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Carbon Monoxide as a Therapeutic for Airway Diseases: Contrast and Comparison of Various CO Delivery Modalities

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Abstract

The quest to find novel strategies to tackle respiratory illnesses has led to the exploration of the potential therapeutic effects of carbon monoxide as an endogenous signaling molecule and a cytoprotective agent. Further, several studies have demonstrated the pharmacological efficacy of CO in animal models of respiratory disorders such as acute lung injury and pulmonary hypertension. Because of the gaseous nature of CO and its affinity for multiple targets, CO's controlled delivery has been a challenge. Past studies have employed different delivery modalities including CO gas, HO-1 inducers, and CO donors, sometimes leading to substantive variations of the resulting pharmacological effects for various reasons. Herein, this review summarizes and analyzes the differences among the profiles of various CO-delivery modalities in terms of their efficacy, dosing regimen, and pharmacokinetics in airways models. We believe that analysis of these issues will help in understanding the fundamental roles of CO in airways and eventually contribute to its development as a medicine for respiratory diseases.

Graphical Abstract

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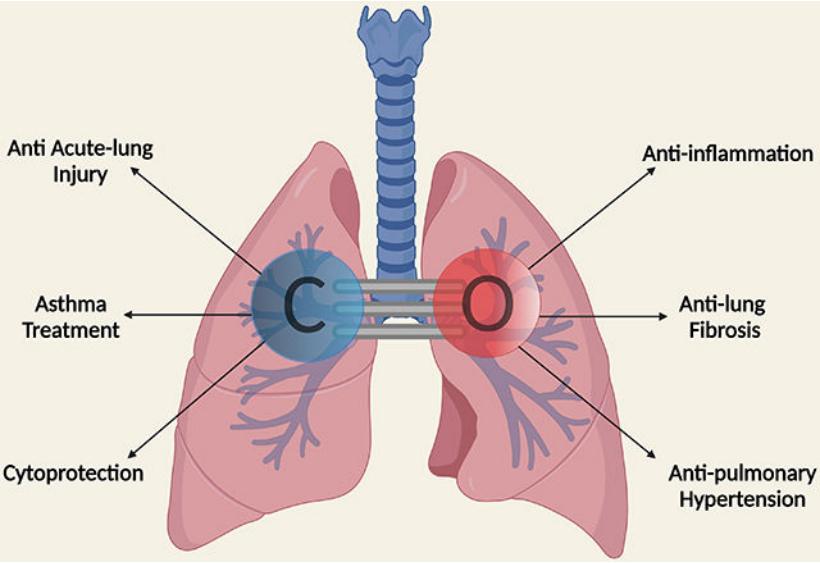

Keywords

Respiratory system; CO; HO-1 inducers; CO-RMs; cytokines

1. Introduction

Carbon monoxide (CO) is an endogenously produced gaseous molecule with important signaling roles in cellular physiology.[1, 2] CO is mainly produced during heme degradation by heme oxygenase enzymes (HO-1, HO-2),[3, 4] of which HO-1 represents the major inducible isoenzyme responsive to various forms of chemical and physical stress, whereas HO-2 is constitutively expressed. Though widely known as a poisonous gas, CO, like other recognized endogenous gaseous signaling molecules such as nitric oxide (NO) and hydrogen sulfide (H_2S), manifests its toxicity dose-dependently at elevated non-physiological levels. Although similar in molecular weight, CO exhibits far less biological reactivity than the other known gaseous mediators, in that it binds solely to biological iron centers. Under normal physiological conditions, humans can harbor mid- to high-micromolar concentrations of CO in the form of carboxyhemoglobin (COHb, ≤2%) in the blood.[2] For smokers, this concentration can approach millimolar levels (routinely 8-9%).[5, 6] Since the first report of the ability of CO to activate the soluble form of guanylyl cyclase (sGC) in 1994,[7] extensive studies over the past three decades have provided strong evidence supporting the pleiotropic effects of CO in antioxidation,[8] anti-proliferation,[9] anticoagulation,[10] anti-inflammation,[10, 11] antiapoptosis[12] and organ protection.[2, 4, 13, 14] At the molecular level, CO exerts its effects by binding to proteins containing transition metals in specific redox states, especially hemoproteins in the ferrous state.[15] Such examples include hemoglobin (Hb), myoglobin, cytoglobin, neuroglobin, inducible nitric oxide synthase (iNOS), cytochrome *c* oxidase, cytochrome p-450, and nuclear receptors such as REV-ERBα/β.[15, 16] Other non-heme putative (and possibly indirect) targets of CO include the K_{ATP} ion channel, the epithelial sodium ion channel, Ca^{2+}-activated K^+ (BK_{ca})

channel, and metalloproteinases containing zinc.[3, 15] At therapeutic doses, the signaling functions of CO can lead to a variety of beneficial effects.[2]

Among its pleiotropic effects, the beneficial roles of CO in airway pathologies are considered very intriguing and exciting. Because of the widely known role of CO as an inhalation poison (at high levels), its beneficial effects in the pulmonary system are counter-intuitive and present unique research opportunities. Many publications have described the physiological roles[17-19] of CO and its therapeutic functions in the respiratory system,[16, 17, 20] including pulmonary preservation,[21-24] hyper-inflammation,[11] lung ischemia-reperfusion (I/R) injury,[23, 25-30] lung transplantation,[12, 31-36] cardiopulmonary bypass (CPB),[37-40] ventilator-induced lung injury (VILI),[41, 42] acute lung injury (ALI) caused by infection[43] including sepsis and endotoxin-induced injury,[44-48] ALI caused by hemorrhagic shock,[49-51] and inhalation of particulate matter.[52] Much of the therapeutic effects of CO have been previously reviewed in different contexts.[13, 17, 20, 53-56] This review aims to provide a succinct and novel discussion of the application of CO in various delivery forms in the airways with emphasis on the comparison of doses required to elicit therapeutic effects. In the field of CO-based therapeutics, traditionally there are three methods of achieving CO delivery. First, inhaled CO (iCO) is the most widely used method to deliver pure CO. Many animal model studies based on iCO have been conducted. However, CO inhalation may have limitations related to the inconvenience of delivering a gas, which include challenges in controlling dosage and potential safety concerns to lab and clinical personnel. Second, there are CO donors available for delivering defined amounts of CO *via* various routes of administration,[13, 57] including intraperitoneal (i.p.), intravenous (i.v.), and oral (p.o.). Some of the most common CO donors used in animal model studies include metal-based CO-releasing molecules (CO-RMs) in the form of immobilized carbonyls[13, 58-66] and organic CO prodrugs.[13, 67-71] Third, there are known inducers of HO-1, which are used as "CO surrogates," with the assumption that HO-1 induction subsequently leads to elevated CO production. However, the functions of HO-1 are known to exceed that of CO production alone. On one hand, heme degradation also generates labile iron, which may cause redox imbalance and must be sequestered or reutilized; as well as biliverdin and its metabolite bilirubin, which have distinct biological activities including antioxidant effects.[72] Further, HO-1, in a truncated form, independently of heme degradation, can function as a transcription factor after nuclear localization.[73, 74] Thus, the effects of HO-1 overexpression are considered pleiotropic in that they potentially impact more processes and targets than the generation of CO alone.

Recently, there have been reports of intrinsic chemical reactivities and CO-independent activities of some of the most commonly used CO-RMs,[2, 75-87] which led to the proposal by Poole *et al.*, for a complete "reappraisal" of these CO donors.[75] We have also characterized distinct chemical reactivities of select CO-RMs.[88, 89] Such results suggest that these molecules may have functions that transcend the delivery of CO. Given the discussions about the three modalities of "CO delivery," clearly there are substantive differences. However, in the CO field, results from the experiments using different "CO delivery" approaches often are collectively attributed to the effects of CO as the active component. In considering the development of appropriate therapeutics based on CO, it is important to analyze the similarities and differences among various approaches by comparing the

specific details, such as delivery forms, dosage, and efficacy as well as their effect on the pharmacokinetics of CO, whenever possible. Therefore, in this review, we will summarize the literature to present experimental results based on delivery approaches and analyze the data accordingly.

2. Comparison of the therapeutic effects of CO delivered by different modalities

The respiratory system is critical to sustain life owing to its primary function in gas exchange. The large surface area and delicate histological structures of the lungs render them vulnerable to various stress factors due to their direct exposure to environmental elements during the breathing process. Hazardous elements such as air pollution,[90] smoking,[91, 92] and pathogens[93] can readily induce inflammatory pathogenesis to the airway tissues. Emphysema, chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis, lung carcinoma, pneumonia, and acute respiratory distress syndrome (ARDS) rank among the common airway pathologies. There is mounting evidence showing that CO can attenuate inflammation and oxidative stress in these conditions.[13] Over a period of decades, CO has found several therapeutic applications in airways through studies using cell culture and animal models.[17, 19] Hyperoxia-induced lung injury resembles pathophysiological changes in human ARDS, such as inflammatory damage to the alveoli and apoptosis of lung cells, and is therefore often used as a model of ALI. One of the pioneering studies by Otterbein *et al.*[94] found that lethal hyperoxia (>98% O_2)-induced lung injury in rats was completely prevented by co-inhaling 250-500 ppm CO gas in the oxygen, while all control animals died within 72 h after exposure to hyperoxia without CO. These results suggest the potential of using CO as an anti-inflammatory and anti-apoptotic agent for therapeutic effects in ALI/ARDS. Subsequently, there have been a large number of studies using CO for treating airway pathologies. Table 1 summarizes such studies based on various CO delivery modalities.

In the following sections, we analyze the data in detail, and whenever possible, provide a contextual comparison of the activity and efficacy of different delivery forms of CO.

2.1. Studies Using CO Gas and CO-RMs

The majority of studies on the airway protective effects of CO have been conducted using CO gas, providing a high degree of assurance that the observed activities are CO-dependent. Therefore, the discussions also start with the results from CO gas inhalation followed by studies employing CO-RMs in the experiments. Such discussions can serve as good reference points for comparison against other approaches. As an intuitive and concise way to highlight research progress in this field, brief descriptions of the findings from each study are summarized in Table 1. Selected examples are described in depth in the following section.

2.1.1. Anti-Inflammatory Effects of CO—Inflammation represents a set of measures taken by the biological system in response to insults by diverse stimuli. In acute conditions, such measures help in the repair and healing of the damaged tissue and in fighting against

harmful xenobiotics.[114] However, prolonged and uncontrolled inflammatory conditions often lead to acute and/or chronic diseases and disorders.[115] Respiratory diseases such as COPD and asthma exhibit a high degree of inflammation, often leading to poor quality of life and death.[115] Therefore, measures to keep inflammation in check are considered of primary importance. Anti-inflammatory effects of CO have attracted researchers to investigate the feasibility of its therapeutic application in respiratory disease (Table 1, Entry 1-16).

Respiratory infection caused by pathogens such as *Streptococcus pneumoniae, Pseudomonas aeruginosa*, and *Haemophilus influenzae* provokes immune responses leading to the development of inflammation in the lungs.[116] In this aspect, CO has shown impressive properties in treating respiratory inflammation by preventing bacterial growth[117] and modulating cellular signaling pathways.[118] In one study[96] of the effects of CO against *Streptococcus pneumoniae*-induced inflammation in a baboon (nonhuman primate) model (Table 1, Entry 2), CO (200 to 300 ppm) was administered for 60 to 90 minutes through inhalation in a manner to maintain the COHb level below 10%. CO administration was done 48 h after *S. pneumoniae* infection. A significant elevation was noted in the level of specialized pro-resolving mediators (SPM) such as plasma lipoxins (78.56 ± 11.8 *vs.* 53.66 ± 13.2 pg/ml) and the eicosapentaenoic acid-derived resolvins (27.66 ± 7.8 *vs.* 18.46 ± 5.9 pg/ml) compared to pre-CO exposure. SPMs are known for their anti-inflammatory activities such as clearance of debris and prevention of PMN recruitment at the site of inflammation.[119] Further, in this study, CO also reduced the level of pro-inflammatory mediator TxB2 to 1.56 ± 0.2 pg/ml in comparison to 2.46 ± 0.1 pg/ml (pre-CO exposure). Thereby, CO acts in diverse ways to treat inflammation. In using CO-RMs as CO donors, one study[99] describes the effect of CORM-2 against *Pseudomonas aeruginosa*-induced inflammation in mice (Table 1, Entry 9). Pre-treatment of mice with 8 mg/kg CORM-2 for 2 h resulted in an approximately 3-fold decrease in the levels of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, and ICAM-1. At the same time, >3-fold decrease in the level of pro-inflammatory interleukin IL-8 was also noted. Of interest, a different reactivity profile of CO-RMs against *P. aeruginosa* has been reported by Motterlini *et al.*[117, 120] where CORM-2[117] and CORM-3,[120] both ruthenium-based CO-RMs, showed significant bactericidal activity while CORM-A1[117] (a non-ruthenium, boron-based CO-RM) elicited bacteriostatic effects. Another breakthrough study by Poole *et al.*[75] delineated cytotoxic activity of CORM-3 against *E. coli* as originating from ruthenium interaction with thiols, not from CO. Therefore, a wide range of mechanisms of action was seen among different CO delivery forms to achieve anti-inflammatory activity against pathogen-induced inflammation. However, pharmacological effects of CORM-2 in the above-discussed experiments require more thorough investigations due to the reported[75, 87] reactivity of the ruthenium cations as discussed earlier.

Apart from the distinctive chemical profiles of CO-RMs, a contrast in a dose-response relationship has also been observed with CO gas in different experimental models. A study by Zhou *et al.*[12] assessed the effects of CO on brain-dead mice (Table 1, Entry 17). Abrupt and severe elevation of intracranial pressure following brain death subsequently results in ALI.[121] In such conditions, donor lungs are not suitable for transplantation. This study examined the effects of CO on brain death-induced ALI models. In the study,

brain death was produced by inflating a balloon catheter inserted into the intracranial cavity and injecting saline at 20 μl/min through it. Further, brain-death was confirmed by complete cessation of electrocerebral activity as assessed by electroencephalogram. After confirmation of brain death, inhalation of 250 ppm CO for 2 h alleviated lung injury *via* inhibition of pro-inflammatory (IL-6, TNF-α) and proapoptotic caspase-3 mRNA expression. Mean serum levels of IL-6 were 100 ± 26 pg/mL in the CO-treated group *vs.* 130 ± 34 pg/mL in the untreated group, while mean serum TNF-α levels were suppressed by CO (247 ± 92 pg/mL) as compared with that of the air-treated group (389 ± 109 pg/mL). In higher mammals, a study by Mitchell *et al.*[98] probed the anti-inflammatory role of CO in cynomolgus macaques (Table 1, Entry 6). Exposure to LPS (15 mg/m^3, 5 min) led to a substantial pro-inflammatory response. Two different doses of CO (500 and 250 ppm, 6 h each) were administered (pre-treatment) to assess the therapeutic doses required to prevent animals from LPS-induced inflammatory damage. At 500 ppm, a 67% reduction in neutrophil levels was observed *vs.* no effect at 250 ppm. Further, 500 ppm of CO reduced the level of TNF-α by 40% in BAL, while surprisingly no significant effects were observed on the levels of IL-6 and IL-8 compared with air-exposed animals. From a basal level of ~4.4% COHb, exposure to 500 ppm CO resulted in an increase of COHb level to an average of 34%, while an increase to 25% was seen in the case of 250 ppm inhalation for 6 h. Interestingly, despite achieving 25% COHb in the systemic circulation, neither therapeutic effects nor toxicity was observed. In contrast, 250 ppm of CO showed pronounced efficacy in other experimental animals such as pigs[37, 38] and mice[95] along with the fact that >20% COHb is known to cause certain signs of toxicity in humans.[122] Along this line, a study by Bathoorn *et al.*[113] applied 100-125 ppm CO to human subjects (COPD patients, Table 1, Entry 36). In this report, inhalation of 100-125 ppm CO for 2 h every day for 4 consecutive days showed no significant reduction in the number of neutrophils in sputum samples of stable-COPD patients compared with placebo group (p-value = 0.34). Interestingly, the number of sputum eosinophils decreased (p-value = 0.07) in the same studies. Both eosinophils and neutrophils belong to the class of granulocytes of white-blood cells responsible for defense against xenobiotics. As compared to the placebo group (0.2% median COHb), inhalation of 100 ppm CO led to a median COHb level of 2.6% while 125 ppm CO to 3.1%. Of course, one cannot directly compare results across species. However, these data do bring out the intricacies of variations among species in terms of dose-response relationship and efficacy.

NLRP3 (nucleotide-binding domain and leucine-rich repeat protein-3) inflammasome activation is a commonly used experimental model[123] to study pro-inflammatory changes in response to various stimuli such as microbial infections,[124-126] ATP,[127] LPS,[125, 127] environmental irritants such as particulate-matter (PM),[124] and silica.[128] Once activated, the NLRP3 inflammasome causes the release of pro-inflammatory interleukins[123] such as IL-1β, IL-18, and pyroptosis.[129] In addition, the roles of NLRP3 in inducing pro-inflammatory responses in airway diseases such as asthma[130-134] have been studied. To understand the anti-inflammatory effects of CO in various biological systems, NLRP3 inflammasome activation has proven to be very useful. A research study by Jung *et al.*[91] describes the actions of CO in an NLPR3 inflammasome model (Table 1, Entry 5). In this study, bone marrow-derived macrophages (BMDMs) were challenged with LPS (10

ng/mL) for 6 h followed by ATP (2 mM) for 1 h with or without CO gas (250 ppm). CO treatment caused >80% reduction in the level of IL-1β and IL-18 compared to the air-treated group. In another experiment, CO treatment resulted in a decrease in the number of depolarized mitochondria (19.8%) with respect to air treatment (35.8%). As reported, pro-inflammatory stimuli elicit higher production of mtROS, which is then associated with the lowering of mitochondrial membrane potential ($\Delta\Psi_m$), and subsequent NLRP3 inflammasome activation.[135, 136] Having examined salutary effects of CO gas in NLRP3 inflammasome model, a study by Kim *et al.*[102] provides detail analysis of properties of CORM-2 and CO gas using the same model (Table 1, Entry 12). CORM-2 pre-treatment for 30 mins augmented LPS (1 μg/ml)-induced expression of pyrin, a negative regulator of NLRP3, in a human leukemic cell line (THP-1) in a dose- and time-dependent manner. CORM-2 at concentrations of 10, 20, and 40 μM upregulated pyrin mRNA expression. The effect of 250 ppm of CO gas was also comparable to CORM-2 (20 μM) on the level of pyrin in cell culture studies as observed by increase in pyrin mRNA expression by ~2.5-fold at the 12 h time-point. In addition, a dose- and time-dependent effect was observed on the inhibition of IL-1β secretion *via* induction of IL-10 mRNA transcription by CORM-2 or CO gas, individually. The effect of 20 μM CORM-2 on the secretion of IL-10 was more pronounced (~3-fold) than 250 ppm CO in the time-dependency assay. However, direct comparison can be challenging because of the untoward chemical reactivities of ruthenium cation present in the CO-RMs as discussed above.[75, 88, 89]

2.1.2. Protection against Pulmonary Hypertension—Pulmonary hypertension (PH) is a clinical condition characterized by high (≥25 mm Hg) mean pulmonary arterial pressure (PAP) under resting conditions.[137] Commonly, patients with congenital heart disease, high blood pressure, emphysema, HIV infection, and liver disease are at an increased risk of developing PH.[137, 138] As a result of this condition, oxygen concentrations in blood vessels in the lungs decrease significantly.[139] Given the vasomodulatory properties[140, 141] of CO, experiments have been conducted to treat PH using CO (Table 1, Entry 31-33). One of the earliest studies by Zuckerbraun *et al.*[108] demonstrated the beneficial effect of CO in hypertensive rats (Table 1, Entry 31). In this study, monocrotaline sodium-treated rats developed pulmonary arterial hypertension (PAH) over a period of 6 weeks. Ventilation with 250 ppm CO for 1 h led to an increase in basal COHb level from 1.2 ± 0.5% to 19 ± 1.5%. Further, early treatment with 250 ppm CO at 1-14 days for 1 h/d significantly prevented PH as assessed by a ~57% reduction in mean PAP. Other indicators of progressive PH such as RV mass and ratio of RV to body weight (RV/BW) were also reduced by ~60% and ~67%, respectively, compared to air treatment. After the development of PAH, CO administration at 15-28 days and 29-42 days showed substantial improvement in PAH and prevented further development. In addition, histopathological analysis of the pulmonary arteriole displayed increased medial expansion of the smooth muscle indicating a remarkable decrease in pulmonary arterial hyperplasia after 6 weeks of treatment. Thereby, these results demonstrated promise of using CO in PH therapy. Recently, a study by Pak *et al.*[109] assessed the direct effects of CORM-2, CORM-3, and CO gas on pulmonary vasomodulation in isolated ventilated and perfused mouse lungs (Table 1, Entry 32). Under hypoxia, CO gas (10% CO, 5% CO_2, 1% O_2, balance N_2) reduced PAP significantly (~25%) when applied 5 mins prior to the hypoxic ventilation. In contrast,

pre-treatment with CORM-2 and CORM-3 showed no significant effect on PAP. However, under normoxia, CORM-2 and CORM-3 and their inactive forms (iCORM-2 and iCORM-3) caused a rise in PAP in a concentration-dependent manner. The experiments used CORM-2 and iCORM-2 at 0.5, 5, 50, and 500 μM in the study, while the doses of CORM-3 and its inactive form used were 10, 50, and 500 μM. Another study[110] was conducted to examine the effects of CORM-3 on PH in mice subjected to hypoxia (9%) for 15 days. An oral dose of CORM-3 (50 mg/kg/day) was given to mice during hypoxia. In contrast to the results of Pak *et al.*,[109] this study reported strong vasomodulatory effects of CORM-3 on PAP in mice as indicated by measuring RSVP. Administration of a single oral dose of 50 mg/kg/day CORM-3 prevented the development of PH as indicated by a significant reduction in RSVP (~50%), RV hypertrophy index (~17%), and pulmonary artery muscularization (~20%) of the chronically hypoxic mice. Thereby, these data reflect contrasting pharmacological profiles of some of the commonly used CO delivery forms.

2.1.3. Application of CO in Lung Transplantation—Lung transplant is the last resort in end-stage pulmonary diseases and often involves reperfusion[31] and surgery-related injuries as well as infection.[142] Considering the potential application of CO as an anti-inflammatory and cytoprotective agent, CO has been explored widely in pre-/post-operative organ transplant care (Table 1, Entry 25-30). Earlier experiments on CO treatment of donors for the transplantation of organs such as lung,[26, 143] kidney,[144] islets,[145] and liver[146] have shown significant beneficial effects and improved graft survival. Recently, Meng *et al.*[34] used 500 ppm CO with a mixture of 40% O_2 + remaining N_2 to inflate rat lungs before transplantation and examined pulmonary graft function under cold ischemic conditions (stored at 4 °C, 3 h). Pulmonary graft function evaluated by oxygenation showed that CO exposure enhanced oxygenation of the graft (381 ± 58 mmHg) compared to the control group (308 ± 78 mmHg) treated with 40% O_2 and 60% N_2. Further, exposure to CO ameliorated ischemia-induced lung injury by reducing serum concentrations of IL-8 (279 ± 46 pg/mL *vs.* 456 ± 63 pg/mL in the control group) and TNF-α (377 ± 59 pg/mL *vs.* 520 ± 91 pg/mL in the control group). CO exposure also elevated the concentration of superoxide dismutase to 64 ± 17 U/mg protein as compared to the control (40 ± 9 U/mg protein).

In another report, a 250-ppm dose instead of 500 ppm was used to examine the effects of CO in lung transplantation. In a post-operative application study, Goebel *et al.*[39] administered CO (250 ppm, 1 h) to study its anti-inflammatory effects in a pig model (Table 1, Entry 18). CO inhalation 1 h after CPB led to an increase of 8-10 folds in COHb level from baseline and reduced the expression of pro-inflammatory factors TNF-α (521 ± 77 pg/mL *vs.* CPB 821 ± 97 pg/mL) and IL-6 (304 ± 81 pg/mL *vs.* 860 ± 153 pg/mL). The level of IL-10 was highly elevated by CO treatment after 1 h CPB (278 ± 40 pg/mL *vs.* CPB 63 ± 20 pg/mL). Further, Zhou *et al.*[36] compared the effects of 250 ppm of CO to 500 ppm in brain-dead rat lung donors (Table 1, Entry 28). Brain-dead rats inhaled either 250 or 500 ppm of CO with 40% O_2 for 2 h. The CO treated group showed an improved anti-inflammatory response over the control group. For example, the concentration of IL-6 in the control group was 138 ±44 pg/ml as compared to 104 ± 20 pg/mL and 76 ± 17 pg/mL in groups treated with 250 ppm and 500 ppm of CO, respectively. Furthermore, a 250 ppm CO dose reduced TNF-α level to 46 ± 8 pg/mL while a 500-ppm dose reduced TNF-α to 30 ± 8 pg/mL as

compared to 60 ±11 pg/mL in the control group. The concentration of anti-inflammatory IL-10 was enhanced by CO treatment– 210 ± 53 pg/mL and 297 ± 55 pg/mL in 250 ppm and 500 ppm dosing groups, respectively, compared to the control group (88 ± 26 pg/mL). The effects were more pronounced with 500 ppm than 250 ppm. These observations reflect dose-dependent effects of CO gas in pre-/post-operative lung transplant care.

2.1.4. Protection Against Lung Injury—The American Thoracic Society and the European Society of Intensive Care Medicine classify Acute Respiratory Distress Syndrome (ARDS) as a severe form of ALI.[147-149] ARDS is characterized by the acute onset of respiratory failure, severe hypoxemia (PaO_2/FiO_2 ratio ≤200 mmHg), hypercapnia, and increased lung permeability.[150] ALI manifests inflammatory reactions in response to diverse stimuli such as sepsis, hyperoxia, pathogens, physical and chemical agents.[151-153] CO has shown a high degree of effectiveness against ALI[153] (Table 1, Entry 17-24). One particular example worth noting is the protective effect of CO against ventilation-induced ALI (VILI). Faller *et al.*[41] investigated pre-treatment and co-treatment of CO in mice undergoing mechanical ventilation for 6 h (Table 1, Entry 19). As a control, ventilation with air for 6 h provoked strong pulmonary inflammation, exemplified by transmigration of inflammatory cells (e.g., neutrophils), release of pro-inflammatory cytokines (e.g., IL-1β), and histological changes to the lung indicated by an increase in alveolar wall thickening. The study found that 1 h pre-treatment with spontaneous breathing of 250 ppm CO followed by 6 h of air ventilation did not attenuate VILI-related inflammation. However, co-treatment with CO during 6 h air ventilation produced a significant protective effect against VILI. Among different co-treatment regimens, continuous ventilation with 250 ppm of CO throughout a 6 h ventilation period offered the highest protective effect. In BAL, IL-1β level was the same as the non-ventilated group; neutrophil infiltration decreased by 75%. Alveolar wall thickness and VILI score also improved significantly. At the end of the persistent co-treatment during 6 h ventilation, COHb was found to be 29.7 ± 0.6%. Apart from this, the study also included other co-treatment regimens such as CO exposure for the initial 1 or 3 h at the onset of 6 h air ventilation (early phase) or CO exposure starting at 3 or 5 h time point to the end of 6 h air ventilation (late phase). Significant protective effects were observed after these dosing regimens, though to a lesser extent than the persistent co-treatment experiment. COHb levels in the early phase co-treatment groups were similar to the background level of the non-ventilated control group (about 5%), presumably due to the short CO-exposure time and wash-out effect. In the late phase co-treatment groups, COHb levels were measured to be 29.1 ± 0.2% and 24.6 ± 0.6% for 1 h and 3 h CO exposure, respectively. The results demonstrated that adding low-dose CO in the air during mechanical ventilation could offer protective effects against VILI. The protective effect of short-term exposure to CO in the early phase of ventilation was almost as good as the long-term co-treatment, however, with much less systemic CO exposure dosage and presumably less systemic toxicity.

Another study by Kanagawa *et al.*[50] showed that CO was able to prevent lung injury in rats as a result of HSR (Table 1, Entry 22). In the study, rats were ventilated with 250 ppm CO for 1 h before the onset of hemorrhagic shock and 3 h post-resuscitation. The COHb level after 1 h rose to 19.40 ± 0.88% in CO-treated animals compared with 1.75%

± 0.26% in control group (air-treated). CO inhalation lessened HSR-induced injury as demonstrated by a ~4-fold reduction in the accumulation of neutrophils in the lung after HSR. CO reduced gene expression of TNF-α (~30%) and iNOS (~50%) in comparison to air-treated rats. Further, a significant elevation (~1.5-fold) in the level of IL-10 was noted in the CO inhalation HSR group. In another HSR-induced ALI model, Kumada *et al.*[49] applied CORM-3 in rats (Table 1, Entry 24). In the study, 4 mg/kg of CORM-3 was administered intravenously immediately after resuscitation. CORM-3 conferred protective effects as reflected in the reduction in the number of inflammatory cells in BAL by ~40% compared to the untreated group. Further, mRNA expression of TNF-α and iNOS were ~2-fold lower in the −3 treated group than the control group. Expression of IL-10 mRNA was found to be elevated by ~2-fold by CORM-3 treatment. These experiments suggest effectiveness of both CO gas and CORM-3 in HSR induced ALI. However, more research involving different types of CO-RMs will help establish the relative efficacy among different CO-RMs and CO gas.

3. Upregulation of HO-1 for the treatment of airway disease

Many studies have shown the protective effects of HO-1 activation on various airway pathologies, including attenuation of lung inflammation through activation of HO-1 by rosiglitazone,[154, 155] endotoxin-induced lung injury through activation of HO-1 by dimethyloxalylglycine (DMOG),[46] limb-ischemia induced lung injury through activation of HO-1 by cobalt protoporphyrin (CoPP),[156] TNF-induced lung injury[104, 105] and LPS-induced lung injury[101] through activation of HO-1 by CORM-2; TNF-induced lung injury through activation of HO-1 by mevastatin;[57] and endotoxin-induced lung injury through HO-1 activation by hemin.[48, 157] A concise summary of a range of HO-1 inducers along with their pharmacological effects in different experimental models is provided in Table 2. Further, selected studies are discussed in detail in the following sections.

Recently, Shi *et al.*[157] investigated the effect of HO-1 upregulation in LPS-challenged isolated macrophages cells and in rats (Table 2, Entry 7). In cellular studies, hemin (20 μM) pre-treatment for 1 h enhanced viability of LPS (1 μg/ml) treated RAW 264.7 cells to 76.7 ± 9.3% from 54.3 ± 6.9% of the control group. Of note, HO-1 enzymatic activity (nmol bilirubin/h/mg protein/h) was determined by monitoring the amount of bilirubin formed through heme degradation in 1 h and normalized with HO-1 protein concentration by using commercial HO-1 ELISA kits. Further, hemin administration (50 mg/kg, i.p.) in rats 1 h before LPS (5 mg/kg)-induced injury increased the survival rate of rats to 90% at 6 h as compared to 75% in the LPS-treated group. Additionally, histological analyses showed that hemin treatment attenuated lung injury in septic rats by preventing alveolar wall thickening, leukocyte infiltration, and hemorrhage.

Furthermore, a study[48] compared the effects of CORM-2 and hemin on RAW 264.7 cells and in rats (Table 2, Entry 8). In LPS (1 μg/ml)-stimulated RAW 264.7 cells, pre-treatment with CORM-2 (100 μM, 1 h) decreased the level of ROS to 116.62 ± 3.54% of the control group without LPS stimulation as compared to 132.21 ± 8.07% of the control group without CORM-2 treatment. In addition, hemin (20 μM, 1 h) pre-treatment reduced ROS levels to 118.08 ± 3.48% from 133.83 ± 7.56% in the LPS-group with respect to the control group.

The ROS suppression effects of both CORM-2 and hemin were reversed by administration of zinc protoporphyrin IX (10 μM, 0.5 h), an HO-1 inhibitor. Such results support a strong role of HO-1 in the observed pharmacological effects. Another interesting study by Zhang *et al.*[160] used multiple CO-RMs and a HO-1 inducer to inhibit inflammation in 16HBE14o- cells (Table 2, Entry 9). In these experiments, poly-L-arginine (PLA) was employed to challenge 16HBE14o- cells to elicit a pro-inflammatory response. Cells were stimulated by PLA (1 μM) with or without hemin (10 μM). Hemin treatment (2 h prior to PLA exposure) resulted in upregulation of HO-1 by several fold. It was found that hemin attenuated apical secretion of IL-6 and IL-8 by 37.39 ± 8.89% and 70.12 ± 7.45%, respectively *via* HO-1 upregulation. Under the same experimental setting, effects of CORM-A1, CORM-2, and CORM-3 were also evaluated. Upon incubation for 30 min prior to PLA exposure, all the CO-RMs (10, 30, and 100 μM) significantly diminished the release of IL-6, while IL-8 suppression was only significant with CORM-2 and CORM-3 treatment. It is important to mention that among the three CO-RMs used, only CORM-A1 is a transition metal-free compound. Nevertheless, the results highlight the differential reactivity of ruthenium-based and non-ruthenium-based CO-RMs in biological studies. Furthermore, in the current study it was also found that CORM-2 treatment 30 min prior to PLA exposure reduced the mRNA expression of HO-1 and HO-2 by ~50% in comparison to both PLA exposed and control groups. However, western blot studies showed that protein concentrations of both HO-1 and HO-2 remained unaffected in the cells. Surprisingly, iCORM-2 also had a similar effect in cellular studies. In contrast, there are studies that described the upregulation of HO-1 by CORM-2 (*e.g.*, Table 1- Entry 12,[101] 16,[104] and 17[105]). Such results raise discussion of issues related to the inherent activity of certain CO-RMs beyond CO release alone. Further, the results also suggest the need for appropriate control compounds capable of providing results without interference from transition metals.

4. Conclusion and Future Directions

CO has been extensively studied for its beneficial properties in models of airway disease. It is clear that CO has beneficial effects at the appropriate dose. However, delivery methods seem to make quite a difference as well; and the issue goes beyond the difference in route of delivery. In this context, inhalation offers the purest form of CO, though dosage control and safety risks are of concern. Stimulation of HO-1 over-expression offers a means of enhanced endogenous CO production. However, HO-1 expression leads to more biological sequelae than CO production. Further, even in the context of CO production, the correlation of HO-1 over-expression and CO production is affected by availability of heme and location of HO-1. In using different forms of CO donors, there are known differences compared with inhaled CO and among different CO donors. For example,[109] CORM-2 and CORM-3 were ineffective in reducing PAP under hypoxia, while CO gas showed significant efficacy under the same conditions. In another study, CORM-3 displayed effectiveness in controlling PH.[110] Of further concern are potential CO-independent effects coming from the “carrier” portion of various CO donors. For example, the “inactive” forms of CO-RMs have shown uncharacteristic biological effects in many studies.[109, 160] In some cases, such effects were even comparable to their CO-releasing counterparts in terms of potency.[160] Such outcomes coupled with literature precedents clearly point to CO-independent effects

of certain CO-RMs.[75, 88, 89] We hope the summary of the beneficial effects of CO in the airway and the discussions of the differential effects depending on the delivery forms in use will emphasize the therapeutic potential of CO and highlight the need for further in-depth studies to clarify potential confusion in the field. Further progress in CO donor development will require identification and full characterization of CO-dependent *vs.* CO-independent effects. Along this line, there is a clear need to think beyond CO-release alone and to take into consideration pharmaceutical developability issues for eventual human use. Continued development of iCO for clinical use awaits further safety and efficacy trials in pulmonary and non-pulmonary indications.

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Table 1.

Effects of CO on airway protection (ND = not determined; NC = no change)

Entry	CO delivery form	Study description	Changes in HO-1 and CO	Experimental Observations	Other comments
Anti-inflammation					
1	CO gas	CO facilitates inflammation resolution[95]	HO-1: ↑ COHb: ND	CO accelerated resolution of inflammation *via* the specialized pro-resolving mediator/HO-1 axis. CO was effective at both 250 and 500 ppm.	The studies were conducted in the context of understanding the mechanisms of CO effects on airway inflammation.
2	CO gas	Anti-inflammatory effect of CO in a baboon model.[96]	HO-1: ND COHb: ↑	CO (200-300 ppm, 60-90 min) restored levels of specialized pro-resolving mediators (SPMs) and reduced levels of pro-inflammatory mediator thromboxane B2 (TXB2) in *Streptococcus pneumoniae*-induced acute inflammation.	CO was administered in a manner to retain the COHb level below 10%.
3	CO gas	CO and cardiopulmonary bypass (CPB)-induced inflammation in pigs[38]	HO-1: ND COHb: ↑	Pre-treatment with CO (250 ppm) for 1 h markedly attenuated the concentrations of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and effector caspases, factors responsible for the development of inflammation. CO caused a noticeable increase in the level of the anti-inflammatory cytokine (IL-10) and transcription factors (NF-κB and AP-1).	Combination therapy of pulmonary perfusion (20% of the systemic blood flow) and CO produced significant anti-inflammatory results in a pig model. Peak COHb levels were 10.0 ± 2.9% 1 h post-CO inhalation.
4	CO gas	Mechanistic study of CO action against CPB-induced inflammation[37]	HO-1: ND COHb: ↑	Pigs receiving 250 ppm CO for 1 h prior to the CPB showed increased expression of heat shock protein (Hsp)-70 and Hsp-90α. In addition, an inhibitory effect was noted on the expression of pro-inflammatory (i.e., IL-6) cytokines.	Abolition of protective effects of CO by quercetin (an Hsp inhibitor) supports Hsp-mediated actions of CO. After 1 h CO inhalation, COHb mean baseline level elevated from 1.1 to 10.4.
5	CO gas	CO downregulates inflammasome-mediated immune responses[97]	HO-1: ND COHb: ND	CO (250 ppm) inhibited the activation of caspase-1 and secretion of pro-inflammatory cytokines, IL-1β and IL-18. Further, CO also prevented mitochondrial dysfunction by decreasing mitochondrial reactive oxygen species (mtROS) generation and preventing loss of mitochondrial membrane potential. The studies were conducted in bone marrow-derived macrophages and in mice.	Preservation of mitochondrial function by CO leads to anti-inflammatory effects.
6	CO gas	Anti-inflammatory therapy in cynomolgus macaques[98]	HO-1: ND COHb: ↑	LPS exposed animals showed a 67% reduction in neutrophilia after 500 ppm CO inhalation for 6 h. Exposure at 250 ppm for 6 h did not improve inflammation significantly.	250 ppm CO showed efficacy in other animal models (e.g., Entries 4, 5, 6, and 9 in Table 1). At 500 ppm for 6 h, COHb level increased to an average of 34%, while 250 ppm for 6 h led to COHb of 25% from a basal level of ~4.4%.
7	CO gas	LPS-induced endotoxic shock and multi-organ failure[45]	HO-1: ND COHb: ND	1 h of pre-treatment with 250 ppm CO substantially reduced the mortality rate from 14% as compared to 80% in air-exposed rats. CO attenuated the level of inducible nitric oxide synthase (iNOS) expression in lung and alveolar macrophages. Paradoxically, CO augmented iNOS expression in liver tissues.	CO conferred protection in various organs *via* differential regulation of iNOS.
8	CO gas	Mechanistic studies of the anti-inflammatory action of CO in VILI[42]	HO-1: ↓ COHb: ↑	In mice, 250 ppm CO reversed the increase of concentration of neutrophils and total cell count in bronchoalveolar (BAL) fluid at 4 and 8 h of ventilation. Inhibitory effects on the production of IL-1β,	Surprisingly, the application of CO prevented VILI-induced expression of HO-1 in this study. CO ventilation caused an increase of the mean COHb

Entry	CO delivery form	Study description	Changes in HO-1 and CO	Experimental Observations	Other comments
				monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β in lung cells were observed.	level to 25% from the 3% baseline.
9	CORM-2	*Pseudomonas aeruginosa* induced inflammation[99]	HO-1: ND COHb: ND	Pre-treatment with CORM-2 (50 μM) for 2 h exerts anti-inflammatory effects in human pulmonary alveolar epithelial cells (HPAEpiCs) by suppressing the expression of pro-inflammatory factors, including ROS generation, IL-6, and prostaglandin E2. In mice, CORM-2 (8 mg/kg) attenuated levels of pro-inflammatory cytokines such as IL-6, IL-1β, IL-8 and TNF-α.	There have been many recent studies of CO-independent effects of Ru-based CO-RMs such as CORM-2. [75, 88, 89] Such findings may affect the interpretation of the results.
10	CORM-3	Lung inflammation[100]	HO-1: ND COHb: ND	CORM-3 (0.15 mM) treatment enhanced expression of CD206 and Ym-1 while reduced expression of iNOS in cell studies. CD206 and Ym-1 are biomarkers of the anti-inflammatory alveolar macrophage (M2) phenotype.	In this study, CORM-3 did not affect TNF-α expression in contrast to Entry 24, Table 1.
11	CORM-2	LPS-induced inflammation[101]	HO-1: ↑ COHb: ND	CORM-2 (8 mg/kg, 24 h) pre-treatment prevented the expression of intercellular adhesion molecule-1 (ICAM-1) and reduced leukocyte count in BAL in mice and in human tracheal smooth muscle cells (HTSMCs). Concentrations of 10, 25, and 50 μM CORM-2 for 1 h were used in the *in vitro* experiments.	CORM-2 (50 μM) induced gene expression of HO-1 in a time-dependent manner.
12	CORM-2 and CO gas	Anti-inflammatory effects of CO in cell culture and animal models[102]	HO-1: ND COHb: ND	In cell culture, CORM-2 stimulated pyrin production, reduced the levels of caspase-1 and IL-1β, and increased IL-10 in response to LPS treatment. CO (250 ppm) protected mice from lung injury after LPS stimulation with increased levels of pyrin and IL-10 as well as decreased the levels of IL-1β.	The cross corroboration between CORM-2 and CO is an important aspect.
13	CORM-2	Particulate matter (PM)-induced inflammation[52]	HO-1: ND COHb: ND	HPAEpiCs pre-incubated with CORM-2 (50 μM) for 24 h inhibited expression of PM-induced NLRP3 protein and caspase-1. In addition, the elevation of the level of IL-1β, mtROS, and NADPH oxidase in response to PM was attenuated by CORM-2. *In vivo*, a single, intra-tracheal dose (8 mg/kg) of CORM-2 protected lungs from the pro-inflammatory responses induced by PM by inhibiting expression and activation of NLRP3 inflammasome.	CORM-2 showed effectiveness in both *in vitro* and *in vivo* models against PM-induced inflammation.
14	CORM-2	LPS-challenged RAW 264.7 cells and septic mice[103]	HO-1: ND COHb: ND	CORM-2 reduced inflammation by preferentially downregulating the expression of iNOS over cyclooxygenases-2 (COX-2). Induction of high-mobility group box 1 (HMGB1) and iNOS is associated with the progression of sepsis.	The study focused on mechanistic investigations of CO action.
15	CORM-2	TNF-α-induced lung inflammation[104]	HO-1: ↑ COHb: -	CORM-2 (8 mg/kg; i.p.) administration 24 h prior to TNF-α exposure (0.25 mg/kg) accelerated recovery of inflammation in mice. In HPAEpiCs, CORM-2 (50 μM for 24 h) decreased vascular cell adhesion molecule-1 (VCAM-1) expression. CORM-2 upregulated the production of HO-1 mRNA and protein in a dose- and time-dependent fashion.	100 μM CORM-2 treatment lowered cell viability to about 60% at 72 h, as determined by MTT assay. However, there is a recent report on the effect of CORM-2 on MTT itself.[88]
16	CORM-2	TNF-α-induced lung inflammation[105]	HO-1: ↑ COHb: ND	In mice, CORM-2 (10 mg/kg; i.p., 1 h) reduced inflammation by diminishing	This study investigates the mechanism of CORM-2-induced HO-1 expression in a

Entry	CO delivery form	Study description	Changes in HO-1 and CO	Experimental Observations	Other comments
				expression of ICAM-1 and leukocytes count in BAL.	TNF-α-triggered inflammation model.
Protection Against Lung Injury					
17	CO gas	ALI in brain-dead mice[12]	HO-1: ND COHb: ND	250 ppm CO in 40% O_2 and 60% N_2 (2 h) alleviated lung injury *via* inhibition of pro-inflammatory (IL-6, TNF-α) and proapoptotic caspase-3 mRNA expression.	The studies were conducted to show the applicability of CO in lung transplantation in brain death donors.
18	CO gas	Postconditioning of lungs with CO post-CPB[39]	HO-1: ND COHb: ↑	Post-CPB, 250 ppm CO for 1 h ameliorated CPB-induced lung injury in pigs. CO application attenuated pro-inflammatory mediator (i.e., IL-6 and IL-1β) expression in the experiments while IL-10 concentrations were significantly elevated.	The study showed the efficacy of CO in postconditioning of lungs in higher mammals. Previous studies were conducted in small animals (Entries 12, 15, and 16 of Table 1). COHb increases of 8-10 fold from the baseline were observed after 1 h inhalation.
19	CO gas	VILI[41]	HO-1: ND COHb: ↑	In mice, 250 ppm CO administered for 6 h offered excellent protection against VILI as assessed by alveolar wall thickness, edema, and cytokine secretion. In contrast, 1 h pre-treatment or 3 h delayed treatment of CO (250 ppm) did not yield comparable results.	This study suggests that a longer exposure time to CO was more effective than short exposure. After 6 h ventilation, COHb levels were 29.3 ± 0.6% in CO group compared to 5.9 ± 0.3% in air-treated group.
20	CO gas	Pneumococcal pneumonia-induced ALI[43]	HO-1: NC COHb: ↑	In baboons, 200 ppm CO for 1 h alleviated ALI as examined by lung wet/dry ratio, total cell counts in BAL, necrosis, edema, etc.	In contrast to Entry 6, Table 1 CO produced prominent effects at a relatively lower dose in nonhuman primate models. COHb levels of 6-8% were achieved after a 200 ppm CO dose.
21	CO gas	IRI-induced graft injury[106]	HO-1: ND COHb: ND	Under cold ischemic conditions, rat lungs inflated with 500 ppm CO with or without 3% H_2 ameliorated lung injury by alleviating oxidative stress and inflammation as monitored by wet/dry lung weight ratio, arterial blood gas, and pressure-volume (PV) curves.	CO ventilation in combination with 3% H_2 produced more pronounced effects in comparison to CO exposure alone.
22	CO gas	Hemorrhagic shock and resuscitation (HSR)-induced lung injury[50]	HO-1: ND COHb: ↑	Rats treated with 250 ppm CO for 1 h before the onset of hemorrhagic shock and 3 h post-resuscitation attenuated the pro-inflammatory response by decreasing expression of TNF-α and iNOS and augmenting the production of IL-10.	CO dosing regimen in the study led to blood COHb level of 19.40 ± 0.88% compared with 1.75% ± 0.26% in the control group (air-treated). Despite the high COHb concentration, CO did not cause hypotension in hemorrhagic shock conditions. Of note, it is well-known that CO directly or indirectly can cause vasodilation.[107]
23	CORM-2	LPS-induced ALI in mice[44]	HO-1: ND COHb: ND	CORM-2 pre-treatment with an i.p. dose of 30 mg/kg alleviated inflammation in ALI by modulating secretion of pro-inflammatory cytokines (IL-1β, IL-18), and preventing PMN infiltration in BAL.	CORM-2 conferred a protective effect *via* suppression of thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome pathway in ALI.
24	CORM-3	HSR induced lung injury[49]	HO-1: ND COHb: NC	CORM-3 (4 mg/kg) i.v. to rats immediately after resuscitation conferred significant anti-inflammatory and anti-apoptotic effects. Suppression of TNF-α, IL-1β, iNOS, caspase-3, and upregulation of IL-10 were observed in the study.	CORM-3 did not change hemodynamic status during HSR. This study corroborates results from Entry 19, Table 1.
Lung Preservation in Transplantation					

Entry	CO delivery form	Study description	Changes in HO-1 and CO	Experimental Observations	Other comments
25	CO gas	CO in organ preservation[24]	HO-1: ↑ COHb: ND	Rat lungs ventilated 1 h post-mortem with CO (500 ppm) in 60% O_2 (1 h), followed by 15 min of warm perfusion after 1 h cold storage and 1 h after transplantation. CO ventilation group showed less injury.	Parameters monitored include oxygenation level after transplantation, and HO-1 expression level, IL-6, IL-1β, etc.
26	CO gas	High-pressure CO for graft preservation[22]	HO-1: NC COHb: NC	Rat and canine grafts were either ventilated with CO (1.5 atm) and O_2 (2 atm) mixture or air to compare the effect in preventing alveolar hemorrhage and alleviating IRI. Expression of IL-6 and IL-1β of the CO/O_2-ventilated lungs were significantly lower than the air-ventilated lungs, indicating protective effects.	The recipient canine transplanted with high-pressure CO/O_2 gas mixture preserved lungs did not show significant changes in COHb level.
27	CO gas	Lung transplant[33]	HO-1: NC COHb: ↑	250 ppm CO exposure to donor and recipient rats starting 1 h before procedures and continuous till post-transplantation improved graft function. CO exposure reduced the levels of IL-6, TNF-α, IL-1β, COX-2, iNOS, and ICAM-1 compared to the air-treated group. The IL-10 mRNA level was unaltered.	Anti-inflammatory and anti-apoptotic effects of CO were abrogated by administration of the selective p38 MAPK inhibitor, SB203580, suggesting mediation by p38 MAPK in the therapeutic effects of CO. Following CO exposure for 1 h, COHb level reached 20.5 ± 3.0% from 1.6 ± 0.4%.
28	CO gas	Lung transplant from brain dead rat donors[36]	HO-1: ND COHb: ND	250-500 ppm CO inhalation for 2 h prior to lung procurement augmented anti-inflammatory responses and decreased apoptosis. CO inhibited levels of IL-6, TNF-α, caspase-3, and augmented the level of IL-10 in the study.	This study corroborates findings from the report in Entry 27, Table 1 as the protective effects of CO are mediated by MAPK.
29	CO gas	Application of CO in lung preservation[23]	HO-1: ND COHb: ND	Mouse grafts preserved at 4 °C for 6 h in the University of Wisconsin (UW) solution bubbled with either 5 or 100% of CO improved graft functions. CO suppressed the expression of pro-inflammatory mediators such as IL-6, TNF-α, IL-1β, and inhibited neutrophil infiltration in the study.	*Ex vivo* application of CO in this experimental setting opens up new ways of applying CO without considering safety concerns associated with its inhalation.
30	CORM-2	Allograft rejection[35]	HO-1: ND COHb: ND	Mice pre-treated with CORM-2 (10 mg/kg, i.p.) for 1 h before and 1, 3, and 6 days after orthotopic trachea transplantation showed decreased infiltration of macrophages into graft (number of cells per slice, 103 ± 53) in comparison to vehicle-treated allografts (301 ± 128; $P < 0.01$). However, differences in the number of neutrophils were not statistically significant (P = 0.79) among the two groups (number of cells per slice, 151 ± 144 *vs.* 173 ± 107; P = 0.79).	CORM-2 protects from *bronchiolitis obliterans* in airway graft transplant.
Pulmonary Hypertension (PH)					
31	CO gas	CO reverses PH[108]	HO-1: ND COHb: ↑	CO (250 ppm, 1 h/d) inhalation for 2 weeks reverses pulmonary hypertension in rats as indicated by reduction in pulmonary arterial pressure (PAP), vascular wall thickness, right ventricular (RV) hypertrophy.	Mechanistic studies suggested NO-dependent therapeutic effects of CO in this model. Inhalation of 250 ppm CO for 1 h elevated basal COHb level 1.2 ± 0.5% to 19 ± 1.5%.
32	CO gas, CORM-2, CORM-3	Effect of CO gas and CO-RMs on pulmonary vasculature[109]	HO-1: ND COHb: ND	Isolated and perfused mice lungs ventilated with 10% CO markedly reduced hypoxia-induced PAP. The vascular tone during normoxia decreased, as well. In contrast, CORM-2 and CORM-3 exhibited a dose-dependent rise in PAP in normoxia.	This is one of the few studies that provided direct comparison of the effects of CO gas *vs.* CO-RMs.

Entry	CO delivery form	Study description	Changes in HO-1 and CO	Experimental Observations	Other comments
				Importantly, there was no effect by CORM-2 and CORM-3 seen on hypoxia-induced vasoconstriction.	
33	CORM-3	Hypoxia-induced PH in mice[110]	HO-1: ND COHb: ND	CORM-3 (50 mg/kg) was administered to mice 1 h and 3 h prior to the hypoxic challenge. 1 h pre-treatment partially reduced PH as monitored by right ventricular systolic pressure (RVSP), while 3 h pre-treatment completely eliminated the hypoxia-induced increase in pressure.	In addition to a p53-dependent mechanism, CORM-3 alleviated hypoxic vasoconstriction-induced PH by increasing cGMP levels in lung cells.
Clinical Trials in Humans					
34	CO gas	Phase II clinical trials in idiopathic pulmonary fibrosis (IPF) patients[111]	HO-1: ND COHb: ↑	IPF patients were administered 100-200 ppm CO, 2 h, twice weekly for 12 weeks. Measurement of changes in the serum concentration of matrix metaloproteinase-7 (primary study endpoint), a biomarker for the progression of IPF, did not indicate significant differences between CO-treated and control groups.	CO dosing resulted in only a modest increase in COHb levels than the control group (1.75% ± 1.78% to 2.62% ± 2.70%). The percentage of respiratory adverse effects in CO-treated and placebo groups were 37% and 38%, respectively. The study showed the tolerability of the discussed dosing regimen in humans.
35	CO gas	Phase I clinical trials in ARDS patients[112]	HO-1: ND COHb: ↑	CO administered in mechanically ventilated ARDS patients at dose of 100 or 200 ppm for 90 min, up to 5 consecutive days. On day 2 of the treatment schedule, an 11% increase in mean plasma mitochondrial DNA (mtDNA) was observed after 100 ppm CO ventilation compared to 400% in air-treated (placebo) group. In the 200 ppm CO dosing group, mean mtDNA level decreased by 88%. This trend continued on subsequent days of the treatment. Further, there was no significant change in the level of IL-1β, IL-6, IL-8, IL-10, and IL-18 among all the treatment groups.	CO ventilation resulted in increased COHb level i.e., 3.48 ± 0.7% at 100 ppm while 4.9% ± 0.28% at 200 ppm inhalation compared to 1.97 ± 0.39% in the placebo group. No adverse or severe adverse events were observed in this study after CO administration in the patients.
36	CO gas	COPD treatment in humans[113]	HO-1: ND COHb: ↑	CO at 100 and 125 ppm was administered to separate groups of COPD patients, 2 h per day for 4 consecutive days. Reduction in the percentage of eosinophils in sputum samples but not neutrophils was observed.	No difference in effects was noted between 100 and 250 ppm CO dose groups. The median COHb values after exposure to 100 ppm and 125 ppm CO were 2.6% and 3.1%, respectively. Comparatively, the placebo group showed 0.2% median COHb.

Table 2.

Effects of HO-1 induction on airway protection

Entry	HO-1 Inducer	Study Description	Changes in HO-1 and CO	Experimental Observations	Other comments
1	DMOG	LPS-induced lung injury[46]	HO-1: ↑ COHb: -	Stimulation with DMOG activated HIF-1/HO-1 signaling pathways, reduced Golgi stress, and attenuated ROS production as well as associated lung inflammation and injury as a result of LPS exposure.	Such results are consistent with what has been observed with CO gas treatment.
2	Hemin	HSR-induced lung injury[158]	HO-1: ↑ COHb: -	Heme arginate (30 mg of hemin/kg, i.v.) treatment for 18 h prior to HSR markedly reduced inflammation in lungs, kidney, and liver as assessed by measuring parameters such as TNF-α, iNOS, and dry/wet weight ratio (for lungs only) in rats.	The protective effect was more prominent in the lungs, where HSR-induced injury was more serious than liver and kidney due to relatively low HO-1 and high TNF-α expression.
3	Rosiglitazone	Rosiglitazone application in LPS-mediated lung inflammation[154]	HO-1: ↑ COHb: -	Rosiglitazone rescued LPS-challenged HPAEpiCs (30 μM dose) and murine (0.1 mg/kg, i.p.) by attenuating VCAM-1 expression and leukocyte secretion in BAL.	The study was conducted in the context of understanding the mechanism of rosiglitazone-induced HO-1 expression.
4	Rosiglitazone	LPS-induced inflamamtion[155]	HO-1: ↑ COHb: -	Rosiglitazone relieved inflammation by acting against ICAM-1 expression and monocyte adhesion in the lung. Experiments were conducted both HPAEpiCs and in murine models.	Mechanistic study.
5	CoPP	Limb ischemia-reperfusion (LIR)-induced lung injury[159]	HO-1: ↑ COHb: -	CoPP (5 mg/kg) injection protected mice from inflammatory damage due to ischemia. Parameters monitored were histological examinations, alveolar wall thickening, presence of red blood cells, and neutrophil in BAL. Expression of HO-1, nuclear factor erythroid 2-related factor (Nrf-2), BTB, and CNC homology 1 (Bach1) was also examined.	CoPP strengthens the anti-inflammatory response by upregulating Nrf2.
6	Mevastatin	Application of statins in pulmonary inflammation[105]	HO-1: ↑ COHb: -	Mevastatin (MVS) (0.1 mg/kg, 1 h) attenuates ICAM-1 expression and leukocyte concentration in BAL in murine models stimulated by TNF-α. Further, MVS (30 μM) treatment *in vitro* also produced anti-inflammatory effects in a similar manner.	MVS stimulates HO-1 expression in HPAEpiCs *via* generation of ROS in an NADPH oxidase (Nox)-dependent manner.
7	Hemin	Endotoxin-induced ALI[157]	HO-1: ↑ COHb: -	Hemin (50 mg/kg) pre-treatment 1 h before LPS challenge conferred protective effects in mice through reduction of hemorrhage, alveolar wall thickening, and leukocyte infiltration. Hemin pre-treatment improved survival rate of LPS-challenged mice (~90%) compared to the group without hemin administration (75%).	HO-1 induction controls mitochondrial dynamics to protect from ALI.
8	Hemin	LPS-induced ALI[48]	HO-1: ↑ COHb: -	Pre-treatment of RAW 264.7 cells with either CORM-2 (100 μM) or hemin (20 μM) for 1 h reduced ROS production stimulated by LPS. Further, hemin (50 mg/kg) ameliorated inflammation by reducing alveolar wall thickening, inflammatory cell infiltration, and hemorrhage in rats.	This study examined the effect of a HO-1 inducer and CORM-2 on ALI. A detailed description is provided in the text.
9	Hemin,	Polypeptide-induced inflammation[160]	HO-1: ↑ COHb: ND	Hemin (10 μM) administration produced an anti-inflammatory effect by suppressing IL-6 and IL-8. Further, in the same study, CORM-A1, CORM-2, CORM-3 inhibited the level of IL-6 and IL-8 induced by poly-L-arginine (PLA) exposure in the human bronchial epithelial cell line 16HBE14o-.	Hemin and PLA increased the expression of HO-1 in cells. CORM-2 suppressed mRNA expression of HO-1 induced by PLA, whereas protein

Entry	HO-1 Inducer	Study Description	Changes in HO-1 and CO	Experimental Observations	Other comments
				CORM-2 was the most potent. The effect was concentration-dependent (10, 30, and 100 μM).	expression of HO-1 remained unaltered.