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Carbon monoxide: a new pharmaceutical agent?*

Le monoxyde de carbone : un nouvel agent pharmacologique ?

L. Rochette · C. Vergely · F. Rochette · C. Girard

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Abstract Small amounts of carbon monoxide (CO) are continuously produced in mammals. The intracellular levels of this gaseous molecule can markedly increase under stressful conditions following the induction of heme oxygenases (HO), ubiquitous enzymes responsible for the catabolism of heme. The development of a technology concerning the CO-releasing molecules (CO-RMs) that control the delivery and action of CO under different pathological conditions represents a major step forward in the development of CO-based pharmaceuticals with therapeutic applications. CO is important for the homeostatic control of cardiovascular functions. Abnormal metabolism and function of CO contribute to the pathogenesis and development of hypertension. Another vascular disease in which the role of CO has been evaluated is pulmonary arterial hypertension. Important results have been reported in which CO prevents intimal hyperplasia by arresting hyperproliferative vascular smooth muscle cells as well as increased mobilization and recruitment of bone-marrow-derived progenitor cells. Transplantation has been a field of research, in which most studies have investigated the beneficial properties of CO-RMs. CO gas and CO-RMs have produced promising results in the preservation of organs for transplantation. The anti-inflammatory properties of CO and CO-RMs have been demonstrated in a multitude of animal models of inflammation, suggesting a possible therapeutic application for inflammatory diseases. Despite therapeutic benefit in animal model studies, the efficacy of CO in humans remains unclear. Further studies are expected to better understand the pharmacokinetics as well as long- and short-term effects of CO-RMs. ***To cite this journal: Réanimation 21 (2012).***

Mots clés Monoxyde de carbone · Molécules libérant du monoxyde de carbone · Pharmacologie · Cardiovasculaire

Résumé Du monoxyde de carbone (CO) est continuellement produit en petites quantités chez les mammifères. Le niveau intracellulaire de cette molécule gazeuse augmente de façon significative en conditions de stress, après induction des hèmes oxygénases (HO), enzymes ubiquitaires responsables du catabolisme de l'hème. Pouvoir disposer de molécules libérant du CO (MLCO), avec la possibilité de maîtrise de ses conditions de production et d'action, représente une étape essentielle pour le développement de médicaments à base de CO pouvant déboucher sur des applications thérapeutiques. Le CO joue un rôle important dans le contrôle homéostatique des fonctions cardiovasculaires. Toute altération du métabolisme ou de l'utilisation du CO peut contribuer au développement d'une hypertension. L'hypertension artérielle pulmonaire est l'une de ces conditions pathologiques où le CO joue un rôle mécanistique significatif. Des données importantes ont été publiées, démontrant que le CO prévient l'hyperplasie intimale en stoppant la prolifération des cellules musculaires lisses et en inhibant l'augmentation de mobilisation et de recrutement de cellules souches progénitrices dérivant de la moelle osseuse. La transplantation est un domaine de recherche où plusieurs études ont montré le bénéfice de ces MLCO. Le CO et les MLCO sont à l'origine de résultats prometteurs dans le champ de la préservation des organes pour la greffe. Les propriétés anti-inflammatoires du CO et des MLCO ont été retrouvées dans de nombreuses études, au travers de modèles animaux d'inflammation, suggérant ainsi de possibles applications dans le traitement des pathologies inflammatoires. Néanmoins et malgré un bénéfice thérapeutique établi expérimentalement, l'intérêt du CO chez l'homme reste incertain. Des études à venir sont encore attendues pour permettre un

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progrès des connaissances tant pour la pharmacocinétique que pour les effets à court et à long terme des MLCO. ***Pour citer cette revue : Réanimation 21 (2012).***

Keywords Carbon monoxide · Carbon monoxide-releasing molecules · Pharmacology · Cardiovascular

Introduction

Small amounts of carbon monoxide (CO) are continuously produced in mammals. The intracellular levels of this gaseous molecule can markedly increase under stressful conditions following induction of heme oxygenases (HO), ubiquitous enzymes responsible for the catabolism of heme. Activation of HO pathway is part of a complex homeostatic adaptation of cells to the redox imbalance, and it is evident that increased CO production reflects an active involvement of this by-product in the cytoprotective response. CO is an important signaling mediator possessing vasodilatory properties, which are achieved by activation of the guanylate cyclase/cyclic guanylate monophosphate (cGMP) pathway as well as large-conductance potassium channels.

The development of a technology concerning the CO-releasing molecules (CO-RMs) that control the delivery and action of CO under different pathological conditions represents a major step forward in the development of CO-based pharmaceuticals with therapeutic applications.

The studies published in the last few years indicate that CO-RMs possess effective vasodilatory, anti-ischemic, and anti-inflammatory activities. Their pharmacological action may be associated with other important cytoprotective effects provided by small amounts of CO released. It has been reported that these compounds were able (1) to inhibit hypertrophy in cardiac myocytes and (2) to protect the organs subjected to ischemia and reperfusion.

Cellular targets of CO and oxidative stress

In the human body, the predominant endogenous source of CO is the oxidative degradation of heme (iron protoporphyrin IX) by heme oxygenases. Most heme is derived from senescing red blood cells and a small fraction comes from the degradation of other heme proteins such as myoglobin. Under pathophysiological conditions, there is an additional production of nonheme CO by the intestinal bacteria [1].

CO is the diatomic oxide of carbon and it is a chemically stable molecule. The water solubility of CO is very low at standard temperature and pressure. CO cannot react with water without substantial energy input. Unlike the high reactivity of nitric oxide (NO), which by itself is a free radical, CO does not contain free electrons. This stable chemical nature dictates that CO would not by itself give out reactive oxygen species. However, CO may be involved in oxidative stress indirectly, especially under pharmacological and toxicological conditions. Toxic and presumed subtoxic CO exposures are associated with significant oxidative and nitrosative stress (Fig. 1). This CO-induced increase in oxidative stress was not related to hypoxic stress from the formation of carboxyhemoglobin or by circulating platelets or neutrophils. However, CO-dependent lipid peroxidation was prevented or reduced by inhibition of xanthine oxidase or superoxide dismutase (SOD) and iron chelators. It has also been shown that intracellular H_2O_2 production in brain was increased by high concentrations of CO, followed by increases in hydroxyl radical production, and decreases in the reduced to oxidized glutathione (GSH/GSSG) ratio in mitochondria [2].

It has been suggested that CO may activate p38 mitogen-activated protein kinase (MAPK) to inhibit mitochondrial pathway-dependent apoptosis but prevent the phosphorylation of extracellular signal-regulated kinase (ERK)/MAPK pathway to augment death receptor pathway-dependent apoptosis [3].

Properties and sources of endogenous CO

In addition to uptake of exogenous gas, cells and tissues produce significant amounts of CO as an elimination product of cellular metabolism, largely from heme degradation catalyzed by microsomal HO (Reviews: [4–7]). Heme serves as a vital cofactor in oxygen transport proteins (hemoglobin, myoglobin) and enzymes involved in vital cellular processes such as respiration, inflammation, or drug metabolism [8]. Hemo–protein turnover leads to the production of CO as a necessary consequence of heme utilization. Nicotinamide adenine dinucleotide phosphate (NADPH), O_2, and flavoprotein

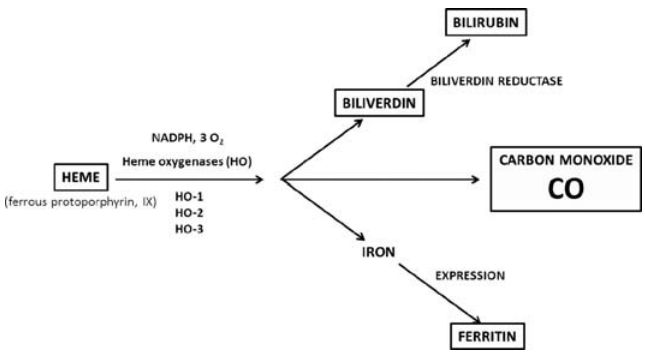

Fig. 1 Carbon monoxide (CO) metabolic pathway. HO: heme oxygenase; NADPH: nicotinamide adenine dinucleotide phosphate

reductase (cytochrome P450 reductase) are also required for turnover.

The production of CO is mediated by the HO enzyme, which exists as a macromolecular complex in the endoplasmic reticulum together with cytochrome c reductase and biliverdin reductase. There are **three HO isoenzymes**, namely HO-1, HO-2, and HO-3, of which only HO-1 is inducible. Although HO-1 plays an essential role in the degradation of hemoglobin (Hb)-derived heme, it is also a stress protein known as heat shock protein 32 (HSP32). Generally, HO-1 is thought to play a cytoprotective role against oxidant insults. Recent studies have suggested that the generation of Fe(II) by HO can result in pro-oxidant effects under some conditions.

The expression of HO-1 is ubiquitous in mammalian tissues and can be particularly up-regulated in the spleen and liver. The expression of the molecule increases after exposure to heme derived from senescent erythrocytes or from hemoproteins. Other inducing factors include nutrient depletion, hyperoxia, hypoxia, lipopolysaccharides (LPS), H_2O_2, NO, metals or organic chemicals.

The HO-2 isoform is expressed in the central nervous system, endothelial cells, and interstitial cell. Like HO-1, it is thought to be cytoprotective [9]. HO-3 has been cloned from rat brain, suggesting a neural function. This enzyme is structurally similar to HO-2, but is less efficient at degrading heme. A special interplay between NO and CO is suggested by the fact that HO-2 can act as a "sink" for NO.

In humans, the metabolism of CO is complex: CO is produced in cells and tissues through endogenous metabolic processes. Human blood contains CO, which originates in part from the degradation of the oxygen carrier hemoglobin. In humans, endogenous CO arises principally from the action of HO enzymes, which catalyze, as we reported, the rate-limiting step in heme degradation. For every mole of CO formed by the mechanism, one mole of ferrous iron is released and one mole of the linear tetrapyrrole biliverdin-IX is produced. The latter undergoes further metabolism to bilirubin-IXa by biliverdin reductase. The HO enzymes play an important physiological role in hemoglobin turnover in reticuloendothelial tissues such as the spleen, kidney, and liver where senescent erythrocytes are destroyed.

CO, carbonate radical, and modulation of intracellular reactive oxygen species

A variety of microorganisms possess the ability to oxidize CO to CO_2. In aerobic bacteria, the oxidation is catalyzed by CO oxidase, an inducible molybdenum-containing enzyme that couples the oxidation of CO to the reduction of oxygen. Moreover, heart cytochrome c oxidase possesses the ability to convert CO to CO_2 [10,11].

$$CO + H_2O \rightarrow CO_2 + H_2$$

or

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \text{ (water–gas shift reaction)}$$

In biological systems, where multiple free radicals were present, the recombination reaction of $^{\bullet}NO_2$ with another radical is very fast.

$$^{\bullet}NO_2 + O_2^{\bullet-} \rightarrow O_2NOO^-$$

Its conjugative acid, peroxynitrous acid is a strong oxidant, so it is a highly toxic compound.

It has been reported that the reaction between peroxynitrite and carbon dioxide may form carbonate radical anion and nitrogen dioxide. Therefore, under physiological conditions, where the concentration of CO_2 is high, rapid reaction of peroxynitrite with CO_2 occurs.

$$ONOO^- + CO_2 \rightarrow [ONOOCO_2^-] \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$$

Carbonate radical is involved in the activity of Cu-Zn-SOD. $CO_3^{\bullet-}$ has a much longer half-life than $^{\bullet}OH$ and can, therefore, diffuse further from the enzyme active site and can oxidatively modify distant cellular targets. During the myocardial reperfusion, the increased flux of free radicals [12,13], which react with $^{\bullet}NO$ enhances the formation of $ONOO^-$. These peroxynitrite ions are decomposed as previously mentioned. $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ are formed within the heart and may exert deleterious effects. Pro-oxidant properties have been described following the inhalation of toxic doses of CO, possibly by neutrophil recruitment [14]. In return, low doses of CO protect against hyperoxia-induced endothelial cell apoptosis by inhibiting reactive oxygen species (ROS) formation [15].

Recent studies implicate ROS-dependent signaling in the stimulation of mitochondrial biogenesis by CO, through redox-dependent activation of the nuclear respiratory factor 1 (NRF1) [16]. A role for autophagic regulatory proteins in CO-dependent cytoprotection has been recently reported [17]. This study uncovers a new mechanism for the protective action of CO via mitochondrial ROS formation.

Interactions of HO/CO and NOs/NO

NO and CO share common pathways that may be relevant to understand (Reviews: [1,18]). NO and CO have a high affinity for Fe. However, while both CO and NO bind Fe(II) avidly in hemoproteins, CO, unlike NO, does not bind to Fe(III) hemoproteins.

In contrast with CO, which exists as a stable neutral molecule, NO can be found in distinct redox-related states that have different reaction specificities. These forms of NO include nitric oxide ($^{\bullet}NO$), nitroxyl anion (NO^-) and the nitrosonium ion (NO^+). In addition to the reaction with Fe and other transition metals, NO can react with dioxygen, superoxide, and thiol groups. Unlike CO, which has a long half-life, the biological half-life of $^{\bullet}NO$ is in the order of

seconds, and it reacts with superoxide to generate peroxynitrite ($ONOO^-$). As we reported, this latter species is highly reactive and rapidly decomposes to form cytotoxic hydroxyl radical ($^{\bullet}OH$).

In terms of the chemical interaction between CO and NO, it is known that CO can stimulate NO release from proteins and the production of peroxynitrite. Piantadosi has suggested that CO could cause redistribution of NO in cells, which is consistent with different equilibrium constants of these molecules for metal binding [19]. Despite NO having a higher affinity for heme Fe, a paradoxical effect of CO is its ability to displace NO after a period of time. This is because the association constant of NO for heme Fe is greater than that of CO, while the dissociation constant for NO is also greater than that for CO. Thus, as NO is displaced by CO, CO remains bound to heme for a longer period of time.

The effector functions of CO and NO on the same molecule can be markedly different. An example is their effect on Hb. It is well known that CO binds to the heme of Hb and prevents oxygen dissociation (e.g., CO poisoning), whereas NO binds not only to heme but also to *S*-nitrosylates, the thiol groups that may be involved in regulating respiration.

Rationale for a therapeutic role for CO

Cardiovascular disorders

The technology is now in place to bring CO to clinical applications in the form of inhaled gaseous therapy or through the use of potentially parenteral and orally active CO-RMs (Reviews: [4,5,20,21]). There is an abundance of preclinical evidence in large and small animals showing the beneficial effects of CO, administered as a gas or as a CO-RM, in cardiovascular disease [22].

CORM-1 and CORM-2 are transition metal carbonyls whose solubility is restricted to organic solvents; consequently, dimethyl sulfoxide (DMSO) has been the classic vehicle used to test these compounds in vitro and in vivo and cannot be used in clinical scenario.

By contrast, CORM-3 and CORM-A1 represent the examples of water-soluble CO releasers (Fig. 2). The two compounds are fundamentally different in terms of chemical structure and rate of CO liberation: CORM-3 is a ruthenium-based tricarbonyl complex that releases CO rapidly ($t_{½} < 1$ min), whereas CORM-A1 is a boron-containing carboxylic acid that, under physiological conditions, releases CO with a slow kinetic ($t_{½} = 21$ min). This chemical difference dictates the way CO causes vasorelaxation and hypotension. CORM-3 elicits a rapid vasodilatory effect in vitro and in vivo; in return, CORM-A1 promotes mild vasorelaxation. In addition, CORM-3 induced vasorelaxation in aortas depend primarily on cGMP and endothelium; in contrast, the vasodilatory effect, mediated by CO slowly liberated from CORM-A1 involves guanylate cyclase and potassium channel activation mechanism, is endothelium-independent.

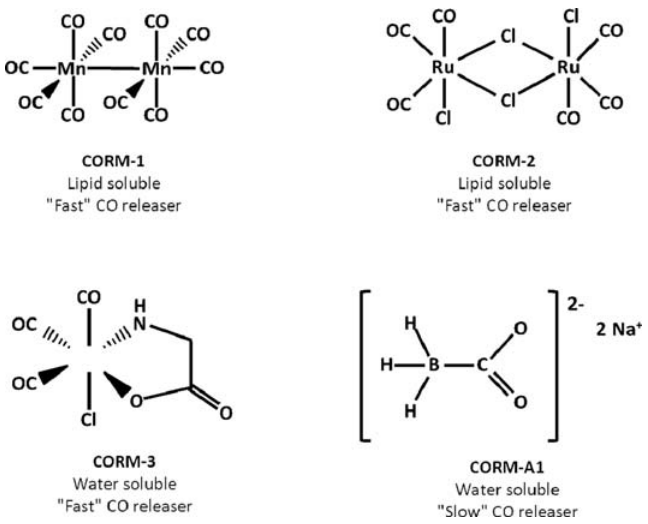

Fig. 2 Chemical structure of lipid-soluble and water-soluble carbon monoxide (CO)-releasing molecules (CO-RMs)

Both HO-1 and HO-2 contribute to the endogenous production of CO in the cardiovascular system [23]. HO-2 expressed in endothelial and smooth muscle layers of arterial and venous blood vessels has been reported to generate CO that intrinsically modulates vascular tone. Depending on the types of blood vessels, HO-2 may become critical in regulating the production of CO and vascular tone.

CO is important for the homeostatic control of cardiovascular functions. Abnormal metabolism and function of CO contribute to the pathogenesis and development of hypertension [24–26]. Hypertension is characterized by increased vascular contractility, concomitant increase in oxidative stress, enhanced vascular inflammation, and vascular remodeling. An up-regulated HO/CO system would not only normalize the endogenous CO concentration but also increase the production of biliverdin and bilirubin, two potent antioxidants.

Vascular disease is perhaps the most logical area where CO provides protective effects simply due to the mode of delivery, which involves from hemoglobin directly to the endothelium and smooth muscle. A recent report [27] showed that **aortic transplantation** in HO-1-deficient mice results in 100% mortality within 4 days owing to severe arterial thrombosis. Treatment of HO-1-deficient mice with CO-RM2 improved survival (62% survival at >56 days). Histological analyses showed that CO-RM2 treatment markedly reduced platelet aggregation in the graft, confirming previous data on the antiaggregatory properties of CO-RM3 and CO gas and emphasizing the pleiotropic

properties of these compounds in the resolution of vascular disorders [28].

In a mouse **myocardial infarct** model of coronary artery occlusion, pretreatment with or intravenous infusion of CO-RM3 at the time of reperfusion reduced infarct size, fibrillation, and tachycardia [29–31]. These cardioprotective mechanisms mediated by CO-RMs probably involve mitochondrial potassium channels, as small amounts of CO-RMs are lost in the presence of inhibitors of mitochondrial adenosine triphosphate (ATP)-dependent potassium channels.

Vascular tissues generate CO which, depending on experimental conditions, has been implicated in mediating vasoconstriction as well as vasodilatation. The vasoconstrictor and vasodilatory responses to CO are critically conditioned by redox mechanisms. The vasoconstrictor action is linked to increased oxidant activity. The vasodilatory action is linked to mechanisms involving guanylate cyclase and calcium-activated potassium (K_{Ca}) channels (Fig. 3).

It may be anticipated that acute administration of CO would elicit vasodilatation, because biliverdin/bilirubin would be present when redox balance is in equilibrium. Plasma bilirubin has been shown to have a large capacity to combat oxidative stress; therefore, lack of this pigment may reduce the antioxidant capacity of the vessel wall and allow for CO to elicit vasoconstriction. **Therefore, the effects of CO may be largely dependent on environmental redox balance or, in some cases, experimental conditions**. The debate as to whether CO of vascular origin functions as a vasodilator or vasoconstrictor has been fueled by conflicting reports in the literature.

The mechanism associated with CO-induced vasoconstriction, which appears to involve the generation of $O_2^{\bullet-}$ and potentially downstream ROS, has not been fully elucidated. ROS are known to lead to the generation of nonenzymatic metabolites of arachidonic acid known as isoprostanes that are capable of constricting vessels.

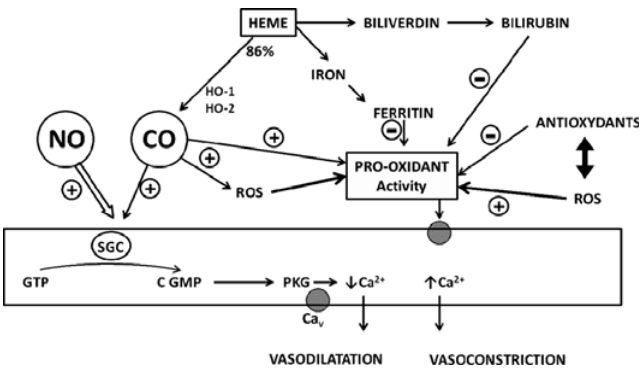

Fig. 3 Vasoregulatory actions of carbon monoxide (CO). cGMP: cyclic guanosine monophosphate; GTP: guanosine-5'-triphosphate; PKG: cGMP-dependent protein kinase; SGC: soluble guanylate cyclase

The cellular and molecular mechanisms responsible for the vasorelaxant effects of CO at physiologically relevant concentrations are not restricted to guanylate cyclase activation. CO directly enhances the activity of big-conductance calcium-activated potassium channels (BK_{Ca}) in rat vascular smooth muscle cells through a cGMP-independent mechanism.

Another vascular disease in which CO has been evaluated is **pulmonary arterial hypertension** for which there is no cure. Pulmonary arterial hypertension is caused by an increased expansion of vascular smooth muscle in the pulmonary arterioles and leads to right heart hypertrophy. In rodent models of established pulmonary arterial hypertension, inhaled CO restored thickened pulmonary arteries and the right heart to normal size and pressures by a mechanism involving endothelial-derived NO to induce apoptosis of the hyperproliferative vascular smooth muscle cells.

Important results had been reported in which CO prevents intimal hyperplasia by arresting hyperproliferative vascular smooth muscle cells and increased mobilization and recruitment of bone-marrow-derived progenitor cells [32].

Organ transplantation

Transplantation has been the main area in which most studies have been made concerning the beneficial properties of CO-RMs. CO gas and CO-RMs have produced promising results in the preservation of organs for transplantation. Currently used cold storage procedures can limit but not completely avoid ischemic injury and graft dysfunction in patients receiving transplants. CO, either as a gas (saturated solution) or in the form of CO-RMs, can act as protective adjuvant of the preservation solutions.

Beneficial effects of HO-1 modulation have been described in xenotransplantation models [33], where HO-1 gene expression appears functionally associated with xenograft survival. In a mouse-to-rat heart transplant model, the effects of HO-1 up-regulation could be mimicked by CO administration, suggesting that HO-derived CO suppressed graft rejection. The authors proposed that CO suppressed graft rejection by different actions such as inhibition of platelet aggregation and endothelial apoptosis. The ability of CO to suppress inflammation is likely involved in xenograft transplant models in which 400 ppm CO for 2 days prevented rejection for up to 50 days. In other study where CO-RM3 have proved to be effective in organ transplantation was reported by Bagul et al. [34]. In an experimental model of non-heart-beating donor kidney in pigs, low concentrations of CO-RM3 ameliorated a loss in renal blood and urine flow. The modulatory effects of CO on platelet aggregation, vasodilation, and proinflammatory

cytokines all potentially contribute to the favorable outcome in xenograft transplantation.

Inflammation

The anti-inflammatory properties of CO and CO-RMs have been demonstrated in a multitude of animal models of inflammation, suggesting a possible therapeutic application for inflammatory diseases. Increased accumulation of neutrophils, expression of intercellular adhesion molecule 1 (ICAM-1), and activation of nuclear factors in septic mice were also attenuated by systemic administration of CO-RM2, an effect that seems to be associated with decreased production of ROS and NO.

Similar anti-inflammatory effects of CO have now been demonstrated in models of ischemia–reperfusion (I/R) injury of the heart and kidney. CO protected against liver I/R injury via activation of the p38 MAPK [35]. Homozygous *ho-1* null mice (*hmox-1*$^{-/-}$) displayed increased mortality in a model of lung I/R injury. Inhalation of CO (1,000 ppm) partially compensated for the HO-1 deficiency in *hmox-1*$^{-/-}$ mice, and improved survival following I/R [36]. In this model, the authors propose that the protection provided by CO involved the enhancement of fibrinolysis via the cGMP-dependent inhibition of plasminogen activator inhibitor-1 (PAI-1) expression.

The second category of effects of CO concerns the effects on apoptosis. In endothelial cells, hepatocytes, and cardiomyocytes, CO is antiapoptotic, preventing cell and tissue injury, whereas in T cells (that attack and destroy cells or tissue), cancer cells, or fibroblasts, CO imparts proapoptotic effects [37,38]. It has been reported in this area its ability to influence cellular proliferation. CO blocks the proliferation of cancer cells, effector T cells, hyperproliferative smooth muscle cells of hyperplastic intima following vessel trauma. Pulmonary I/R caused by temporal clamping of the pulmonary artery induced the biochemical features of apoptosis in experimental rodent lungs. The protective effect of CO pretreatment on mice subjected to lung I/R in vivo was associated with caspase-3 activation and depended on the activation of p38α MAPK, markers of apoptosis [39,40].

Protective effects of CO have been studied in sepsis. Sepsis-induced death associated with Enterococcus faecalis infection in mice is also reversed by treatment with CO-RM2 in wild-type and HO-1-deficient animals [41]. Recent studies have explored the protective effects of CO and CO-RM in vitro and in vivo in several models of acute respiratory distress syndrome (ARDS). Findings report the possible applications of CO and CO-RMs in acute lung injury and ARDS [42]. Despite the therapeutic benefit in animal model studies, the efficacy of CO in humans remains unclear.

Conclusion: can CO be considered as a therapeutic agent?

As reported, CO is produced in cells and tissues through endogenous metabolic processes. Human blood contains CO, which originates in part from the degradation of the oxygen carrier hemoglobin. In humans, endogenous CO arises principally from the action of HO enzymes. CO and CO-RMs exhibit a wide range of biological effects resulting in specific responses that involve a number of intracellular pathways and targets that encompass inflammation, apoptosis, and cellular proliferation. Both CO gas and CO-RMs have potent anti-inflammatory effects as they are able to decrease the production of inflammatory mediators and to attenuate the levels of NO and tumor necrosis factor-α (TNF-α).

One question that arises with regard to HO is whether CO generated endogenously during HO activity similar in concentrations to that administered exogenously by CO gas or by a CO-RM. Numerous studies show a role for HO-1-derived CO as a protective agent, but the therapeutic concentrations may be extremely different. This is a challenging issue to resolve because HO-1-derived CO is probably at higher concentrations in the tissue, is localized in distinct intracellular compartments. Notably, the effects of HO-1-derived CO on cellular respiration appear identical to that observed with exogenously delivered CO at a concentration otherwise shown to impart cellular benefits.

As with all new agents, a wide therapeutic window and strategies to minimize potential toxic effects are the ultimate objectives. One challenge, however, for which development of the gas versus the CO-RMs differs, is in the parent molecule of the CO-RM. CO gas is simple, relatively nonreactive and easy to administer. Further studies are expected to lead to a better understanding of the pharmacokinetics and long- and short-term effects of the CO-releasing compounds.

Conflict of interest : none.

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