressed in the forebrain, which is a CO-dependent regulator of the circadian rhythm [59,60,63].

CooA represents the prototypic CO sensor present in a wide variety of bacteria [64]. In the purple nonsulfur bacterium *R. rubrum*, in the presence of CO, CooA promotes the transcription of genes involved in CO oxidation [61,62]. Both chains of CooA contain a *N*-terminal sensory domain and a *C*-terminal DNA-binding domain, connected by the long C-helix [65]. The inactive "off state" of CooA has the His and Pro residues as the two axial ligands of the heme-$Fe2^{+}$-atom. In contrast, in the "on state," which is active in DNA binding, the Pro residue is replaced by CO [61,62]. In addition to CO binding, the active on state of CooA has two major structural differences from the off state: (*i*) the repositioning of the long C-helices at the dimer interface and (*ii*) the concomitant reorientation of the hinge region between the DNA- and effector-binding domains within each monomer [61].

CooA acts also as a redox sensor [61]; indeed, CO is able to bind only the reduced form of CooA, this causing a protein conformational change(s) necessary for DNA binding and thereby for CooA-dependent gene transcription [60]. Recent studies suggest that changes in the heme ligation alter the structure stability of the heme domain and of the dimer interface, without altering the stability of the DNA-binding domain [66].

The mammalian NPAS2, together with its binding partner brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1), a bHLH transcription factor, binds DNA as an obligatory heterodimer [63,67]. The transcriptional activity of the NPAS2-BMAL1 heterodimer is modulated by CO and by the cellular redox balance (*i.e.*, the NADPH/NADP molar ratio) [59,63]. The NPAS2 monomer contains two hemes located in the PAS domains (named PAS-A and PAS-B) and representing the CO-binding sites; both hemes are six-coordinated in the "resting state" [60,63]. Remarkably, CO binding to the heme-PAS domains, leading to the "inactive state," inhibits the DNA binding activity of holo-PAS2 and leads to the formation of inactive BMAL1 homodimers instead of active NPAS2-BMAL1 heterodimers [63]. *In vivo*, this results in the negative modulation of the day–night cycle [67]. A model of the pathway involves light-induction of heme and iron homeostasis-related transcripts including heme oxygenase 2 (HMOX2) and cytochrome p450 oxidoreductase. HO-2 thus generates CO as a signal, the redox state of the cell being influenced by the NADPH/NADP molar ratio [68]. Inhibition of membrane NADPH oxidase by CO, and the subsequent downregulation of the $O_2^{\bullet -}$ production, has been implicated in the anti-proliferative and anti-inflammatory effects of CO [42].

Recently, the pseudo-cytochrome p450 cystathionine β-synthase (CBS), a H_2S-producing enzyme, has been postulated to be a CO-specific sensor [69]. Notably, CBS plays a key role in cysteine metabolism, and its malfunction leads to homocysteinuria [15,69,70]. Human CBS is a multimeric protein composed of subunits containing a pyridoxal 5′-phosphate (PLP) cofactor at its active site, catalyzing the synthesis of cystathionine from homocysteine and serine [71,72]. Mammalian enzymes also contain a heme group, whose removal low down but do not eliminate human CBS activity [69]. However, the heme may play a regulatory role in modulating the CBS activity [69]. In fact, the hexa-coordinated carbonylated derivative of CBS is inactive, whereas the penta-coordinated nitrosylated form does not affect the CBS action(s) [73]. Moreover, CBS inhibition by CO requires the ferrous form of the heme-$Fe2^{+}$-atom, indeed CO does not bind to the ferric form, which retains the enzymatic activity [69]. Thus, the heme-$Fe2^{+}$-atom redox state also regulates human CBS activity and the inhibitory role of CO [69]. CO binding to human CBS is an anti-cooperative process characterized by a slow and possibly multistep tautomeric shift of PLP from the ketoenamine to the enolimine form associated with the loss of CBS activity. The affinity of CO for CBS is in

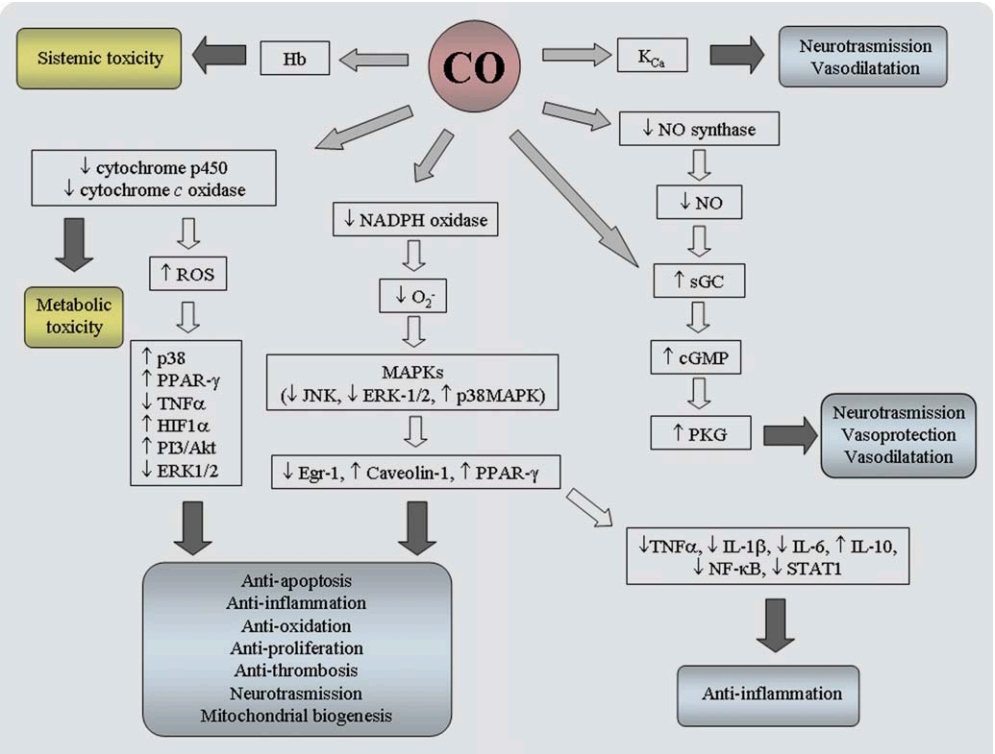

Fig. 1. Schematic representation of CO molecular targets, signaling pathways, and major protective effects against tissue injury (↓, decreased expression; ↑, increased expression). The systemic and metabolic toxicity effects of CO are highlighted. For details, see the text. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the physiologically relevant micromolar range, this indicating that endogenous CO may act as an inhibitor of human CBS [69]. The CO-induced inhibition of human CBS is a delayed response, the inactivation rate being modulated by pH and buffer concentration. The delayed response could protect against the transient increase of CO level, while lowering of pH would sensitize CBS to CO inhibition [69].

CBS has been also reported to act as a CO-responsive regulator of bile excretion in rats [73]. In fact, CO inhibits H_2S production thereby modulating biliary HCO_3^- excretion via H_2S-sensitive ion channels, by shutting down the CBS activity. The heme-based regulation of the CBS activity may also control H_2S production in other tissues such as the brain, where H_2S acts as a neuromodulator [74–76].

4. CO signaling

The primary molecular target of CO is the heme-iron center of heme-proteins, including catalase, cyclooxygenase, cytochrome *c*, COX, cytochrome p450, prostaglandin endoperoxide synthase, sGC, Hb, Mb, NADPH oxidase, nitric oxide synthase (NOS), NPAS2, peroxidases, prostaglandin H synthase, and tryptophan dioxygenase [12] (Fig. 1). High CO concentrations cause hypoxemia by competitive binding to the O_2-binding sites of Hb, the CO affinity being approximately 200–250 higher than that of O_2 [7,12]. On the contrary, low CO levels can influence several signaling pathways, including those regulated by sGC and/or MAPKs [16–20] (Fig. 1).

sGC, the physiological NO receptor, catalyzes the conversion of guanosine-5′-triphosphate (GTP) to the secondary messenger guanosine-3,5-cyclic monophosphate (cGMP), which in turn plays a pivotal role in several physiological processes including vasodilatation and neuronal signal transduction [77–84] (Fig. 1). Vertebrate sGC is a cytosolic heterodimeric heme-protein composed of α1 and β1 subunits. sGC consists of: (*i*) a sensory *N*-terminal domain termed heme-NO/O_2 (H-NOX) involved in heme binding, (*ii*) a PAS domain, (*iii*) a linker coiled-coil (CC) helix, and (*iv*) a *C*-terminal catalytic domain [85–87]. In addition to NO, sGC is slightly activated by CO [88]. Remarkably, CO and exogenous compounds such as 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) and 3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyradazolo[3,4-b]pyridine (BAY 41-2272) synergistically activate sGC to levels approximately similar to those induced by NO [88,89]. Such sGC induction leads to the increase of the cGMP level and to the activation of the cGMP-dependent protein kinase (PKG) phospho-transferase activity, thus affecting several cellular functions based on ion channel, phosphodiesterase, and protein kinase actions [77, 80, 90–95].

Both CO and the downstream effects on cGMP formation have been implicated in a number of neuronal signaling processes, including olfactory neurotransmission [20] (Fig. 1). Furthermore, by activating sGC, CO has been implicated

in the regulation of the vessel tone, in the inhibition of platelet aggregation, and in the inhibition of smooth muscle cell proliferation [96]. It has been hypothesized that CO may be a modulator of cGMP levels by interfering with the sGC/NO signaling system in the brain. Finally, it has been suggested that the neuronal NOS (nNOS)/NO system is not functional in the absence of HO-2, CO itself being sufficient to restore the physiological effects of NO [12,96].

The MAPK pathways, playing a key role in signal transduction pathways activated in response to oxidative stress and inflammation [97], are important downstream targets of CO [17,19,20] (Fig. 1). Indeed, MAPKs constitute hierarchic phosphorylation cascades responsible for transducing inflammatory signals from the cell surface to the nucleus, which may result in cellular activation and production of cytokines that in turn amplify inflammation [98]. The anti-inflammatory activity of CO has been shown to involve the downregulation of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), IL-1β, and IL-6 together with the increase of the anti-inflammatory cytokine IL-10, via the p38 MAPK and c-Jun *N*-terminal kinase (JNK)-MAPK pathways [99,100]. In particular, the p38 MAPK signaling pathway has also been implicated in the antiapoptotic and antiproliferative effects of CO [18,20,97,99,101–103] (Fig. 1).

The interaction of CO with ion channels (in particular with the K^+ channels) constitutes an important mechanism underlying the biological effect(s) of CO (Fig. 1). The superfamily of K^+ channels is composed of voltage-dependent (K_V), ATP-sensitive (K_{ATP}), and Ca^{2+}-activated K_{Ca} channels. Big-conductance K_{Ca} channels (BK_{Ca}) and Ca^{2+}-spark activated transient K_{Ca} have been demonstrated to be activated by CO [12]. The role of BK_{Ca} channels in the vascular tone regulation, in the determination of the action potential duration and frequency, and in the neurotransmitter release has been reported [104]. Consistent with this functional role, notable phenotypes such as hypertension, erectile dysfunction, and urinary incontinence have been associated with the inhibition or down regulation of the BK_{Ca} channel activity [105,106]. Conversely, the upregulation of the channel function in specific cell types may offer protection against some of the aforementioned disorders [107–110]. The BK_{Ca} channel activity is subjected to modulation by a wide spectrum of biologically relevant factors such as Ser/Thr/Tyr phosphorylation, Cys/Met oxidation, steroid hormones, and diatomic gases (O_2, NO, and CO) [111–114]. The PKG-dependent activation of the Na^+/Ca^{2+} exchanger by CO has been proposed [12]: this may increase the submembrane Ca^{2+} concentration in the vicinity of the BK_{Ca} channels with their consequential opening [115]. An activated cGMP pathway may be permissive for BK_{Ca} channels activation by CO [12]. In addition to the PKG-dependent mechanism, it has been suggested that CO directly stimulates BK_{Ca} channels, implicating that the channels themselves are gas sensors [116]. CO, administrated as a gas or as CO-releasing molecules (CORMs) [117], increases the probability of channel opening [118–120] even in cell-free membrane patches [112,114,121], thus suggesting the possibility that CO modulates the channel directly or indirectly through entities intimately associated with the channel proteins, possibly in the same macromolecular complex [116].

CO is able to inhibit O_2 consumption by inhibiting the mitochondrial COX, the terminal electron acceptor of the electron transport chain (ETC; Fig. 1). COX does represents a target and a central mediator of mitochondrial respiration, not only through its natural ligand (*i.e.*, O_2) but also through the binding of CO, NO, and H_2S. COX possesses four redox-active metal centers, all of which can be targeted by gases. Indeed, CO, similarly to NO and H_2S, can bind to the iron center of the COX prosthetic heme, thus inhibiting O_2 binding [15]. The inhibition of COX by CO suppresses oxidative phosphorylation (OXPHOS) and reduces ATP production [15,122]. OXPHOS downregulation alters the redox state of the ETC and produces ROS. Note that ROS may act as signaling molecules controlling cell functions, a process known as mitochondrial redox signaling (MRS). Furthermore, CO upregulates superoxide dismutase 2 (SOD2) expression, thus promoting the conversion of O_2 into the signaling molecule H_2O_2 and further inducing MRS [15] (Fig. 1).

As cell functions depend mostly on mitochondrial OXPHOS, cells increase their number of mitochondria as a function of their cellular energy demands. Mitochondrial biogenesis is a complex process involving the coordinated expression of both mitochondrial and nuclear genes. The CO-induced mitochondrial biogenesis is dependent on MRS [15]. Recently, endogenously produced CO, induced by transfection of the *HO-1* gene, has been reported to induce mitochondrial biogenesis in rat myocardium [123]. HO-1/CO-induced mitochondrial biogenesis requires both H_2O_2 and sGC activity (Fig. 1). The production of H_2O_2 derives from mitochondria and requires the activity of the Akt/protein kinase B (PKB) pathway for the activation of the transcriptional activator peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α) and the transcriptional factor nuclear respiratory factor-1 and -2 (NRF-1 and NRF-2, respectively). NRFs are the transcriptional factors that control almost all the proteins involved in OXPHOS in mitochondria. Moreover, PGC1-α and NRFs are responsible for the transcription of the transcription factor A mitochondrial (Tfam) protein, a molecule controlling mitochondrial DNA replication and transcription of mitochondria-encoded genes [15]. Remarkably, CO-induced mitochondrial biogenesis accompanies the HO-1 and SOD2 upregulation [124], this suggesting the involvement of mitochondrial oxidative stress [15] (Fig. 1).

Remarkably, CO also inhibits cytochrome p450 and NADPH oxidase cytochrome b_{558} activity, these heme-proteins being involved in free radical production, oxidative stress, and oxidative stress-induced apoptosis [125,126] (Fig. 1).

Additional candidates that function as downstream target molecules of CO signaling include the tumor suppressor protein caveolin-1 (cav-1), the anti-inflammatory nuclear regulator early growth response transcription factor-1 (Egr-1), the hypoxia-inducible factor 1 (HIF1α), the 70-kD heat shock protein (Hsp70), the interferon regulatory factor (IRF), the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), the NOS/NO system, the phosphatidylinositol-3-kinase

(PI3K)-Akt, PPAR-γ, and the signal transducers and activators of transcription (STAT) [20,21,42,127–137] (Fig. 1).

Notably, the upregulation of cav-1 expression has been observed in senescent cells, as well as in mice, rats, and humans [138]. Collectively, the interaction between cav-1 expression and p38 MAPK activity may control a variety of key biological programs, such as cell proliferation, differentiation, and senescence [131,138,139] (Fig. 1). Because trace amounts of exogenous CO treatment can cause upregulation of cav-1 expression and p38 MAPK activation, it has been speculated that CO modulates cell growth or differentiation. Indeed, CO inhibits the vascular smooth muscle cells proliferation and neointima formation during balloon injury in rats, by modulating the sGC-cGMP/p38 MAPK/cav-1 signaling pathways [20,131].

As mentioned above, many of the cytoprotective effects of CO depend on the activation of p38 MAPK [99,140]. Remarkably, several MAPKs, including p38 MAPK, may play a functional role in the initiation and propagation of the ventilator-induced lung injury (VILI) [141]. Mechanical ventilation at a moderate tidal volume causes a significant and time-dependent inflammatory response, reflected both by the infiltration of macrophages and neutrophils into the airways and by an increased production of cytokines and chemokines (*i.e.*, IL-1β, MIP-1β, and MCP-1) [142,143]. These proinflammatory mediators have been described not only to attract neutrophils into the lung [144] but also to severely exacerbate VILI [145]. CO exerts major anti-inflammatory effects by downregulating the production of cytokines in response to proinflammatory stimuli in macrophage cell culture, and by reducing the infiltration of macrophages and neutrophils into the lung [99]. Furthermore, the antiinflammatory effects of CO during ventilation are mediated via induction of PPAR-γ, thus preventing the upregulation of the proinflammatory transcriptional regulator Egr-1. Indeed, Egr-1, a zinc-finger transcription factor, represents an important transcriptional regulator that coordinates proinflammatory responses in various cell types, including macrophages [146]. In response to activation by ischemic stress, Egr-1 propagates deleterious responses leading to injury, by controlling the expression of critical genes involved in the regulation of inflammation, coagulation, and vascular permeability [146]. Thus, inhaled CO at low concentrations may represent a promising future therapeutic option for the reduction of VILI [147]. Whether these and other protective effects of CO application observed in animal models of lung injury can be extrapolated to the treatment of patients is the subject of ongoing clinical trials (www.clinicaltrials.gov) [147].

Interestingly, the upregulation of stress proteins such as HO-1 and Hsp70 has been shown to limit inflammatory responses in many models [148–150]. Moreover, CO treatment *in vivo* has been shown to induce HO-1 in the liver [129] and Hsp70 in the lung [130]. The upregulation of heat shock factor-1 and Hsp70 expression by CO has been proposed to mediate CO-dependent anti-inflammatory effects in a murine endotoxemia model. Both HO-1 and Hsp70 are upregulated in lung tissue in response to mechanical ventilation, and the application of CO during mechanical ventilation prevents the induction of both genes [147]. These data are consistent with the role of HO-1 or Hsp70 as inducible stress proteins that are upregulated as a consequence of systemic stress and inflammatory tissue injury [26]. Therefore, it has been suggested that both HO-1 and Hsp70 are markers of cellular or organ injury, whose expression is precluded by anti-inflammatory protection afforded by exogenous CO treatment [147].

The HO-1/CO system acts as an antioxidant, protects endothelial cells (EC) from apoptosis, regulates the vascular tone, attenuates inflammatory response in the vessel wall, and participates in angiogenesis and vasculogenesis. Remarkably, the oxidative stress leads to EC and vascular smooth muscle cell (VSMC) dysfunction, leading to the increase of the vessel tone, of the cell growth, and of the gene expression. These conditions are responsible for the creation of a prothrombotic/proinflammatory environment [151] (Fig. 2). Therefore, EC integrity and activity occupy a central position in the pathogenesis of cardiovascular diseases, and cardiovascular disease risk conditions converge in the contribution to oxidative stress. Subsequent formation, progression, and obstruction of atherosclerotic plaque may result in myocardial infarction, stroke, and cardiovascular death. The HO-1/CO system efficiently acts to recover the damaged tissues in IR injury, hypertension, and atherosclerosis, mainly by improving endothelial precursor cell (EPC)/EC functions and inhibiting proliferation of smooth muscle cells (SMC) [151] (Fig. 2). HO-1 has been demonstrated to stimulate cell cycle progression and proliferation in vascular endothelium, mainly by stimulating the synthesis of the vascular endothelial growth factor (VEGF) from vascular cells. HO-1 expression can protect EPC from oxidative injury and stimulate their homing to injured regions for promoting angiogenesis, such a positive feedback being relevant in EPC function. Additionally, HO-1 can directly affect cell viability by blocking programmed cell death [151] (Fig. 2).

CO has been also shown to regulate cell cycle, indeed it decreases proliferation of VSMC, of airway SMC, and of pancreatic stellate cells, while it increases proliferation of EC [152]. Indeed, CO may specifically contribute to the re-endothelialization of the vessel wall at sites of vascular injury inducing the production of angiogenic mediators [*e.g.*, VEGF, IL-8, and stromal cell derived factor-1 (SDF-1)] and decreasing the levels of the anti-angiogenic mediators [*e.g.*, VEGF receptor 1 and soluble endoglin (sEng)]. These events promote EC proliferation, migration, and the antiapoptotic response [151] (Fig. 2). Remarkably, BV is also able to stimulate the induction of proangiogenic factors in human keratinocytes cells (*e.g.*, VEGF and IL-8), whereas Fe^{2+} antagonizes the antiangiogenic effects of the high molecular weight kininogen (HKa) [151] (Fig. 2).

HO-1 and CO contribute to the neo-vascularization and to the cardiac regeneration after myocardial infarction. In particular, CO promotes cardiac regeneration by increasing the accumulation of c-kit+stem/progenitor cells into the infarct area; moreover, it promotes both vasculogenesis and formation of new cardiomyocytes by increasing the expression of HIF1α, SDF-1α and VEGF-B in the infarct area. In

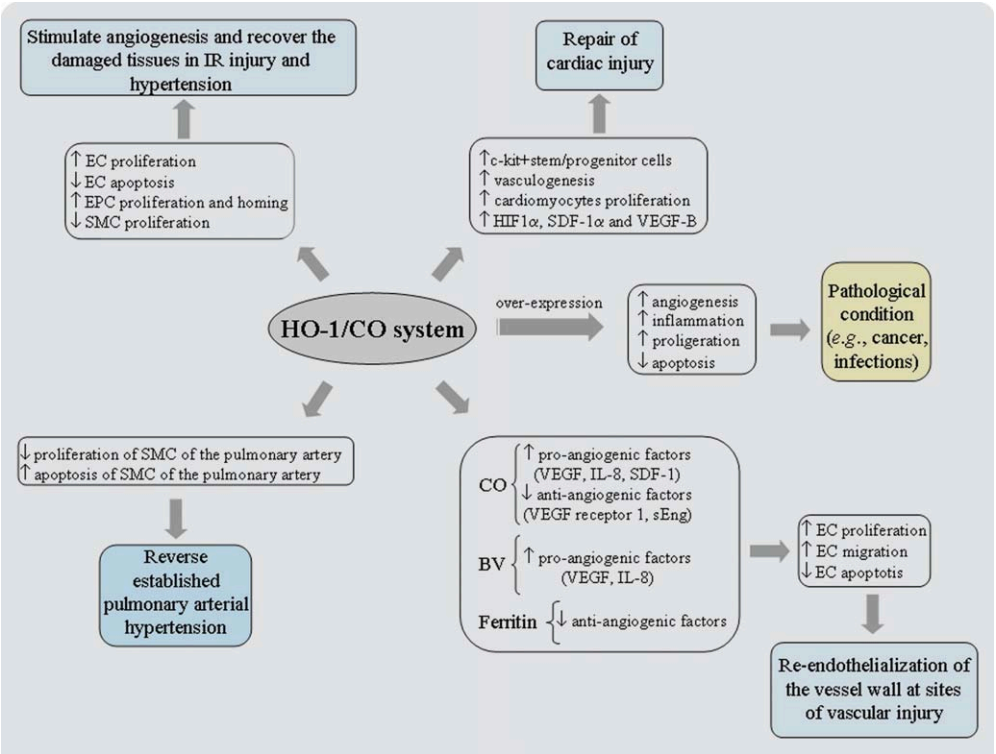

Fig. 2. Schematic representation of the "pro" and "anti" physiologic effects of the HO-1/CO system. Remarkably, overexpression of the HO-1/CO system may be responsible of several pathological conditions, such as cancer (↓, decreased expression; ↑, increased expression). For details, see the text. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

contrast, HO-1 induces angiogenesis presumably by a CO-independent mechanism, as HO-1 increases the expression of SDF-1α only. Thus, modulation of the HO-1/CO axis may provide a novel tool for the repair of cardiac injury [152] (Fig. 2).

However, CO does not always exert cytoprotective activity. The protective or detrimental effects of CO may depend on several factors, including the type of cells, the concentration of CO produced or administered, and the tissue-specific signaling transduction pathway(s) that may be involved in its biological activity [151,153]. The exposure to low concentrations of CO enhances apoptosis in porcine granulosa cells and SMC, both *in vitro* and *in vivo* [41]. Exposure to CO also increases Fas/CD95-induced cell death in Jurkat T cells, which correlates with CO-induced upregulation of the proapoptotic protein Fas-associated protein with death domain (FADD), as well as the activation of caspase-8, -9, and -3, while simultaneously downregulating the antiapoptotic protein BCL-2. Therefore, in contrast to other studies showing the antiapoptotic effects of CO, Fas/CD95-induced cell death in Jurkat T cells is augmented by exposure to CO partially occurring via inhibition in the activation of the extracellular signal-regulated kinase (ERK) MAPK [154]. Furthermore, CO has been demonstrated to reverse established pulmonary arterial hypertension *in vivo*, an incurable disease characterized by a progressive increase in pulmonary vascular resistance, leading to heart failure [134]. This occurs in part suppressing growth and increasing apoptosis of the smooth muscle cells of the pulmonary artery. The ability of CO to exert its remodeling effects requires the expression and function of eNOS/NOS3. Indeed, NO acts in part as the effector molecule to induce cell death of pulmonary artery smooth muscle cells while preserving the endothelial cells [134] (Fig. 2).

CO may increase the formation of ROS and pro-inflammatory molecules producing noxious effects in certain organs (*e.g.*, the brain). Indeed, it has been shown that CO increases the formation of pro-inflammatory prostaglandins by activating COX in rat hypothalamic explants and in primary culture of rat hypothalamic astrocytes, suggesting that CO stimulates pro-inflammatory responses at least in the brain. Moreover, CO has been shown to reduce cellular levels of antioxidants (*e.g.*, glutathione) by increasing mitochondrial ROS formation through the CO binding to the cytochrome a3 residing in complex IV, indicating that CO may cause oxidative tissue damages [151].

Overactivation of the HO/CO system may have detrimental effects to normal homeostasis and, in particular, HO-1 may contribute to tissue injury under certain unfavorable circumstances. In particular, an excessive upregulation of HO and an increase of its end products (*i.e.*, free iron, BV, and CO) may have a pro-oxidant, pro-apoptotic, pro-inflammatory, and pro-proliferative effects. The excessive activation of the mammalian HO/CO system may therefore be associated with several clinical conditions, as well as exacerbate the virulence and pathogenic effects of certain microbial infections [41] (Fig. 2).

In some tissues and under certain conditions, HO activity can be considered to be detrimental in the development

and progression of cancer [41]. In fact, it has been speculated that the cytoprotective effects of HO in cancer cells may facilitate the evasion of oxidative stress, promoting resistance to several therapies and increasing tumor survival, growth, invasiveness, and metastasis [41,155]. Remarkably, upregulation of HO-1 expression has been reported in several tumors, including adenocarcinoma, cerebral glioblastoma and astrocytoma, chronic myeloid leukemia, hepatoma, lymphosarcoma, malignant vertical growth melanoma, oligodendroglioma, oral squamous cell carcinoma, prostate cancer, and renal cell carcinoma [41,155] (Fig. 2).

The adverse affects due to HO activation are strictly associated with the release of free ferrous iron (which is a pro-oxidant that induces oxidative stress and tissue injury), BV and CO (whose increased cellular levels may promote or exacerbate pathological conditions, depending on the tissue involved and circumstances). Many diseases, including hemolytic, inflammatory, and oxidative conditions, have been linked to abnormal CO metabolism and functions [58,156] (Fig. 2).

Chronic overexpression of HO-1, and the attendant liberation of intracellular free iron and CO, may contribute to the aberrant patterns of brain iron deposition and mitochondrial insufficiency described in age-related neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [41] (Fig. 2).

5. Conclusions and perspectives

Exogenous CO is poisonous to humans, impairing the O_2 carrying capacity of Hb [1,7,8,12]. However, eukaryotic organisms use endogenous CO, produced during heme degradation by the highly conserved HO enzymes [2–4,12,26,27], as a neurotransmitter and as a physiological signal molecule [12,21,152]. Moreover, diverse microorganisms, including aerobic and anaerobic bacteria and anaerobic archaea, use exogenous CO as a source of carbon and energy for growth [10,11,22]. Overall, the HO-dependent endogenous CO production in almost all the three domains of life (*i.e.*, archea, bacteria, and eukaryotes) seems to support the CO key role in many biological processes [10,11,22].

In the last years, progresses have been done in the comprehension of the HO/CO system. Endogenous CO has been recently demonstrated to act as a cytoprotective and homeostatic molecule with important signaling roles in both physiological and pathological conditions. Indeed, accounting for the antiapoptotic, anti-inflammatory, antioxidant, antiproliferative, and vasodilator effects of HO-1 and HO-1 end-products *in vitro*, CO delivery may impact on several pathophysiological conditions [12,20,21,26,157]. To date, it is openly debated how CO exposure modulates the cell molecular machinery in order to induce an homeostatic response after a stress stimulus [21]. Remarkably, CO interacts with several cellular hemoprotein targets, although the functional significance of such interactions is still unclear [11–13,15]. Remarkably, CO, delivered either as a gas or from CORMs, displays therapeutic potential in inflammation, sepsis, lung injury, cardiovascular diseases, transplantation, and cancer, this is supported by preclinical evidences, in large and small animal models [157,158]. The technology is now focused in bringing CO to clinical applications in the form of inhaled gaseous therapy or through the use of potentially parenteral and orally active CORMs (www.clinicaltrial.gov). However, considerable work is yet needed to correlate these exogenous effects with those generated endogenously [157,158].

Finally, the interplay between CO and other gases, such as O_2, NO, and H_2S, appears pivotal in several pathophysiological conditions, including: (*i*) mitochondrial respiration; (*ii*) vasodilatation, angiogenesis, and vascular remodeling; (*iii*) inflammation; and (*iv*) oxidative and nitrosative stress [15,122,159]. However, molecular mechanisms of gas actions are difficult to be established in detail. Indeed, (*i*) heme-proteins, playing a pivotal role in gas-generation, -signal transduction, and -interaction, provide binding sites where gases can interrelate; (*ii*) gas-producing, -sensing, and -action sites are often in physical proximity, lowering down the free-gas concentration; and (*iii*) the free and bound gas concentrations are difficult to be determined [15,27,39,159,160].

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