Carbon monoxide reduces pulmonary ischemia–reperfusion injury in miniature swine

Hisashi Sahara, MD, Akira Shimizu, MD, PhD, Kentaro Setoyama, DVM, PhD, Masayoshi Okumi, MD, PhD, Manei Oku, MD, Emma Samelson-Jones, MD, and Kazuhiko Yamada, MD, PhD

Objectives: Carbon monoxide is produced endogenously as a by-product of heme catalysis and has been shown to reduce ischemia–reperfusion injury in a variety of organs in murine models. The aims of this translational research were to establish an in situ porcine lung model of warm ischemia–reperfusion injury and to evaluate the cytoprotective effects of low-dose inhaled carbon monoxide in this model.

Methods: Warm ischemia was induced for 90 minutes by clamping the left pulmonary artery and veins in 8 Clawn miniature swine (Japan Farm CLAWN Institute, Kagoshima, Japan). The left main bronchus was also dissected and reanastomosed just before reperfusion. Four animals were treated with inhaled carbon monoxide at a concentration of approximately 250 ppm throughout the procedure. Lung function and structure were serially accessed via lung biopsy, chest x-ray films, and blood gas analysis.

Results: Carbon monoxide inhalation dramatically decreased the lung injury associated with ischemia and reperfusion. Two hours after reperfusion, the arterial oxygen tension of the carbon monoxide–treated group was 454 ± 34 mm Hg, almost double the arterial oxygen tension of the control group (227 ± 57 mm Hg). There were fewer pathologic changes seen on chest x-ray films and in biopsy samples from animals in the carbon monoxide–treated group. Animals in the carbon monoxide–treated group also had fewer inflammatory cell infiltrates and a markedly smaller increase in serum concentrations of the proinflammatory cytokines interleukin 1β , interleukin 6, and high-mobility group box 1 after ischemia–reperfusion injury.

Conclusions: The perioperative administration of low-dose inhaled carbon monoxide decreases warm ischemia–reperfusion injury in lungs in miniature swine. This protective effect is mediated in part by the downregulation of proinflammatory mediators. (J Thorac Cardiovasc Surg 2010;139:1594–601)

Ischemia–reperfusion injury (IRI) in the lungs is characterized by nonspecific alveolar damage, lung edema, and hypoxemia within 72 hours of transplantation.¹ Despite advances in donor and recipient perioperative management, surgical technique, and lung preservation, IRI continues to be the most common cause of primary graft failure. Between 15% and 33% of lung transplants are affected by IRI, frequently leading to recipient death after prolonged mechanical ventilation.² Furthermore, patients with lung IRI who survive the perioperative period remain at an increased risk of both acute and chronic rejection.^{3,4} The shortage of available organs for transplantation has increasingly led to the use of organs from extended-criteria donors that are more susceptible to severe IRI. As a result, the clinical

impact of IRI has expanded. Strategies that reduce IRI have the potential to expand the use of marginal organ donors and improve both the short- and long-term outcomes of transplantation.

Tissues exposed to stressful stimuli upregulate protective genes such as heme oxygenase-1,⁵ which has been shown to decrease both nonspecific inflammation and alloantigen-specific immune responses to IRI⁶ and transplantation.⁷ Carbon monoxide (CO) has recently been shown to have the same protective effects as the upregulation of heme oxygenase-1. Although CO is commonly recognized as an environmental air pollutant that displaces oxygen in the blood and causes tissue hypoxia,⁸ it is also produced endogenously in response to heat or oxidant stress by the metabolism of heme by heme oxygenases to CO, iron, and biliverdin.⁹ Furthermore, the inhalation of low-dose CO at concentrations between 20 and 500 parts per million (ppm) has been shown to exert cytoprotective effects in murine models of IRI and organ transplantation.¹⁰ CO delivered by CO-releasing molecules^{11,12} or in soluble form via a preservation solution¹³ has been reported to exert similar protective effects in murine IRI models.

Whereas CO has been shown to inhibit lung IRI in a variety of organs in murine models, the beneficial effects of CO on IRI in large animals have only been reported in

From the Xenotransplantation Surgery Section, Frontier Science Research Center, Kagoshima University, Kagoshima, Japan.

This research was supported by a Grant-in-Aid for Young Scientists (to H.S.) and the Kagoshima University Research Awards (to K.Y.).

Disclosures: None.

Received for publication June 2, 2009; revisions received Aug 15, 2009; accepted for publication Sept 7, 2009; available ahead of print Nov 12, 2009.

Address for reprints: Kazuhiko Yamada, MD, PhD, Professor, Frontier Science Research Center, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima, 890-8520, Japan (E-mail: kyyamada@m2.kufm.kagoshima-u.ac.jp).

0022-5223/\$36.00

Copyright © 2010 by The American Association for Thoracic Surgery

doi:10.1016/j.jtcvs.2009.09.016

Abbreviations and Acronyms

CO	= carbon monoxide
COHb	= carboxyhemoglobin
CXR	= chest x-ray
HMGB1	= high-mobility group box 1
IRI	= ischemia–reperfusion injury
Pao ₂	= arterial oxygen tension

a porcine model of cardiopulmonary bypass¹⁴ and an ex vivo perfusion of an isolated kidney.^{15,16} CO has not yet been studied in an in situ porcine pulmonary IRI model. Translational research using an accepted and relevant preclinical species is necessary before the powerful therapeutic implications of this murine research can be applied in a clinical setting. In this study, we demonstrate that the inhalation of low-dose CO dramatically decreases warm IRI to native lungs in miniature swine, in part through the downregulation of proinflammatory mediators.

MATERIALS AND METHODS

Animals

Male and female Clawn miniature swine between 5 and 9 months of age and weighing 10 to 15 kg were obtained from the Japan Farm CLAWN Institute (Kagoshima, Japan). All animal care and procedures were performed in accordance with the guidelines of the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The study protocol was approved by the Ethical Committee of the Faculty of Medicine at Kagoshima University in Japan.

CO Inhalation

CO (1%) in oxygen was directed into the isoflurane vaporizer and delivered to the animals via the inhaled anesthesia machine. A CO analyzer (CO checker YZ-005A; Yazaki Co Ltd, Tokyo, Japan) was used to continuously measure CO levels in the inhaled gas and maintain a CO concentration of 250 ppm at all times. The percentage of carboxyhemoglobin (COHb) in arterial blood was measured by a CO-Oximeter (CCX1 with CO-Oximeter; Nova Biomedical, Tokyo, Japan).

Experimental Design

Eight miniature swine were divided evenly into 2 groups: a CO-treated group and a control group. In the CO-treated group, animals were continuously treated with inhaled CO from the time of intubation until 2 hours after reperfusion. The time of CO inhalation before the left bronchus was clamped was approximately 150 minutes, with a total CO inhalation time of 360 minutes. Total surgical time was approximately 7 hours for both experimental groups.

IRI

After anesthesia, the animals were mechanically ventilated with a mixture of 100% oxygen and 1% to 3% isoflurane at a tidal volume of 15 to 20 mL/kg and a respiratory rate of 12 to 15 breaths per minute (MD-705 XL; Senko Medical Instrument Mfg Co, Ltd, Tokyo, Japan). A positive end-expiratory pressure of 5 cm H₂O was added at the time of reperfusion. A left thoracotomy was performed in the fifth intercostal

space. After the intravenous administration of heparin, 300 IU/kg, warm ischemia was induced by clamping the left pulmonary artery, veins, and left main bronchus. Simultaneously, the left main bronchus was dissected, thereby collapsing the left lung. After 90 minutes of ischemic time, the left main bronchus was reanastomosed. The pulmonary vessels were then unclamped to permit reperfusion, and a thoracotomy tube was placed. Open lung biopsy specimens of the left lung were obtained 2 hours and then 2, 7, 28, and 56 days after reperfusion. So that sampling error would be minimized, relatively large specimens (ie, 15–20 g) were obtained from the inferior lingular segment and anterior/lateral basal segment of the left lung.

Assessment of Lung Injury by Blood Gas Analysis

Arterial blood was obtained before the operation and then 10, 30, 60, and 120 minutes after reperfusion through a left carotid arterial line. Blood was analyzed with a CO-Oximeter.

Assessment of Lung Injury by Chest X-Ray (CXR) Films

CXR films were taken 2, 7, 28, and 56 days after IRI. CXR films were evaluated by a blinded radiologist using the composite 4-point scoring system described by Murray and associates¹⁷ in 1988: 0 = no alveolar consolidation, 1 = alveolar consolidation in one quadrant, 2 = alveolar consolidation in two quadrants, 3 = alveolar consolidation in three quadrants, and 4 = alveolar consolidation in all four quadrants.

Assessment of Lung Injury by Histopathologic Examination

Biopsy samples were analyzed by a blinded pathologist using light microscopy and transmission electron microscopy. Samples analyzed by light microscopy were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin and elastica–Masson Goldner stain. A semiquantitative score was calculated on the basis of the evaluation of cell infiltration, intra-alveolar edema, fibrin exudation, and hemorrhage. The morphologic changes were graded on the basis of the score described by Müller and colleagues¹⁸: 0 = normal; 1 = mild; 2 = moderate; and 3 = severe changes. Twenty fields of the slides at ×400 were scored and calculated in each lung sample without prior knowledge of the clinical or histologic findings. For electron microscopy, tissue was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H7500 electron microscope (Hitachi, Tokyo, Japan). For electron microscopy, a semiquantitative score was calculated on the basis of the evaluation of swelling of endothelial cells and alveolar epithelial cell desquamation. Twenty fields of the slides at ×5000 were scored in each lung sample using the same grading system as used for light microscopy.

Measurement of Serum Interleukin (IL) 1 β , IL-6, and High-Mobility Group Box 1 (HMGB1) Levels

The serum concentrations of IL-1 β , IL-6, and HMGB1 were measured frequently perioperatively and then 1, 2, 4, and 7 days after reperfusion by enzyme-linked immunosorbent assay as described by the manufacturer (IL-1 β , IL-6: R&D Systems, Minneapolis, Minn; HMGB1: Shino-Test Corporation, Sagamihara, Japan).

Statistical Analysis

Results were expressed as means $\pm$ SEM. Group comparisons were performed by the Student *t* test, the Mann–Whitney *U* test, or analysis of variance, as appropriate.

RESULTS

CO Inhalation Increases COHb Levels

Baseline COHb levels were $0.5\% \pm 0.2\%$. With the inhalation of CO at approximately 250 ppm in the CO-treated group, COHb levels rose rapidly and stabilized at $12.1\% \pm 0.3\%$. When CO treatment was discontinued 2 hours after reperfusion, COHb levels dropped rapidly (Figure 1). COHb levels in the control group remained at baseline throughout the experiment. Low-dose CO inhalation had no apparent effect on animal behavior after the operation. Blood pressure (systolic/diastolic $105/69 \pm 6$ mm Hg in the CO-treated group vs $112/79 \pm 16$ mm Hg in the control group 2 hours after reperfusion) or liver or renal function (alanine aminotransferase/creatinine: 21 ± 8 IU/L 1.0 ± 0.0 mg/dL in the CO-treated group vs 29 ± 4 IU/L 0.8 ± 0.2 mg/dL in the control group 1 day after reperfusion).

CO Inhalation Improves Short-Term Oxygenation After IRI

Arterial oxygen tension (P_{aO_2}) was used to characterize the effect of IRI on lung function.¹⁹ In the control group, 90 minutes of warm ischemia resulted in a significant decrease in P_{aO_2} , from 513 ± 22 mm Hg before ischemia to 227 ± 57 mm Hg 2 hours after reperfusion ($P = .032$; Figure 2, A). Moreover, despite successful extubation and recovery from anesthesia, 1 animal died of severe pulmonary edema 4 hours after reperfusion after discharging a significant amount of foamy, bloody sputum. The clinical diagnosis of pulmonary edema was later confirmed by pathologic examination. In sharp contrast, animals in the CO-treated group had no significant change in P_{aO_2} despite 90 minutes of warm ischemia (P_{aO_2} 481 ± 18 mm Hg before ischemia, P_{aO_2} 454 ± 34 mm Hg 2 hours after reperfusion; $P = .56$) (Figure 2, B). All animals in the CO-treated group survived the experimental period.

CO Inhalation Inhibits the Development of Pulmonary Infiltrates on CXR Films

CXR films of animals in the control group revealed decreased lung expansion and increased pulmonary infiltrates 2 days after reperfusion in the left lung only (Figure 3, A). In contrast, the CXR films of the CO-treated group showed well-expanded lungs bilaterally with few infiltrates 2 days after IRI (Figure 3, B). The quantitative CXR score of the left lung 2 days after reperfusion was 1.0 ± 0.4 in the CO-treated group and 2.7 ± 0.3 in the control group. No significant differences between the CXR films of the groups were observed 7, 28, or 56 days after IRI.

CO-Treated Animals Had Fewer Histopathologic Changes by Light Microscopy

Pathologic examination of biopsy specimens taken 2 hours after reperfusion in the control group showed focal

FIGURE 1. Percent carboxyhemoglobin (COHb) in the blood during and after carbon monoxide (CO) inhalation.

cellular infiltration, including neutrophils, slight intra-alveolar edema, mild hemorrhage, and fibrin exudation (Figure 4, A). Two days after reperfusion, there was significant damage to alveolar endothelial cells with intra-alveolar edema, hemorrhage, and fibrin exudates (Figure 4, B). Neutrophil infiltration was evident in both the alveolar capillaries and the small pulmonary arterioles (Figure 4, C). Fibrin exudates that form as a result of alveolar wall damage were also seen in the control group (Figure 4, D). In contrast, lung biopsy specimens from the CO-treated animals 2 hours after reperfusion showed almost normal lung histologic features (Figure 4, E). Only mild intra-alveolar edema (Figure 4, F) and cellular infiltration (Figure 4, G) were observed 2 days after reperfusion. Fibrin exudates were significantly decreased in the CO-treated group compared with in the control group 7 days after reperfusion (Figure 4, H). Histopathologic scores of biopsy samples are detailed in Table 1.

CO Inhalation Protects Vascular Endothelial Cells From IRI

Changes in cell morphology that result from IRI were evaluated by electron microscopic examination of lung biopsy samples taken 2 hours and 2 days after reperfusion. In biopsy samples from the control group, neutrophil infiltrates were seen in the alveolar capillaries, and endothelial cells showed marked swelling with vacuolization and disorganization of intracellular architecture 2 hours after reperfusion (Figure 5, A). Two days after IRI, biopsy specimens from the control group showed multiple patchy areas of alveolar wall thickening with deposits of collagen fibrils in the alveolar walls. Damaged alveolar capillaries, fluid and blood leakage into the alveolar space, intra-alveolar fibrin exudates, marked interstitial edema, and neutrophil infiltration into alveolar capillaries and surrounding interstitium were seen (Figure 5, B). In contrast, biopsy specimens from CO-treated animals revealed only mild swelling of endothelial cells with mild edema of alveolar walls 2 hours

FIGURE 2. Arterial blood gas analysis before and after 90 minutes of warm ischemia. A, In the control group, PaO₂ decreased from 513 ± 22 mm Hg before ischemia to 227 ± 57 mm Hg 2 hours after reperfusion (*P* = .032). B, In the carbon monoxide (CO)-treated group, there was no significant change in PaO₂ after ischemia–reperfusion injury (481 ± 18 mm Hg before ischemia; 454 ± 34 mm Hg 2 hours after reperfusion; *P* = .56).

(Figure 5, C) and 2 days (Figure 5, D) after reperfusion. Near-normal ultrastructure was maintained after IRI. Histo-pathologic scores of biopsy samples evaluated by electron microscopy are detailed in Table 2.

CO Inhalation Inhibits the Production of Proinflammatory Mediators in Response to IRI

Serum cytokine levels were measured to characterize the inflammatory response to IRI and evaluate the effect of CO on that response. Sequential measurements of systemic early-phase cytokine levels (IL-1β, IL-6) showed that the concentrations of these proteins increased after IRI in both groups, peaking 1 day after reperfusion in the control group and 2 hours after reperfusion in the CO-treated group. The concentration of IL-1β 2 days after reperfusion in the CO-treated group was significantly lower than in the control

group (9 ± 4 pg/mL vs 43 ± 14 pg/mL; *P* = .044; Figure 6, A). The concentration of IL-6 in the control group increased substantially to 130 ± 43 pg/mL 1 day after reperfusion. In contrast, the concentration of IL-6 in the control group at the same time was only 34 ± 9 pg/mL (*P* = .055). IL-6 levels in both groups declined 2 days after reperfusion (2.1 ± 1.5 pg/mL in the CO-treated group vs 30 ± 9 pg/mL in the control group; *P* = .019; Figure 6, B). HMGB1 has recently been reported to be an early mediator of inflammation and cell injury after IRI. The peak concentration of HMGB1 in the control group (14 ± 6 ng/mL) was observed 2 days after reperfusion (Figure 6, C). In contrast, the HMGB1 concentration 2 days after reperfusion in the CO-treated group was only 0.4 ± 0.1 ng/mL (*P* = .037; Figure 6, C). The concentrations of IL-1β, IL-6, and HMGB1 returned to baseline by 4 days after reperfusion (data not shown).

FIGURE 3. Representative chest x-ray films taken 2 days after ischemia–reperfusion injury. A, In the control group, the films revealed diffuse pulmonary infiltrates in the left lung. B, In the carbon monoxide–treated group, few pulmonary infiltrates are seen.

FIGURE 4. Histologic analysis by light microscopy of representative lung biopsy specimens from the control (A–D) and carbon monoxide (CO)-treated groups (E–H). The control group showed focal cellular infiltration, slight intra-alveolar edema, mild hemorrhage, and fibrin exudation 2 hours after reperfusion (A); damaged alveolar endothelial cells, intra-alveolar edema, hemorrhage, and fibrin exudates 2 days after reperfusion (B); neutrophil infiltration in both the intra-alveolar space and small pulmonary arterioles 2 days after reperfusion (C); and massive alveolar exudates and focal hyaline membrane formation 7 days after reperfusion (D). In dramatic contrast, lung biopsy specimens in the CO-treated animals revealed nearly normal structure 2 hours after reperfusion (E); mild swelling of the alveolar wall (F) and neutrophil infiltration 2 days after reperfusion (G); and reversion to near-normal histology 7 days after reperfusion (H). A, B, D to F, and H: Elastica-Masson Goldner stain (EMG; $\times 200$). C and G: Hematoxylin and eosin stain (H&E; $\times 600$).

DISCUSSION

Graft quality has a major impact on the short- and long-term outcomes of transplantation. The donor's baseline health and cause of death, as well as the conditions of organ procurement, storage, and transplantation, all affect susceptibility to IRI. In transplanted lungs, IRI takes the form of nonspecific inflammatory changes that result in cellular structural damage, the increased expression of MHC antigens²⁰ and proinflammatory cytokines,²¹ and the activation of proapoptotic pathways in the graft, all of which begin before organ transplantation.²² The prevention of IRI in the graft is important for both early and long-term graft function, particularly when using organs from marginal donors.

In this study, we established a clinically relevant large animal model of standardized lung ischemia and reperfusion that evaluates the functional and biochemical aspects of IRI in vivo. We induced 90 minutes of warm ischemic injury to one lung, with concurrent dissection of the associated main bronchus. This injury led to a reduction in P_{aO_2} in the control group from 513 ± 22 mm Hg before ischemic injury to 227 ± 57 mm Hg after ischemia, a 50% reduction that persisted for 2 hours after reperfusion in the control group. IRI in the control group was associated with the development of significant pulmonary infiltrates on CXR films and widespread tissue damage, as well as the rapid death of 1 animal from severe pulmonary edema. In contrast, the continuous administration of inhaled low-dose CO to the naïve lung throughout the operation, and to the ischemic lung both before and after IRI, led to significant improvements in oxygenation and cellular integrity. CO inhalation also reduced the num-

ber of pulmonary infiltrates and the elevation of serum concentrations of the proinflammatory cytokines IL-1 β , IL-6, and HMGB1 after IRI.

Recent work has demonstrated that constitutive heme oxygenase expression and the endogenous generation of CO help maintain the integrity of cells and the physiologic functions of organs via anti-inflammatory, antiapoptotic, and antiproliferative effects.¹⁰ CO also exerts potent protective effects on blood vessels; it optimizes microcirculation by promoting vasodilation and inhibiting platelet activation.¹⁰ Furthermore, increased levels of CO—as a result of enhanced endogenous production through heme oxygenase-1 induction or the exogenous delivery of CO by gas inhalation, CO-releasing molecules, the pro-drug methylene chloride, or in soluble form in a preservation solution—have been shown to exert cytoprotective effects in more than

TABLE 1. Histologic scores of lung biopsy specimens based on light microscopy

	Two hours after reperfusion		Two days after reperfusion	
	Control (n = 4)*	CO-treated (n = 4)	Control (n = 3)*	CO-treated (n = 4)
Infiltrating cells	1.6 ± 0.2	$0.5 \pm 0.0^\dagger$	2.3 ± 0.4	1.1 ± 0.1
Intra-alveolar edema	1.0 ± 0.4	$0.3 \pm 0.0^\dagger$	1.7 ± 0.7	0.5 ± 0.2
Fibrin exudation	1.0 ± 0.3	$0.2 \pm 0.1^\dagger$	2.2 ± 0.4	0.5 ± 0.2
Intra-alveolar hemorrhage	1.7 ± 0.3	$0.4 \pm 0.2^\dagger$	2.0 ± 0.3	0.8 ± 0.1

CO, Carbon monoxide. Data are shown as mean $\pm$ SEM. *One animal died of severe pulmonary edema 4 hours after reperfusion. $^\dagger P < .05$ versus control group.

FIGURE 5. Histologic analysis by electron microscopy of representative lung biopsy specimens. A, In the control group 2 hours after reperfusion, electron microscopy revealed edema of alveolar walls, neutrophil infiltration in the alveolar capillaries, and marked swelling of endothelial cells. B, Two days after reperfusion, the alveolar walls were thickened, with fluid and blood leakage into the alveolar spaces. Damaged and destroyed alveolar capillaries are seen. C, In the carbon monoxide (CO)-treated animals, pathologic changes were limited to mild swelling of endothelial cells and edema of the alveolar walls 2 hours and 2 days (D) after reperfusion. Original magnification: A, ×5000; B and D, ×4000; and C, ×6000.

a dozen small animal models of organ transplantation, including models of lung, heart, kidney, small intestine, liver, and islet cell transplantation.¹⁰ In this study, we showed that low-dose CO inhalation limits acute IRI to lungs in a preclinical large animal model.

Experimental and clinical data suggest that IRI during transplantation occurs in a biphasic pattern: the early phase occurs within 15 minutes of graft reperfusion and depends primarily on donor characteristics, such as the number of resident alveolar macrophages in the graft; the delayed phase occurs over the ensuing 24 hours and depends primarily on recipient factors, including the infiltration and activity of recipient neutrophils and lymphocytes.^{23,24} Abundant pulmonary resident macrophages are a source of multiple proinflammatory mediators, including IL-1 β , tumor necrosis factor- α , and IL-6. These cells can cause immediate direct injury to cells and can prompt neutrophil activation, migration, and extravasation into the alveolar interstitial space. The decrease in serum levels of IL-1 β and IL-6 reported in our study suggests that the protective effects of CO are mediated in part by the downregulation of the proinflammatory mediators normally produced by resident macrophages.

CO effectively limits the proinflammatory cytokine/chemokine response of macrophages to ischemia and reperfusion, resulting in decreased paracrine activation of other constituent lung cell populations and a decrease in the recruitment of circulating inflammatory cells.

The importance of reactive oxygen species such as superoxide anion and hydrogen peroxide has been recognized in lung IRI. Reactive oxygen species are highly unstable and react with the lipid component of the cell membrane, which can range from increased permeability to cell lysis.¹

TABLE 2. Histologic scores of lung biopsy specimens based on electron microscopy

	Two hours after reperfusion		Two days after reperfusion	
	Control (n = 4)*	CO-treated (n = 4)	Control (n = 3)*	CO-treated (n = 4)
Swelling of endothelial cells	1.9 $\pm$ 0.2	1.0 $\pm$ 0.2†	1.8 $\pm$ 0.2	0.7 $\pm$ 0.1
Alveolar epithelial cell desquamation	0.4 $\pm$ 0.1	0.1 $\pm$ 0.0†	1.2 $\pm$ 0.4	0.0 $\pm$ 0.0

CO, Carbon monoxide. Data are shown as mean $\pm$ SEM. *One animal died of severe pulmonary edema 4 hours after reperfusion. †P < .05 versus control group.

ET/BS

FIGURE 6. Serum levels of inflammatory cytokines. Two days after reperfusion, serum cytokine levels were significantly lower in the carbon monoxide (CO)-treated animals than in the control animals: A, IL-1 β : 9 ± 4 pg/mL versus 43 ± 14 pg/mL; $P = .044$; B, IL-6: 2.1 ± 1.5 pg/mL versus 30 ± 9 pg/mL; $P = .019$; C, HMGB1: 14 ± 6 ng/mL versus 0.4 ± 0.1 ng/mL; $P = .037$. Data are expressed as the mean $\pm$ SEM. * $P < .05$ between the CO-group (solid bars) and control group (open bars). IL, Interleukin; HMGB1, high-mobility group box 1.

One important mechanism that leads to the production of reactive oxygen species during lung ischemia/reperfusion depends on the nicotinamide adenine dinucleotide phosphate system, which is present mainly on activated neutrophils, monocytes/macrophages, and endothelial cells and catalyzes the reduction of oxygen into hydrogen peroxide and superoxide anion.¹ The protective effect of CO on inflammatory cells such as neutrophils or macrophages and vascular endothelial cells could reduce the production of reactive oxygen species during IRI in this model. To evaluate functional characteristics of the microvasculature by measuring microvascular reactivity directly would be very useful in further understanding the mechanism of CO activity on endothelial cells.²⁵ In addition, the strong affinity of CO for the heme moiety of nicotinamide adenine dinucleotide phosphate oxidase could modulate reduction–oxidation signaling and reduce the production of reactive oxygen species.²⁶

Extracellular HMGB1 is a potent inflammatory stimulator that acts by promoting cytokine production by monocytes, macrophages, dendritic cells, and endothelial cells.²⁷ It is released into the extracellular milieu either passively from damaged or necrotic cells or actively by secretion from activated monocytes and macrophages. We found that CO inhalation almost eliminated the increase in serum levels of HMGB1 seen in the control group. Further studies are necessary, however, to determine the mechanism of HMGB1 secretion in this model. We are currently carrying out experiments to determine whether direct inhibition of HMGB1 expression prevents IRI in this model.

CO binds hemoglobin with an affinity that is 240 times greater than that of oxygen, thereby interfering with the oxygen delivery system of the body. COHb levels correlate well with clinical symptoms of CO poisoning in humans; levels of 10% to 30% can cause minor transient symptoms including headache, dizziness, and shortness of breath; death occurs at COHb levels between 50% and 80%.⁸ In murine studies, no deleterious side effects have been reported from short- or long-term CO inhalation at dosages between 20 and

500 ppm (COHb $\sim$ 10%–25%).¹⁰ Similarly, our study and others have shown no adverse effects of CO inhalation at the dosages of 250 ppm with 10% to 15% COHb in miniature swine.¹⁴ One study in humans reported that inhalation of 500 ppm CO for 1 hour, which correlated with COHb levels of about 7%, produced no clinical symptoms.²⁸ Short-term treatment with low-dose inhaled CO in the setting of careful monitoring of COHb levels may be clinically applicable, but only after further studies in large animals establish a clinically acceptable risk profile for short-term, low-dose CO inhalation in humans.

CO inhalation by donors and recipients during the perioperative period may decrease IRI during clinical lung transplantation. Donor pretreatment with inhaled CO before organ harvest could be accomplished in a clinical setting, potentially decreasing the effects of warm ischemia and prolonging the tolerable ischemic interval with little risk of adverse effects on the recipient. CO could be easily administered to lung donors after the declaration of brain death or even after cardiac death, during the time between expected circulatory arrest and the infusion of a cold preservative solution. Although it is difficult to conclude from this *in situ* lung IRI model whether we should ventilate the donor or the recipient or both with CO to prevent IRI, experiments in large animal models of lung transplantation would be useful to determine whether treatment with CO of the donor alone improves relevant outcomes.

In conclusion, we have shown that the perioperative administration of low-dose inhaled CO decreases warm IRI in lungs in miniature swine. The rather dramatic effect of size seen in this study, as well as the ease with which CO inhalation could be translated to a clinical setting, suggest the potential for CO inhalation to have a powerful therapeutic effect in reducing IRI in lung transplantation in humans.

We thank Drs Hiroshi Date, Toru Bando, and Adam Griesemer for their helpful advice and review of this manuscript. We also thank Shino-Test Corporation, Sagamihara, Japan, for generously providing the HMGB1 enzyme-linked immunosorbent assay kit II.

References

- de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia–reperfusion–induced lung injury. *Am J Respir Crit Care Med*. 2003;167:490–511.
- Whitson BA, Nath DS, Johnson AC, Walker AR, Prekker ME, Radosevich DM, et al. Risk factors for primary graft dysfunction after lung transplantation. *J Thorac Cardiovasc Surg*. 2006;131:73–80.
- Serrick C, Giaid A, Reis A, Shennib H. Prolonged ischemia is associated with more pronounced rejection in the lung allograft. *Ann Thorac Surg*. 1997;63:202–8.
- Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Jones DR, et al. Ischemia–reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. *Ann Thorac Surg*. 2002;73:1041–7; discussion 7–8.
- Bach FH, Hancock WW, Ferran C. Protective genes expressed in endothelial cells: a regulatory response to injury. *Immunol Today*. 1997;18:483–6.
- Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest*. 1999;104:1631–9.
- DeBruyne LA, Magee JC, Buelow R, Bromberg JS. Gene transfer of immunomodulatory peptides correlates with heme oxygenase-1 induction and enhanced allograft survival. *Transplantation*. 2000;69:120–8.
- von Burg R. Carbon monoxide. *J Appl Toxicol*. 1999;19:379–86.
- Ryter SW, Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med*. 2000;28:289–309.
- Nakao A, Choi AM, Murase N. Protective effect of carbon monoxide in transplantation. *J Cell Mol Med*. 2006;10:650–71.
- Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, et al. Cardioprotective actions by a water-soluble carbon monoxide–releasing molecule. *Circ Res*. 2003;93:e2–8.
- Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R, Motterlini R. Treatment with CO-RMs during cold storage improves renal function at reperfusion. *Kidney Int*. 2006;69:239–47.
- Kohmoto J, Nakao A, Sugimoto R, Wang Y, Zhan J, Ueda H, et al. Carbon monoxide–saturated preservation solution protects lung grafts from ischemia–reperfusion injury. *J Thorac Cardiovasc Surg*. 2008;136:1067–75.
- Lavitrano M, Smolenski RT, Musumeci A, Maccherini M, Slominska E, Di Florio E, et al. Carbon monoxide improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass in pigs. *FASEB J*. 2004;18:1093–5.
- Bagul A, Hosgood SA, Kaushik M, Nicholson ML. Carbon monoxide protects against ischemia–reperfusion injury in an experimental model of controlled nonheartbeating donor kidney. *Transplantation*. 2008;85:576–81.
- Hosgood SA, Bagul A, Kaushik M, Rimoldi J, Gadepalli RS, Nicholson ML. Application of nitric oxide and carbon monoxide in a model of renal preservation. *Br J Surg*. 2008;95:1060–7.
- Murray JF, Matthay MA, Luce JM, Flick MR. An expanded definition of the adult respiratory distress syndrome. *Am Rev Respir Dis*. 1988;138:720–3.
- Muller C, Hoffmann H, Bittmann I, Isselhard W, Messmer K, Dienemann H, et al. Hypothermic storage alone in lung preservation for transplantation: a metabolic, light microscopic, and functional analysis after 18 hours of preservation. *Transplantation*. 1997;63:625–30.
- Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, et al. Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat Med*. 2001;7:598–604.
- Waddell TK, Gorczynski RM, DeCampos KN, Patterson GA, Slutsky AS. Major histocompatibility complex expression and lung ischemia–reperfusion in rats. *Ann Thorac Surg*. 1996;62:866–72.
- Dinarello CA. Proinflammatory cytokines. *Chest*. 2000;118:503–8.
- Bharat A, Narayanan K, Street T, Fields RC, Steward N, Aloush A, et al. Early posttransplant inflammation promotes the development of alloimmunity and chronic human lung allograft rejection. *Transplantation*. 2007;83:150–8.
- de Perrot M, Young K, Imai Y, Liu M, Waddell TK, Fischer S, et al. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. *J Immunol*. 2003;171:4995–5002.
- Fiser SM, Tribble CG, Long SM, Kaza AK, Cope JT, Laubach VE, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. *J Thorac Cardiovasc Surg*. 2001;121:1069–75.
- Yang Z, Sharma AK, Linden J, Kron IL, Laubach VE. CD4 + T lymphocytes mediate acute pulmonary ischemia–reperfusion injury. *J Thorac Cardiovasc Surg*. 2009;137:695–702; discussion 702.
- Taille C, El-Benna J, Lanone S, Boczkowski J, Motterlini R. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *J Biol Chem*. 2005;280:25350–60.
- Dumitriu IE, Baruah P, Manfredi AA, Bianchi ME, Rovere-Querini P. HMGB1: guiding immunity from within. *Trends Immunol*. 2005;26:381–7.
- Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, et al. Effects of carbon monoxide inhalation during experimental endotoxemia in humans. *Am J Respir Crit Care Med*. 2005;171:354–60.