Evaluation of inhaled carbon monoxide as an anti-inflammatory therapy in a nonhuman primate model of lung inflammation

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Carbon monoxide (CO) confers anti-inflammatory protection in rodent models of lung injury when applied at low concentration. Translation of these findings to clinical therapies for pulmonary inflammation requires validation in higher mammals. We have evaluated the efficacy of inhaled CO in reducing LPS-induced lung inflammation in cynomolgus macaques. LPS inhalation resulted in profound neutrophil influx and moderate increases in airway lymphocytes, which returned to baseline levels within 2 wk following exposure. CO exposure (500 ppm, 6 h) following LPS inhalation decreased TNF-α release in bronchoalveolar lavage fluid but did not affect IL-6 or IL-8 release. Lower concentrations of CO (250 ppm, 6 h) did not reduce pulmonary neutrophilia. Pretreatment with budesonide, a currently used inhaled corticosteroid, decreased LPS-induced expression of TNF-α, IL-6, and IL-8, and reduced LPS-induced neutrophilia by ~84%. In comparison, CO inhalation (500 ppm, for 6 h after LPS exposure) reduced neutrophilia by ~67%. Thus, inhaled CO was nearly as efficacious as pretreatment with an inhaled corticosteroid at reducing airway neutrophil influx in cynomolgus macaques. However, the therapeutic efficacy of CO required relatively high doses (500 ppm) that resulted in high carboxyhemoglobin (COHb) levels (>30%). Lower CO concentrations (250 ppm), associated with anti-inflammatory protection in rodents, were ineffective in cynomolgus macaques and also yielded relatively high COHb levels. These studies highlight the complexity of interspecies variation of dose-response relationships of CO to COHb levels and to the anti-inflammatory functions of CO. The findings of this study warrant further investigations for assessing the therapeutic application of CO in nonhuman primate models of tissue injury and in human diseases. The study also suggests that akin to many new therapies in human diseases, the translation of CO therapy to human disease will require additional extensive and rigorous proof-of-concept studies in humans in the future.
knockout mice, in response to hypoxia, develop pulmonary hypertension (5, 35), whereas over expression of HO-1 imparts cytoprotection against tissue injury and prevents pulmonary hypertension (5, 35). This leads to a role for HO-1 as an important vasodilator and may explain why CO has such potent anti-inflammatory activity. CO has also been shown to limit coagulation and thrombosis, thus preventing anoxia and tissue necrosis (26). Additionally, CO can induce NO, leading to increased expression of HO-1 in a cytoprotective-positive feedback loop (37).

The work described herein shows that inhaled CO is protective against inhaled LPS-induced pulmonary inflammation in nonhuman primates. Work by Choi and others (3, 8, 27–29, 36) has shown that CO can play a role in protection against lung inflammation and injury in rodents. Several knockout mice studies have also shown potential mechanisms for this cytoprotection (25). This work is in agreement with mouse models showing the protective role CO could play in human disease through the use of an animal model phylogenetically similar to humans, the nonhuman primate. Furthermore, use of the positive control, inhaled budesonide, in the primate study showed that CO was nearly as efficacious as the steroid at reducing neutrophil influx in the airway, but not as effective in reducing proinflammatory cytokine production. Unlike rodent models, the therapeutic effectiveness of CO in cynomolgous macaques required relatively higher concentrations of CO associated with a higher carboxyhemoglobinemia than would be considered required relatively higher concentrations of CO associated with the therapeutic effectiveness of CO in cynomolgous macaques showing the protective role CO could play in human disease through the use of an animal model phylogenetically similar to humans, the nonhuman primate. Furthermore, use of the positive control, inhaled budesonide, in the primate study showed that CO was nearly as efficacious as the steroid at reducing neutrophil influx in the airway, but not as effective in reducing proinflammatory cytokine production. Unlike rodent models, the therapeutic effectiveness of CO in cynomolgous macaques required relatively higher concentrations of CO associated with a higher carboxyhemoglobinemia than would be considered safe for human application. These studies are the first to examine the therapeutic index and dose-response relationships of CO therapy in nonhuman primates, and warrant further investigations in humans.

METHODS

Male cynomolgus macaques (Macaca fascicularis) were obtained from the breeding colony at Lovelace Respiratory Research Institute. Animals were moved to indoor individual housing in stainless steel cages with wire mesh bottoms. Primates were given a 2-wk acclimation period before any procedures took place. All primates were between 5 and 6 yr of age and weighed ~3.5–5 kg. Primates were fed Harlan Teklad Certified monkey diet (2050C) and tri-weekly fresh fruit enrichment. Water was available ad libitum throughout the study. Environmental conditions were set as follows: 18–26°C, 30–70% humidity, 12:12-h light-dark cycle. Health observations were conducted twice a day by trained animal care staff. Animals were identified by microchip. In the case of procedures that required anesthetics, all food was withheld the morning of the procedure. All animal work described was conducted under approval of the Animal Care and Use Committee at Lovelace Respiratory Research Institute.

Bronchoalveolar lavage. Bronchoalveolar lavage was performed as a baseline 10 days prior to LPS exposure and treatments, as well as 24 and 72 h post-LPS exposure. Prior to study start all primates were chemically restrained with a 10 mg/kg im injection of ketamine followed by inhaled isoflurane (5% to induce and 2% to maintain). During anesthesia, primates were kept warm on a circulating water blanket (Andriot Medical Systems, Loudon, TN). After anesthetic induction, primates were intubated, and a 3.5-mm flexible bronchoscope (Olympus) was inserted through an inline adapter in the anesthetic breathing circuit. The bronchoscope was advanced into the right caudal lung lobe and wedged in a sublobar bronchus. Sterile saline was instilled and aspirated (2 × 10 ml) through the biopsy channel. Bronchoalveolar lavage fluid (BALF) recovery was typically 80% of the instilled volume. Total nucleated and differential cell counts were performed for each sample. BALF was subsequently centrifuged. Supernatants were collected and frozen for cytokine analysis at a later date. Statistical analysis included one-way ANOVA in cases of more than two treatment groups and t-test (Mann-Whitney) when only control vs. treated were analyzed. Prism statistical software was used to analyze the data and statistical significance was determined by a P value < 0.05.

Hematology. Blood was collected 10 days prior to LPS exposure and treatments, as well as 24 and 72 h post-LPS exposure. Blood was collected from the femoral vein into EDTA vacutainers. Complete blood counts were used to determine whether any of the primates were in questionable health or should be excluded from study. Primates were closely monitored until they were alert and exhibited swallow reflex. Once all primates had recovered from the anesthetic, they were allowed food. Water was available ad libitum throughout the study.

Budesonide pretreatment. Budesonide was loaded into Penn-Century Dry Powder Insufflators (Philadelphia, PA). The Penn-Century device was actuated into an expansion chamber connected to an endotracheal tube with simultaneous, forced ventilation delivered through the expansion chamber from an inline resuscitation bag. Delivery from the device was determined to be 50% of the loaded dose and of that 50% yield, 30% of the drug reaches the lung where it carries out its mode of action. Therefore, although 10 mg was loaded into the Insufflator (and each primate received 2 actuations), only ~1.5 mg was delivered to the lung per actuation. In a 4-kg primate this yields a 0.75-mg/kg delivered dose. The average particle size of the budesonide aerosol was determined to be ~5.1 µm with a GSD of 2.8.

Four primates were pretreated with budesonide followed 3 h later with LPS exposure. Primates were anesthetized as above and intubated. The anesthetic breathing circuit was momentarily removed and the Penn-Century Insufflator was connected to the endotracheal tube. Budesonide was administered with the Insufflator using two device actuations each with simultaneous forced manual ventilation to an airway pressure of ~20 cm of water. The primate was then reconnected to the anesthetic circuit and received oxygen until respiration was back to normal. Respiration was monitored by study staff before and after drug delivery to ensure that postdelivery rates were comparably to preexposure rates.

Blood and lavage fluid was collected 24 and 72 h post-LPS exposure. At the 72 h collection, primates were euthanized with an overdose of Euthasol delivered intravenously through a catheter in the saphenous vein. Death was confirmed by bilateral pneumothorax.

Inhaled LPS. All the primates underwent exposure to LPS. LPS was isolated from pseudomonas aeruginosa serotype 10 (Sigma, St. Louis, MO) suspended in sterile water. This exposure took place under chemical restraint (10 mg/kg ketamine and an additional 3 mg/kg Telazol when necessary). During the exposure the animal was kept warm on a circulating water blanket. LPS was administered through a face mask for 5 min at a concentration of 15 mg/ml. Animals were lavaged 24 h later to determine whether this concentration of LPS was sufficient to induce neutrophilia. Animals were also lavaged 2 wk later to ensure that all inflammation had subsided. LPS was aerosolized from a 0.5 mg/ml formulation with a Pari LC plus nebulizer with an inlet pressure of 20 psi. Flow past the primate was maintained at 1.5 times the respiratory minute volume of the animal (~4 l/min). LPS concentration in the aerosol was determined by gravimetric analysis of filter samples. LPS aerosols were collected from the exposure plenum on a type T60A20, 47-mm Pallflex membrane filter (Pall Gelman Sciences, Ann Arbor, MD) at a flow rate of 1 l/min. Filters were then dried and weighed. Particle size of the aerosol was determined using an aerodynamic particle sizer (model 3321; TSI, St Paul, MN). The average particle size of the LPS aerosol was determined to be 3.9 µm with a geometric standard deviation of 1.5.

Inhaled CO. Immediately following recovery from anesthetics used for the LPS exposure, primates were loaded into whole body exposure chambers that contained 250 or 500 ppm CO (n = 6) or control air.
Two primates received CO only and did not undergo LPS exposure prior to CO exposure. Additional primates were also exposed to 6 h of 250 or 500 ppm CO without prior LPS exposure. These two primates were solely used to determine carboxyhemoglobin (COHb) levels following the CO exposure. A pre-CO blood draw was conducted to determine the baseline COHb level in each of the two primates. Primates then received an additional blood draw immediately following the 6-h CO exposure. Samples were analyzed on a Siemens Rapidpoint 405 blood gas analyzer. This analyzer uses a Siemens measurement cartridge that houses all reagents needed.

Hazleton H-2000 (Lab Products, New Jersey) whole body exposure chambers were modified to hold custom-built stainless steel cages (18 “wide×18” deep×23 “high) that keep primates secure during the 6-h CO exposure. CO concentration was monitored by a calibrated infrared analyzer for the entire duration of the exposure. Animals were given enrichment food, and water during the exposure. Primates were conditioned to these cages on three separate occasions prior to the exposure (1 h, 3 h, and 6.5 h). During conditioning, enrichment was given as positive reinforcement. Lavage and blood collections were conducted 24 h and 72 h post-LPS exposure (~18 h post-CO or air exposures). Animals were euthanized at the 72-h time point. See Fig. 1 for study design.

RESULTS

Inhaled LPS induces pulmonary inflammation that clears within 2 wk. Primates exhibited a several hundred-fold increase in the number of neutrophils in the airway at 24 h post-LPS exposure (see Fig. 2A). Macrophage cell numbers were not significantly increased with exposure to LPS (Fig. 2B). However, lymphocytes were induced to enter the airway at 24 h post-LPS (~5-fold increase as seen in Fig. 2C). Both neutrophil and lymphocyte cell numbers were comparable to baseline at 2 wk post-LPS.

CO exposure reduces neutrophilia and alters cytokine profiles induced by inhaled LPS in a dose-dependent manner. Whole body CO or sham air exposure for 6 h was conducted immediately following exposure to LPS (15 mg/m^3 for 5 min). At 24 h post-LPS (18 h post-CO or air chamber exposure) lavage fluid was collected and analyzed for cell counts and cytokines. At the 24-h time point, animals that received LPS and 500 ppm CO had significantly fewer neutrophils in the airway compared with animals that received LPS and sham air. Those primates that received 250 ppm CO for the 6-h duration did not exhibit a decrease in neutrophils in the lung following LPS induction. Primates that were exposed to CO alone without prior exposure to LPS did not exhibit changes in neutrophil numbers compared with baseline measurements (see Fig. 3).

LPS/high-dose CO exposed primates exhibited ~67% reduction in neutrophils compared with LPS/sham air-exposed primates. Additionally, LPS/high-dose CO-exposed primates expressed decreased levels of TNF-α with respect to their sham air exposed counterparts (~40% reduction). Compared with baseline, TNF-α was decreased in lavage fluid supernatants from 500 ppm CO only-exposed primates showing that CO is directly affecting this inflammatory cytokine (Fig. 4A). Interestingly, IL-6 and IL-8 were not reduced with 500 ppm CO exposure following LPS induction of pulmonary inflammation, nor did 500 ppm CO alone decrease these cyto/chemokines below baseline levels (Figs. 4B and 4C).

COHb levels were determined in primates that were exposed to CO alone. Baseline levels were determined to be ~4.4%.
Immediately following the 6-h exposure to 250 ppm CO, COHb levels had increased to \( \sim 25\% \), whereas animals exposed to 500 ppm CO increased to an average of 34. Inhaled budesonide, delivered prior to LPS challenge, reduces neutrophilia and inflammatory cytokines. Induction of neutrophilia by LPS was attenuated by pretreatment with budesonide by insufflation. Since budesonide was the positive control in these studies, delivery prior to the LPS insult was the chosen method even though this is not relevant in real world applications. Direct delivery of dry powder budesonide to lungs of primates 3 h prior to LPS exposure resulted in marked reductions in neutrophils in the airway at 24 h post-LPS exposure (~84% reduction). Seventy-two hours post-LPS, neutrophil numbers had returned to baseline (see Fig. 5). Furthermore, key inflammatory cytokines were reduced with budesonide treatment as expected. IL-6, IL-8, and TNF-\( \alpha \) were all induced by LPS. Budesonide pretreatment reduced these cytokines significantly at 24 h post-LPS exposure (see Figs. 6, A–C). TNF-\( \alpha \) was reduced by 60%, IL-6 by 80%, and IL-8 by 52%. All measured inflammatory cytokines returned to baseline by 72 h post-LPS as seen in Figs. 6A–6C.

DISCUSSION

The pharmacological or exogenous application of CO at low concentration can provide cellular and tissue protection in animal injury models, including rodent models of endotoxemia (25), acute lung injury (22, 28, 11), sepsis (6), and vascular injury (30). CO can promote survival in mice exposed to hyperoxia or systemic LPS challenge (25, 28). Furthermore, CO application can prevent ischemia/reperfusion injury, reduce the incidence of graft rejection during xeno- and allograft transplantation (33), and reverse established pulmonary hypertension in rodents (38). The stress protein HO-1 represents the major source of endogenous CO in higher organisms. HO-1 catalyzes the oxidative degradation of heme into CO, bilirubin-IX\( \alpha \), and ferrous iron (reviewed in Ref. 31). The increased expression of HO-1 has been described in lung disease states involving inflammation or oxidative stress, including asthma, COPD, pneumonia, and acute respiratory distress syndrome (9, 12, 34). Over expression of HO-1 can confer similar protective effects as observed for CO during endotoxin challenge (25). The increased incidence of exhaled CO in humans, presumably as
the result of HO-1 induction in the airways, has been implicated as a potential biomarker of proinflammatory conditions (18). These studies, taken together, suggest that HO-1 and its byproduct, CO, may exert adaptive or protective roles in inflammatory diseases. In the current study, we have evaluated the efficacy of applying exogenous CO immediately following the onset of inflammation. We cannot exclude the possibility that inhalation CO may induce secondary induction of HO-1 in the primate lung.

The tissue protective effects of CO have been attributed to anti-inflammatory and vasoregulatory effects, as well as inhibitory effects on apoptosis, cell proliferation, and thrombosis in situ. In a mouse model of endotoxemia, CO conferred anti-inflammatory protection through the downregulation of proinflammatory (i.e., TNF-α, IL-1β) cytokines, and the upregulation of the anti-inflammatory cytokine IL-10. CO also conferred anti-inflammatory protection in rodents subjected to hyperoxia (28) or ventilator-induced lung injury (11). In the latter study, inclusion of CO during mechanical ventilation reduced ventilation-associated pulmonary neutrophil influx and proinflammatory cytokine production (11). CO (500 ppm) also attenuated pulmonary neutrophil influx in a model of acid-induced acute lung injury, but only at the early phase of inflammation (22). In contrast, several studies report no direct anti-inflammatory benefit of CO in comparable rodent acute lung injury models (7, 10). However, anti-inflammatory effects of CO were recently substantiated in higher animals, using a porcine model of sepsis (16).

While considerable progress has been made in defining the therapeutic potential of CO in animal models, the clinical benefit of therapies involving CO have not been definitively demonstrated in humans. A recent clinical trial has shown therapeutic benefit of CO in reducing proinflammatory cell counts in patients with COPD (1). Bathoorn et al. (1) showed that low CO levels (100–125 ppm for 2 h/day) were sufficient to reduce eosinophil levels in COPD patients. In contrast, a pilot clinical study reported no anti-inflammatory benefit of inhaled CO (500 ppm for 1 h pretreatment, resulting in 7% COHb) in humans subjected to LPS injection (15). The results from a recently completed Phase II trial of inhaled CO in renal transplantation are currently pending. There is therefore a need for additional controlled clinical studies with inhalation CO before conclusions can be reached on its clinical efficacy as an anti-inflammatory agent.

In the present studies, we show for the first time that inhaled LPS (5 min at 15 mg/m²) induces profound neutrophilia in the airway of cynomolgus macaques. The primates did not present any systemic alterations in cell populations as measured by complete blood counts (data not shown) following LPS inhalation, which renders this an ideal model for pulmonary inflammation.

CO inhalation at high concentration (500 ppm) reduced neutrophil influx into the lung by ~67% compared with primates that received sham air following LPS inhalation exposure to LPS (5 min at 15 mg/m²). Budesonide-treated animals had 0.2 × 10⁶ neutrophils/ml lavage fluid compared with 1.2 × 10⁶ neutrophils/ml in primates that were not pretreated with budesonide (n = 4). In either case, neutrophilia was cleared by 2 wk post-LPS exposure. *Statistically significant difference compared with LPS + sham air-exposed primates (P < 0.05).

Fig. 5. Pretreatment with budesonide by insufflation reduces LPS-induced neutrophilia in the lungs of primates. Budesonide-treated animals had 0.2 × 10⁶ neutrophils/ml lavage fluid compared with 1.2 × 10⁶ neutrophils/ml in primates that were not pretreated with budesonide (n = 4). In either case, neutrophilia was cleared by 2 wk post-LPS exposure. *Statistically significant difference compared with LPS + sham air-exposed primates (P < 0.05).

Fig. 6. Budesonide significantly reduces LPS-induced TNF-α, IL-6, and IL-8 expression. Twenty-four hours post-LPS primates that received budesonide pretreatment expressed marked reductions in TNF-α (A), IL-6 (B), and IL-8 (C) compared with animals that were not pretreated with budesonide (n = 4). All proteins returned to baseline levels by 72 h post-LPS. All samples were run in triplicate for cyto/chemokine analysis. *Statistically significant difference compared with LPS + sham pretreatment-exposed primates (P < 0.05).
sure. In contrast to rodent models, no reduction in neutrophil influx was conferred at lower concentrations (250 ppm) of CO. By comparison, the clinically relevant steroid, budesonide, when delivered prior to LPS exposure, reduced neutrophil accumulation in the airways by ~84%. CO (500 ppm) markedly reduced LPS-induced TNF-α production (25). This data is consistent with previous rodent data that demonstrated down-regulation of TNF-α production by CO treatment through regulation of the p38 MAPK pathway (25).

Interestingly, 500 ppm inhaled CO did not alter the expression of two well-known proinflammatory cytokines, IL-6, and IL-8, the latter being a major neutrophil chemoattractant. These results indicate that the inhibitory effect of CO on neutrophil influx is likely IL-8 independent. In contrast, budesonide was effective in reducing not only TNF-α, but also IL-6 and IL-8, which may account for the larger reduction in LPS-induced neutrophilia compared with 500 ppm CO. Due to limitations in the nonhuman primate model, we did not test IL-1β, MCP-1, and MIP-1β, and cannot exclude a role for these proteins in airway neutrophil recruitment. A weakness of the present study is that additional ex vivo end points were not conducted to better elucidate a mechanism for the selective reduction in TNF-α compared with IL-6 and IL-8. Of note, future studies should include ex vivo analysis of tissues at 24 h postexposure as opposed to 72 h post-LPS exposure.

In cynomolgus macaques, COHb levels were determined to exceed 30% following 6 h of 500 ppm CO inhalation. Although COHb levels were high in these primates, exposures to 500 ppm CO were well tolerated with no clinical signs of CO toxicity or poisoning. A lower level of CO exposure (250 ppm for 6 h) resulted in lower COHb levels (~25%) following LPS exposure, which is still relatively higher than that observed in mice exposed to equivalent ambient concentration. Unlike rodents, no anti-inflammatory effect with respect to cytokine production or neutrophil influx was observed at this concentration (250 ppm) in primates. These results indicate that relatively higher levels of CO are needed to show a therapeutic effect in primates compared with mice.

Use of nonhuman primate models such as cynomolgus macaques, represents a logical intermediate step in the transformation of rodent model studies to human clinical research and may provide insight in how to conduct future human studies. Due to potential interspecies differences in lung physiology, including the clearance of inhaled gases, it is not clear whether the dose-response relationship between inhaled CO and the formation of COHb would be identical between humans and nonhuman primates. It may therefore be difficult to predict the therapeutic window for inhaled CO in humans directly from primate studies. It should also be noted that an inhaled CO dose resulting in COHb levels exceeding 30% would be unacceptable for delivery to humans, given the toxicity associated with this level of carboxyhemoglobinemia. Certainly, potential CO toxicity may be a limiting factor in translating the therapeutic potential of CO from animals to humans.

Development of an alternative noninvasive anti-inflammatory treatment for advanced lung disease is of utmost importance, as current therapies for acute lung injury/acute respiratory distress syndrome still permit a high rate of mortality. On the basis of the large volume of animal studies, inhaled CO continues to show potential as an anti-inflammatory therapy. Inhalation therapies have a practical advantage in that they offer a high level of patient compliance. Since most critical care patients are already on oxygen therapy and/or mechanical ventilation, the addition of low concentrations of CO to gas mixtures would not increase physical or monetary burden significantly. Furthermore, since CO is an inert gas it may be administered with a low probability of drug interactions. On the other hand, the current limitations of CO therapy include incomplete understanding of the chronic toxicology of low-dose CO administration in humans, which may include possible extrapulmonary effects such as cardiac- and neurotoxicity.

In summary, the reduction in neutrophil influx with high levels of inhaled CO following LPS exposure in cynomolgus macaques indicates that the potent anti-inflammatory effects of CO observed in rodents can potentially translate to nonhuman primates. Unfortunately, the levels of CO that lead to significant reduction in neutrophilia and cytokine production result in COHb levels that are potentially toxic to humans. These studies also highlight the complexity of interspecies variation (rodents vs. primates) of dose-response relationships between CO dose and COHb levels and the resulting anti-inflammatory effects. Hence, this study warrants further dosing and pharmacokinetic investigations for assessing the therapeutic application of CO in both nonhuman primate models of tissue injury as well as in human diseases. The application of CO therapy to human disease will require additional carefully controlled clinical studies on the safety and efficacy of this experimental therapeutic.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

REFERENCES


