Carbon Monoxide Protects against Ventilator-induced Lung Injury via PPAR-γ and Inhibition of Egr-1

Alexander Hoetzel[1,2], Tamas Dolinay[1], Simone Vallbracht[2], Yingze Zhang[1], Hong Pyo Kim[1], Emeka Ifedigbo[1], Sean Alber[3], A. Murat Kaynar[4], Rene Schmidt[2], Stefan W. Ryter[1], and Augustine M. K. Choi[1,5]

[1]Department of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; [2]Department of Anesthesiology and Critical Care Medicine, University Hospital Freiburg, Freiburg, Germany; [3]Center of Biological Imaging, and [4]Department of Critical Care Medicine and Anesthesiology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; and [5]Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

***Rationale*: Ventilator-induced lung injury (VILI) leads to an unacceptably high mortality. In this regard, the antiinflammatory properties of inhaled carbon monoxide (CO) may provide a therapeutic option.**
***Objectives*: This study explores the mechanisms of CO-dependent protection in a mouse model of VILI.**
***Methods*: Mice were ventilated (12 ml/kg, 1–8 h) with air in the absence or presence of CO (250 ppm). Airway pressures, blood pressure, and blood gases were monitored. Lung tissue was analyzed for inflammation, injury, and gene expression. Bronchoalveolar lavage fluid was analyzed for protein, cell and neutrophil counts, and cytokines.**
***Measurements and Main Results*: Mechanical ventilation caused significant lung injury reflected by increases in protein concentration, total cell and neutrophil counts in the bronchoalveolar lavage fluid, as well as the induction of heme oxygenase-1 and heat shock protein-70 in lung tissue. In contrast, CO application prevented lung injury during ventilation, inhibited stress-gene up-regulation, and decreased lung neutrophil infiltration. These effects were preceded by the inhibition of ventilation-induced cytokine and chemokine production. Furthermore, CO prevented the early ventilation-dependent up-regulation of early growth response-1 (Egr-1). Egr-1–deficient mice did not sustain lung injury after ventilation, relative to wild-type mice, suggesting that Egr-1 acts as a key proinflammatory regulator in VILI. Moreover, inhibition of peroxysome proliferator-activated receptor (PPAR)-γ, an antiinflammatory nuclear regulator, by GW9662 abolished the protective effects of CO.**
***Conclusions*: Mechanical ventilation causes profound lung injury and inflammatory responses. CO treatment conferred protection in this model dependent on PPAR-γ and inhibition of Egr-1.**

Keywords: carbon monoxide; early growth response-1; inflammation; peroxysome proliferator-activated receptor-γ; ventilator-induced lung injury

Mechanical ventilation, routinely required for the clinical treatment of respiratory failure, can cause ventilator-induced lung injury (VILI), and aggravate the progression of acute respiratory distress syndrome. Despite the implementation of protective ventilation strategies (1–3), involving the reduction of tidal volume, the increase of positive end-expiratory pressure, or lung recruitment maneuvers, mechanical ventilation contributes to an unacceptably high mortality in these patients. Therefore, additional strategies must be developed to further decrease the risk of ventilator-associated lung injury.

The development of VILI involves a complex orchestration of biotraumatic events. Ventilation, which imposes mechanical stress (cyclic stretch) on the lung, can cause disruption of the alveolar–capillary barrier, leading to pulmonary edema, increased oxidative stress, and an increase in proinflammatory processes (4). The inflammatory responses are not restricted to the lung but may also affect other organs, leading to multiple organ dysfunction.

Carbon monoxide (CO), a low-molecular-weight gas, when applied at low concentrations, has shown promise as a potential therapeutic agent in animal models of inflammation-associated lung injury, despite its reputation as a lethal asphyxiant and noxious air pollutant. It has been recognized since the 1950s that CO is produced in humans as the result of hemoglobin turnover (5) and that this endogenous production increases during systemic or pulmonary inflammation (6). Endogenous CO arises principally as the by-product of heme metabolism, catalyzed by heme oxygenase (HO) enzymes (7). The inducible isozyme HO-1, a major inducible stress protein, responds to transcriptional regulation by a broad spectrum of chemical and physical agents, including hemodynamic stress, cyclic stretch, and prooxidative and proinflammatory mediators (8–10). Accumulating evidence has indicated that the up-regulation of HO-1 can confer protective effects *in vitro* and *in vivo* that can be mimicked by exogenous application of low-concentration CO (11). On the basis of these observations, over the last decade, efforts have been undertaken to define the therapeutic potential of CO by introducing this gaseous molecule by inhalation or by the pharmacologic application of synthetic prodrugs (8, 12). The

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Mechanical ventilation can cause inflammatory lung injury. The antiinflammatory properties of carbon monoxide may be exploited to reduce lung injury caused by mechanical ventilation.

What This Study Adds to the Field

Carbon monoxide protects against pulmonary inflammation in a murine model of ventilator-induced lung injury by specific antiinflammatory mechanisms, involving peroxisome proliferator-activated receptor-γ and early growth response factor-1.

(*Received in original form August 28, 2007; accepted in final form March 17, 2008*)

Supported by a grant from the Deutsche Forschungsgemeinschaft to A.H. (DFG HO 2464/1-1); by an award from the American Heart Association to S.W.R., (AHA 0335035N) and H.P.K. (AHA 0525552 U); and NIH grants R01-HL60234, R01-HL55330, R01-HL079904, and P01-HL70807 awarded to A.M.K.C.

Correspondence and requests for reprints should be addressed to Augustine M. K. Choi, M.D., Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115. E-mail: amchoi@rics.bwh.harvard.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177. pp 1223–1232, 2008
Originally Published in Press as DOI: 10.1164/rccm.200708-1265OC on March 20, 2008
Internet address: www.atsjournals.org

vast majority of these investigations demonstrated antiproliferative, antiapoptotic, antioxidative, antiinflammatory, and vasomodulatory effects of CO, contributing to tissue protection in various disease models, including septic or oxidative lung injury and organ transplantation (8, 13). Given the observed antiinflammatory properties of CO, we hypothesize that this gas might represent a potential therapeutic tool in protecting the lung from the injurious consequences of mechanical ventilation.

Our laboratory has previously shown that CO inhalation at low concentration (250 ppm) during mechanical ventilation reduced lung injury caused by the combination of high–tidal volume ventilation and bacterial lipopolysaccharide (LPS) treatment in a rat model (14).

In the current study, we have used a mouse model of VILI at physiologically relevant tidal volumes, and demonstrate that the application of low-concentration CO attenuates VILI during mechanical ventilation. We propose a mechanism for the antiinflammatory and tissue protective effects of CO in this model involving the coordinated regulation of proinflammatory (i.e., early growth response [Egr]-1) and antiinflammatory (i.e., peroxisome proliferator-activated receptor [PPAR]-γ) transcription factors. Some of the results of these studies have been previously reported in the form of abstracts (15, 16).

METHODS

Animals

All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. The Animal Care and Use Committee of the University of Pittsburgh approved the protocols. Male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) and male Egr-1–deficient (*egr-1*$^{-/-}$) mice (C57/BL6 background; Taconic, Hudson, NY) were 7–10 weeks of age and weighed 25–29 g, without differences among treatment groups. All animals were anesthetized with ketamine (80 mg/kg, intraperitoneally) and acepromazine (1 mg/kg, intraperitoneally) and placed on a heating pad. A polyethylene catheter was inserted into the left carotid artery for direct blood pressure monitoring as well as for blood gas sampling, and a tracheostomy was established using a 20-gauge catheter. A rodent ventilator (Voltekenterprises, Toronto, ON, Canada) was set to a tidal volume of 12 ml/kg, a frequency of 80 breaths/minute, and a positive end-expiratory pressure of 2 cm H_2O, and connected to the tracheal cannula. Muscular relaxation was achieved by applying pancuronium 2 mg/kg intraperitoneally. A 0.7-ml saline bolus was injected intraperitoneally to compensate for evaporation during ventilation. Animals received ventilation with room air, or room air supplemented with 250 ppm CO for 1, 4, or 8 hours. Control animals were sham operated and ventilated for 5 minutes. After surgical preparation and before the onset of the experiment, all animals were randomized to control, ventilation, or ventilation with CO within the respective experimental set. Blood samples were withdrawn from control animals after 5 minutes and from ventilated animals at 1 hour after onset of mechanical ventilation, and blood gases were measured using an automated blood gas analyzer (ABL5; Radiometer, Bronshoi, Denmark). Pretreatment with GW9662 (5 mg/kg, intraperitoneally; Axxora Life Sciences, San Diego, CA), a specific PPAR-γ inhibitor, was conducted 30 minutes before mechanical ventilation.

Physiologic Measurements

Peak airway pressure, plateau airway pressure (cm H_2O), and arterial blood pressure (mm Hg) were continuously monitored (PowerLab; ADInstruments, Colorado Springs, CO) and recorded every 30 minutes.

Bronchoalveolar Lavage

At the end of each experiment, a bronchoalveolar lavage (BAL) was performed using 1 ml phosphate-buffered saline (PBS). The recovered volume was centrifuged and the supernatant analyzed for protein concentration (Biorad Assay; Biorad, Hercules, CA). Furthermore, the pellet was redissolved in PBS and subsequently the number of total cells as well as the fraction of neutrophils were determined.

Cell Culture

RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (17). Murine bronchial epithelial cells (Beas2B) were cultured as previously described (18). Murine primary lung endothelial cells (MLECs) were isolated as previously described (19) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 6.32 g/L *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid, and 3.3 ml of endothelial cell growth supplements. Cells were cultivated for three passages in humidified incubators at 37°C. For experiments, the cells were placed into a sealed chamber (COY Laboratory Products, Inc., Ann Arbor, MI) and exposed to 21% O_2, 5% CO_2, and 250 ppm CO for 1–6 hours.

Immunoblotting and Immunohistochemistry

See online supplement for details.

Cytokine/Chemokine Measurements

See online supplement for details.

Statistical Analysis

Data represent means + SEM. Statistical analyses were performed using analysis of variance for multiple-group comparison and the Student-Newman-Keuls post hoc test (Sigmastat statistical software; Systat, Inc., Erkrath, Germany). For two-group comparison, the Student *t* test was used to compare parametric data, and the Mann-Whitney rank sum test used to compare nonparametric data. $P < 0.05$ was considered significant.

RESULTS

Effect of CO on Airway and Systemic Blood Pressure

Mechanical ventilation of mice with 12 ml/kg resulted in a mean peak airway pressure of 16 cm H_2O and a mean plateau pressure of 12 cm H_2O (Figures E1A–E1B of the online supplement). CO has been previously demonstrated to relax vascular and airway smooth muscle through cyclic guanosine-3′-5′ monophosphate (cGMP)–dependent pathways (20–23). We therefore investigated the effect of CO application during mechanical ventilation on airway pressure. The application of CO (250 ppm) had no influence on either mean peak airway or plateau pressure (Figures E1A–E1B). Blood gases taken 1 hour after the onset of ventilation revealed a mean pH of 7.29, a P_{O_2} of 102 mm Hg, and a P_{CO_2} of 34 mm Hg, which were not significantly different from control values (data not shown). A carboxyhemoglobin fraction of 3% was observed in both control and ventilated animals, which rose to a mean of 25% in animals that were ventilated in the presence of CO (data not shown). Most interesting, ventilation led to an increase of blood pressure readings over time that was prevented by CO application (Figure E1C). At 4 and 8 hours, ventilated animals exhibited mean blood pressure values of 93 and 85 mm Hg that were decreased in the presence of CO to 80 and 69 mm Hg, respectively.

Effect of CO on Lung Injury

Mechanical ventilation at 12 ml/kg for 4 to 8 hours led to significant increases in protein concentration, total cell count, and the number of neutrophils in the BAL fluid (Figure 1), reflecting a time-dependent progression of VILI. In contrast, CO treatment during ventilation substantially decreased the protein leakage and the recruitment of inflammatory cells into the lung. These findings were confirmed by the histologic examination of lung sections (Figures 2A–2C). Hematoxylin-and-eosin staining of lung tissue sections revealed that mechanical ventilation caused the infiltration of inflammatory cells into the

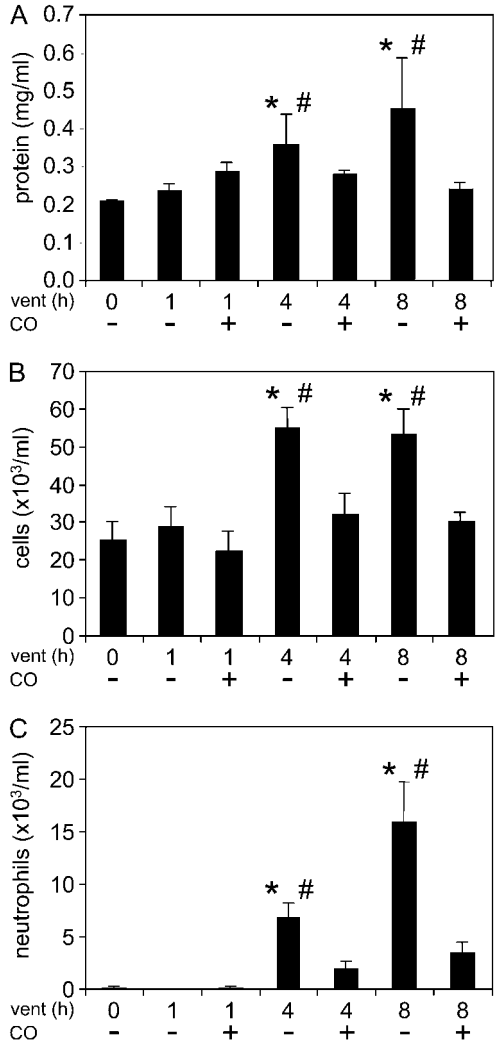

Figure 1. Effect of CO on protein concentration (*A*), total cell count (*B*), and neutrophil count (*C*), measured in the bronchoalveolar lavage (BAL) fluid that was obtained at the end of each experiment. Mice were ventilated for indicated times ± CO (250 ppm). Ventilation led time-dependently to an increase in protein concentration (*A*) and cell (*B*) and neutrophil (*C*) in the BAL fluid that was prevented by application of CO. Data presentation and statistical analysis: n = 5/group; mean + SEM; analysis of variance (ANOVA); $^{*}P < 0.05$ versus control (0 h ventilation), $^{\#}P < 0.05$ versus time-matched CO-treated mice.

interstitium and the formation of edematous thickened septae relative to nonventilated controls. In sharp contrast, administration of CO (250 ppm) during the ventilation significantly reduced these indicators of lung injury (Figure 2C). Moreover, we investigated the regulation of inducible stress proteins HO-1 and heat shock protein-70 (HSP70), which were previously described as being responsive to cellular stretch (9, 24) and, at least *in vitro*, modulated by CO (25). Mechanical ventilation induced HO-1 and HSP70 after 8 hours as demonstrated by representative Western blots (Figure 3A) and corresponding densitometric analysis (Figures 3B–3C). In sharp contrast, application of CO (250 ppm) during ventilation prevented the expression of these genes.

Effect of CO on Neutrophil Transmigration

As shown above, administration of CO prevented neutrophil accumulation in the BAL fluid (Figure 1C). We further investigated whether CO inhibited the neutrophil migration from the lung interstitium to the alveolar space or whether CO inhibited the recruitment of neutrophils into the lung. For that purpose, lung sections were stained with granulocyte-1, a specific marker of neutrophils. As depicted in Figures 2D–2F, mechanical ventilation led to significant number of neutrophils in the interalveolar septae that was not observed in control animals nor in CO-treated animals. These results indicate that BAL measurement of neutrophils reflects the number of neutrophils in the interstitium and that CO prevented the recruitment of neutrophils into the lung.

Effect of CO on Cytokines and Chemokines

The infiltration of neutrophils into the lung on mechanical ventilation is believed to be preceded by up-regulation of a variety of cytokines and chemokines. Therefore, we measured IL-1β, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β in lung tissue homogenates. Figure 4 demonstrates that these cytokines and chemokines were highly up-regulated in response to mechanical ventilation after 4 hours. The administration of CO (250 ppm) completely prevented the production of IL-1β, MCP-1, and MIP-1β after 4 hours of mechanical ventilation.

Role of Egr-1 in the Antiinflammatory Effect of CO

The cytokines and chemokines that attract neutrophils in the lung are well known to be transcriptionally regulated. In that context, Egr-1 represents a major proinflammatory transcriptional regulator whose expression precedes and subsequently regulates cytokine or chemokine production (26). As depicted in the representative Western blot and corresponding densitometric analysis in Figures 5A–5B, Egr-1 was up-regulated as early as 1 hour after the onset of ventilation and preceded the observed increases in cytokine and chemokine levels (Figure 4). CO treatment (250 ppm) prevented Egr-1 up-regulation during mechanical ventilation.

To investigate the functional role of Egr-1 inhibition by CO, we ventilated Egr-1–deficient mice (*egr-1*$^{-/-}$) for 8 hours in the absence or presence of CO (250 ppm). The occurrence of significant lung inflammation in response to mechanical ventilation in wild-type mice (Figures 5C–5D, *solid bars*) was abolished in *egr-1*$^{-/-}$ mice (Figures 5C–5D, *open bars*). Macrophage and neutrophil infiltration of the lung, reflected by the total cell count and neutrophil count in the BAL fluid, did not change in response to ventilation to any further extent and was not modulated by CO. These data were confirmed by the histologic analysis of lung sections. The prevention of increased cellular infiltration was paralleled by a normal appearance of lung tissue despite ventilation when stained with hematoxylin and eosin (Figures 6A–6C). The indices of lung injury that were described in wild-type mice after 8 hours of ventilation as shown in Figures 3A–3C were completely abolished in *egr-1*$^{-/-}$ mice (Table E1). In contrast to wild-type animals, *egr-1*$^{-/-}$ mice responded to mechanical ventilation with only a slight increase in BAL protein that did not reach statistical significance. The levels of BAL cytokines, such as IL-1β, MCP-1, and MIP-1β, were much lower in *egr-1*$^{-/-}$ mice as compared with wild-type mice in response to mechanical ventilation (Table E1). CO inhalation decreased the cytokine response to ventilation in wild-type mice. In *egr-1*$^{-/-}$ animals, in which cytokine production did not respond to ventilation, CO treatment resulted in comparable cytokine levels relative to wild-type mice. Most interesting, HO-1 was slightly increased in *egr-1*$^{-/-}$ mice in response to ventilation (Figures E2A–E2B). However, in contrast to wild-type mice, CO did not down-regulate HO-1 in *egr-1*$^{-/-}$ mice.

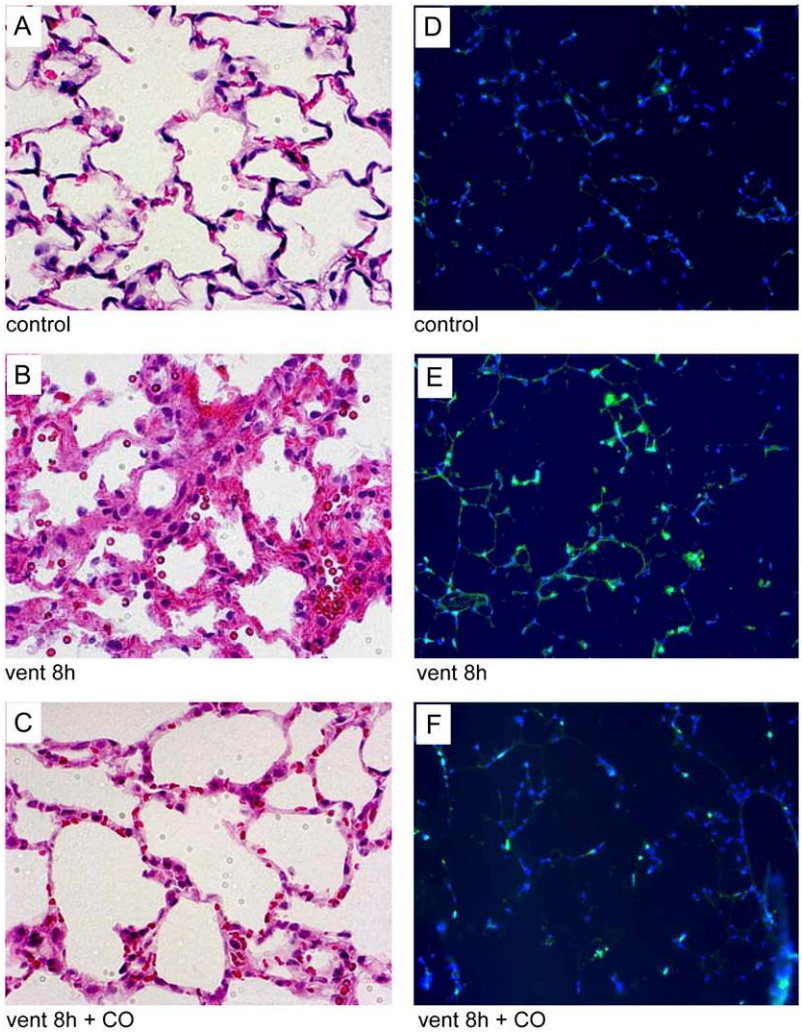

Figure 2. Effect of CO on lung injury and neutrophil infiltration. Left lung lobes were taken from animals without ventilation (*A* and *D*), with ventilation for 8 hours (*B* and *E*), and with ventilation + CO (250 ppm) for 8 hours (*C* and *F*). Lung sections were stained with hematoxylin and eosin and presented in ×400 original magnification (*A*–*C*) or with granulocyte-1, a specific marker for neutrophils, presented in ×120 original magnification (*D*–*F*). As compared with control (*A*), ventilation led to thickening of septae and infiltration of inflammatory cells (*B*), which was prevented in the presence of CO (*C*). Although neutrophils were not detectable in control animals (*D*), ventilation led to significant accumulation of neutrophils within the lung interstitium (*E*), which was prevented in the presence of CO (*F*).

Role of PPAR-γ in the Antiinflammatory Effect of CO

In lung macrophages, PPAR-γ has been demonstrated to act as an antiinflammatory mediator by counteracting the proinflammatory effects of Egr-1 (27). To test whether CO regulates PPAR-γ *in vitro*, mouse macrophages (RAW 264.7), MLECs, and mouse bronchial epithelial cells (Beas2B) were incubated in the presence of 250 ppm CO for 1 to 6 hours. As shown in Figure 7, continuous exposure to CO for 1 hour led to the induction of PPAR-γ in RAW 264.7 cells, but not in Beas2B or MLECs, indicating that the CO-dependent up-regulation of PPAR-γ appears to be cell specific and restricted to macrophages.

To evaluate whether PPAR-γ regulates Egr-1 *in vivo*, mice were pretreated with GW9662, a specific PPAR-γ inhibitor, and ventilated in the absence or presence of CO. The immunoblot of Egr-1 and its densitometric analysis showed a substantial induction of the gene by ventilation (Figures 8A–8B) that was more pronounced as compared with nontreated animals (Figures 5A–5B). Moreover, whereas CO prevented Egr-1 gene expression in nontreated animals, GW9662 pretreatment abolished the CO-mediated effect (Figures 8A–8B).

To test the hypothesis that the protection from VILI afforded by CO during mechanical ventilation involves functional PPAR-γ, lung injury parameters from GW9662-treated animals were analyzed and compared with non-pretreated mice. As shown in Figures 8C–8D, CO reduced the infiltration of macrophages and neutrophils into the lung in ventilated animals (*solid bars*). After the application of GW9662 (*open bars*), ventilation led to a more pronounced total cell or neutrophil count in the BAL fluid and CO failed to reduce both parameters after 8 hours of ventilation. In this context, hematoxylin-and-eosin staining from lung sections of GW9662-treated animals provided further evidence that PPAR-γ inhibition led to a significant degree of lung injury after ventilation (Figures 9A–9C). Moreover, ventilation led to a higher degree of BAL protein and HO-1 expression in GW9662-treated mice (Table E1 and Figure E2). These findings show that the protective effect of CO was abolished when PPAR-γ was inhibited. However, even if cytokine production was not prevented by CO, which is in sharp contrast to noninhibited animals, total cytokine levels had the tendency to be lower in PPAR-γ–inhibited mice.

DISCUSSION

In the current study, we have demonstrated that mechanical ventilation at a moderate tidal volume (12 ml/kg) caused a significant and time-dependent inflammatory response, reflected by the infiltration of macrophages and neutrophils into the airways, as well as the increased production of cytokines and chemokines. Egr-1 was up-regulated within 1 hour after the onset of ventilation. The inflammatory response during ventilation depended on Egr-1 expression, because it was dramatically attenuated in *egr-1*$^{-/-}$ mice. Treatment with CO completely inhibited lung injury as well as attenuated the proinflammatory

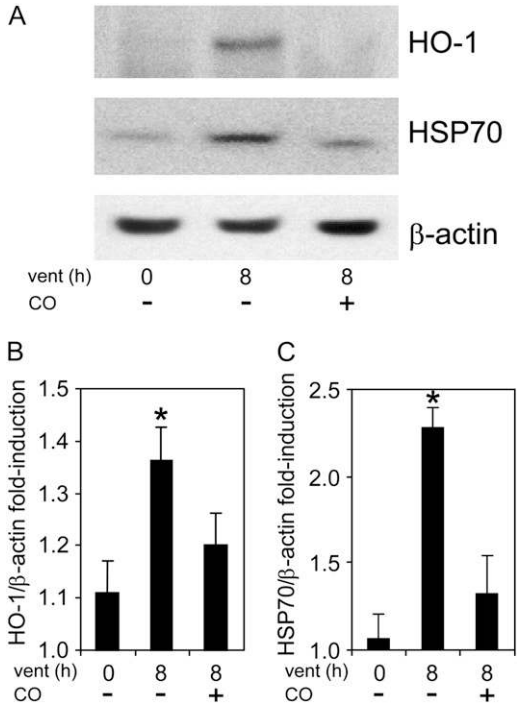

Figure 3. Effect of CO on heme oxygenase (HO)-1 and heat shock protein-70 (HSP70). Lung samples were taken from mice without ventilation (control), with ventilation for 8 hours, and with ventilation for 8 hours in the presence of 250 ppm CO. (*A*) Representative Western blots of HO-1 (*A, upper panel*) or HSP70 (*A, middle panel*). Membranes were stripped and immunoblotted with β-actin to ensure equal loading (*A, lower panel*). Densitometric analysis of all samples were corrected to β-actin and expressed as fold-induction for HO-1 (*B*) and HSP70 (*C*). Ventilation-induced HO-1 and HSP70 were prevented in the presence of CO. Data presentation and statistical analysis: n = 5/group; mean + SEM; ANOVA; $*P < 0.05$ versus control (0 h ventilation).

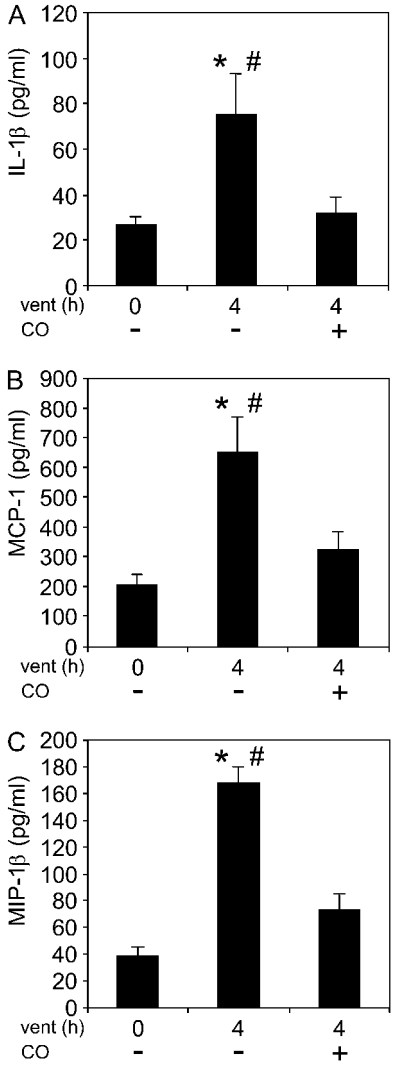

Figure 4. Effect of CO on ventilation-induced cyto- and chemokines. Samples were taken from lung tissue homogenates. (*A*) IL-1β, (*B*) monocyte chemotactic protein (MCP)-1, and (*C*) macrophage inflammatory protein (MIP)-1β were up-regulated in response to 4 hours of mechanical ventilation, which was prevented in the presence of CO (250 ppm). Data presentation and statistical analysis: n = 5/group; mean + SEM; ANOVA; $*P < 0.05$ versus control (0 h ventilation), $^{\#}P < 0.05$ versus time-matched CO-treated mice.

response to mechanical ventilation. The protection afforded by CO was associated with the down-regulation of Egr-1 protein expression and depended on an antiinflammatory transcriptional regulator, PPAR-γ.

We have recently described beneficial effects of CO administration against LPS-induced lung injury in ventilated rats (14). The data reported in this previous study could not be extrapolated directly to the mechanism of VILI or to the specific effects that CO exerts during mechanical ventilation. Animals were pretreated with LPS to model proinflammatory conditions, rendering it difficult to distinguish between LPS-mediated and ventilation-mediated consequences. In addition, rats were ventilated with high tidal volumes (26 ml/kg), which are rarely used in clinical settings. To address CO-dependent effects in a clinically relevant model and to explore their underlying mechanism(s), we established a mouse model using a tidal volume routinely used in clinical practice (12 ml/kg), which allowed us to ventilate animals up to 8 hours without significant mortality.

In our current model, application of CO during ventilation dramatically reduced lung injury that was induced by mechanical ventilation. Specifically, CO prevented the appearance of histologic indices of lung injury, as observed by hematoxylin-and-eosin staining, including alveolar septal thickening, and the infiltration of proinflammatory cells. Moreover, the increase of BAL protein content associated with mechanical ventilation was completely abolished in the presence of CO, indicating reduced protein leakage and preservation of the alveolar–capillary barrier. Furthermore, the inclusion of CO during mechanical ventilation abolished increases in cell count and neutrophil fraction in the BAL fluid that were observed in response to mechanical ventilation alone.

In this context, we sought to determine the molecular mechanisms underlying the apparent protective effect of CO in this model. CO has been previously shown to activate several intracellular signaling pathways that have been associated with its cytoprotective actions (8). First, CO can act as a ligand for soluble guanylate cyclase, although apparently less potently than its cognate gas, nitric oxide, leading to activation of enzymatic activity and the increased production of cGMP (20, 21). Thus, CO can cause a cGMP-dependent relaxation of vascular and airway smooth muscle cells as well as bronchodilation *in vivo* (reviewed in Reference 8). Therefore, we investigated the effect of CO on airway pressure to evaluate whether its reduction by CO might be associated with the observed CO-mediated lung protection. But neither peak nor plateau airway pressure was influenced by CO treatment in our model. In contrast to airway pressure and as previously suggested by clinical studies (28), CO slightly but significantly lowered systemic blood pressure in ventilated animals. However, correlation analysis of blood pressure and inflammatory parameters (data not shown) revealed no interdependence between these values, rendering it unlikely that reduced blood pressure contributed to the protective effects of CO in this model.

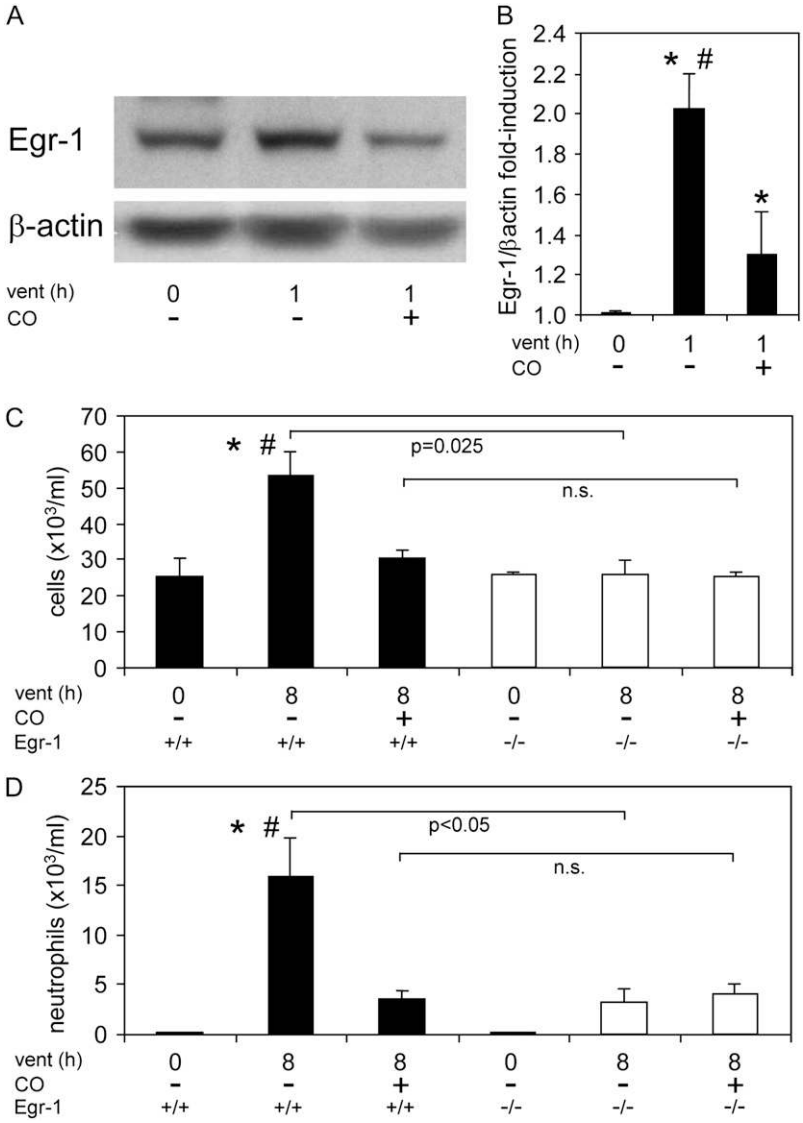

Figure 5. Role of early growth response (Egr)-1 gene in CO-mediated effects. (*A*) Representative Western blot of Egr-1. Lung samples were taken from mice without ventilation (control) or with ventilation for 1 hour ± CO (250 ppm) as indicated. Membranes were stripped and reprobed with β-actin. (*B*) Densitometric analysis of all samples were normalized to β-actin and expressed as fold-induction for Egr-1 (n = 5/group). Ventilation induced Egr-1 as early as after 1 hour, which was prevented in the presence of CO. (*C* and *D*) Total cell count and neutrophil count of bronchoalveolar lavage fluid of wild-type (*solid bars*) and Egr-1–deficient mice (*egr-1*$^{-/-}$; *open bars*) subjected to ventilation for 8 hours ± CO (250 ppm) (n = 3–5/group). Data presentation and statistical analysis: mean + SEM; (*B*) ANOVA; *$P < 0.05$ versus control (0 h ventilation), $^{\#}P < 0.05$ versus time-matched CO-treated mice; (*C*, *D*) Mann-Whitney rank sum test, *P* value as indicated. n.s. = not significant.

Second, the up-regulation of stress proteins such as HO-1 and HSP70 has been shown to limit inflammatory responses in many models (29–31). Moreover, *in vivo*, CO treatment has been shown to induce HO-1 in the liver (32) and HSP70 in the lung (25). The up-regulation of heat shock factor-1 and HSP70 expression by CO has been proposed to mediate CO-dependent antiinflammatory effects in a murine endotoxemia model. As our results suggest, both HO-1 and HSP70 are up-regulated in lung tissue in response to mechanical ventilation. In our current model, the application of CO during mechanical ventilation prevented the induction of both genes. These data are consistent with the role of HO-1 or HSP70 as inducible stress proteins that are up-regulated as a consequence of systemic stress and inflammatory tissue injury, and that remain at basal levels in the absence of cellular stress (reviewed in Reference 8). Therefore, we suggest that HO-1 and HSP70 appear as markers of cellular or organ injury whose expression is precluded by antiinflammatory protection afforded by exogenous CO treatment. We suggest that CO prevented tissue injury in this model by the early down-regulation of inflammatory regulators, rather than by the secondary regulation of stress-protein gene expression.

It is well established that production of cytokines and chemokines is significantly augmented during the development of VILI (33). Ventilation alone led to an increased cytokine and chemokine production as reflected by induced IL-1β, MIP-1β, or MCP-1. These proinflammatory mediators have been described not only to attract neutrophils into the lung (reviewed in Reference 34) but to severely exacerbate VILI (24). Early release of cytokines in response to lung stretch has been displayed in previous studies using high tidal volumes representing overventilation *in vivo* or *ex vivo* (24, 35, 36). As evidenced by our current data, even lower tidal volumes (e.g., 12 ml/kg), as used in our experiments, provoke a significant early proinflammatory response.

We have previously shown that CO exerts major antiinflammatory effects by down-regulating the production of cytokines in response to proinflammatory stimuli in macrophage cell culture (17). In the current study, CO application reduced the production of IL-1β, MIP-1β, or MCP-1 during mechanical ventilation. Interestingly, CO exerted this antiinflammatory effect relatively early within the course of ventilation—that is, after 4 hours.

The production of cytokines or chemokines by macrophages or other inflammatory effector cells is transcriptionally regulated. Because CO abolished early cytokine and chemokine release in the lung, we hypothesized that CO might act via the

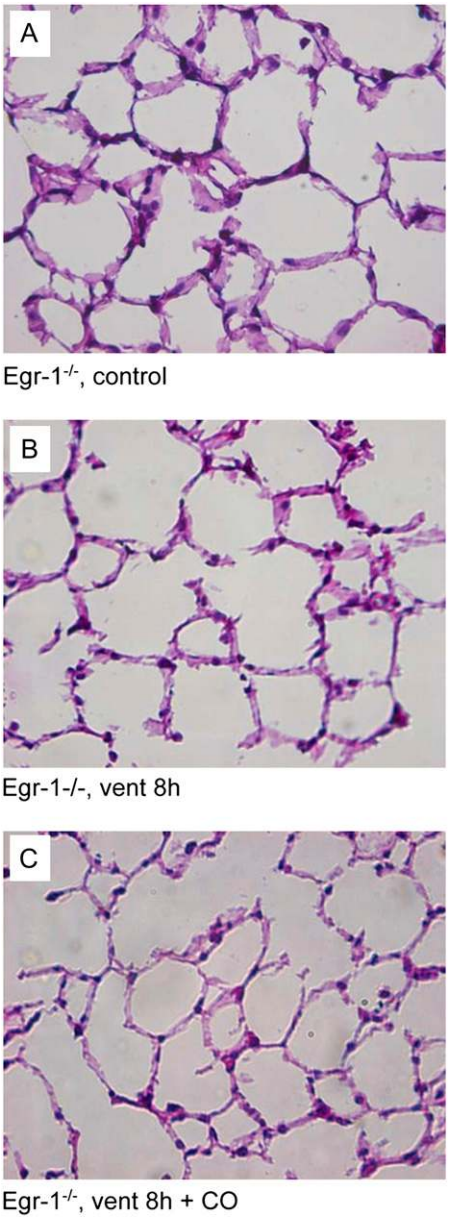

Figure 6. Effect of ventilation and CO on lung injury in early growth response (Egr)-1 gene–deficient mice (*egr-1*$^{-/-}$). Left lung lobes were taken from animals without ventilation (*A*), with ventilation for 8 hours (*B*), and with ventilation + CO (250 ppm) for 8 hours (*C*). Lung sections were stained with hematoxylin and eosin and presented in ×400 original magnification. As compared with control (*A*), neither ventilation (*B*) nor ventilation in the presence of CO (*C*) changed the appearance of lung tissue.

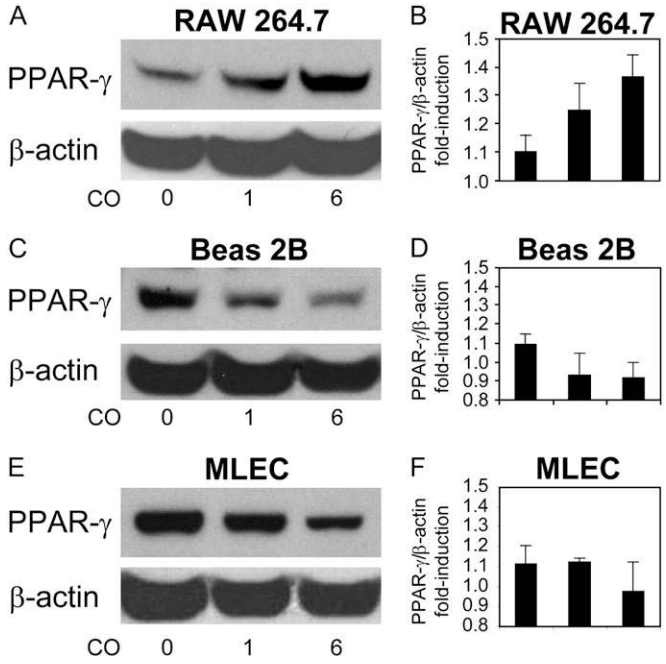

Figure 7. Effect of CO on peroxisome proliferator-activated receptor (PPAR)-γ expression. RAW 264.7 cells, Beas2B cells, and murine primary lung endothelial cells (MLECs) were incubated for 1 or 6 hours with CO. (*A*, *C*, *E*) Representative Western blots of PPAR-γ. Membranes were stripped and reprobed with β-actin. (*B*, *D*, *F*) Densitometric analysis of all samples were normalized to β-actin and expressed as fold-induction for PPAR-γ. CO incubation led to a time-dependent increase of PPAR-γ expression in macrophages (RAW 264.7 cells; *A*, *B*) but not in lung epithelial cells (Beas2B; *C*, *D*) or in MLECs (*E*, *F*). Experiments were repeated three times independently.

modulation of transcriptional regulators. Egr-1, a zinc-finger transcription factor, represents an important proinflammatory transcriptional regulator that coordinates proinflammatory responses in various cell types, including macrophages (26). In response to activation by ischemic stress, Egr-1 propagates deleterious responses leading to injury by regulating critical genes involved in the regulation of inflammation, coagulation, and vascular permeability (26). Our current results demonstrate that Egr-1 is highly up-regulated in the lung as early as after 1 hour after the onset of mechanical ventilation. Egr-1 induction due to mechanical stretch has been described recently in an *in vitro* cellular stretch model (37), and *in vivo* during overventilation (24). Moreover, recent studies have described the modulation of Egr-1 by CO in which LPS application or ischemia/reperfusion led to an up-regulation of Egr-1 in the lung, which was prevented by pretreatment with CO (27, 38). Interestingly, we found that the inclusion of CO at the onset of and concurrently during ventilation, in the absence of pretreatment, prevented Egr-1 up-regulation in the mouse lung. To our knowledge, the functional role of Egr-1 has never been described in the context of VILI. We demonstrate for the first time that *egr-1*$^{-/-}$ mice did not develop pulmonary inflammation in response to mechanical ventilation, and were resistant to VILI relative to ventilated wild-type mice. These data demonstrate that Egr-1 acts as an important factor in the formation of VILI. Our data are further supported by recent studies demonstrating the crucial role of Egr-1 in ischemia/reperfusion injury (38). The *egr-1*$^{-/-}$ mice displayed a marked reduction of injury after temporal ligation of the pulmonary artery followed by reperfusion. Furthermore, and in agreement with our current observations, administration of CO to *egr-1*$^{-/-}$ mice had no further effect on organ injury, most likely because the degree of injury in *egr-1*$^{-/-}$ was almost negligible and comparable to control animals. It is important to note that macrophage and neutrophil development in *egr-1*$^{-/-}$ mice might genuinely be altered (39, 40). Considering this phenotype, the data on *egr-1*$^{-/-}$ support the important role of neutrophil and macrophage infiltration for the development of VILI, which is reduced by the limitation of these proinflammatory cells during CO inhalation.

Although Egr-1 acts as a regulator of proinflammatory gene expression in macrophages, PPAR-γ counteracts Egr-1 to pro-

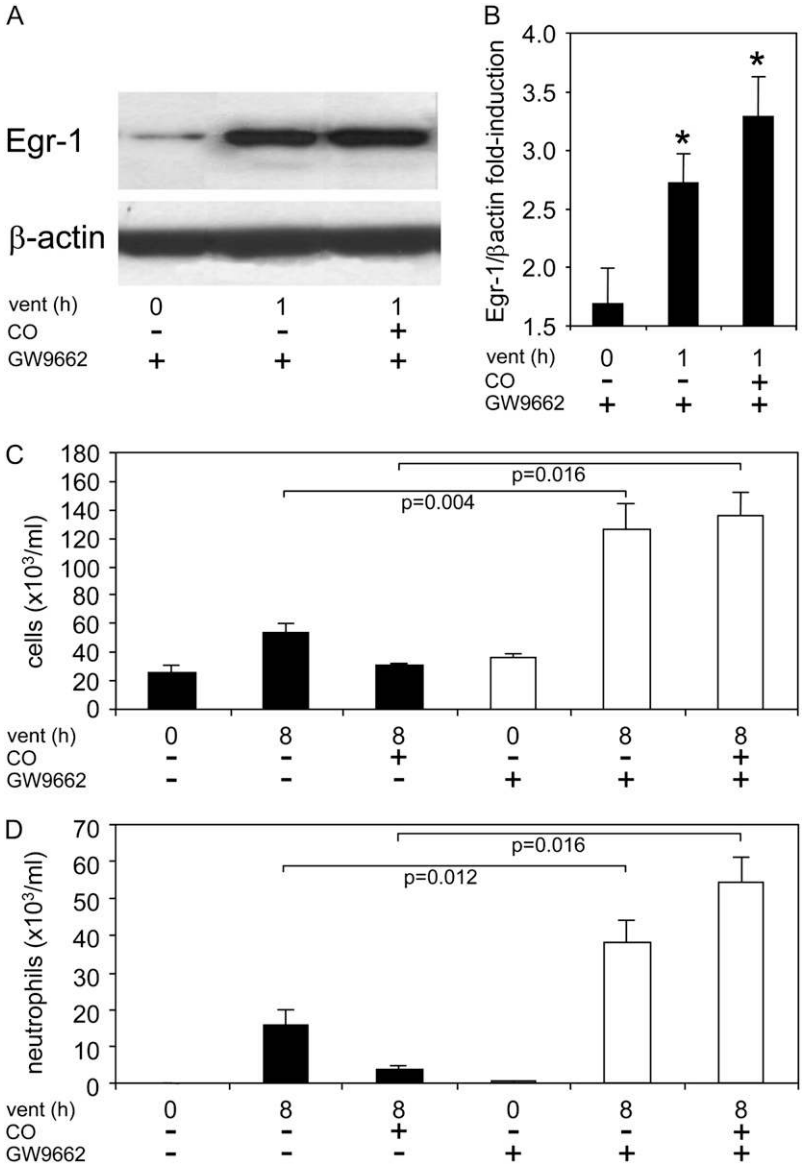

Figure 8. Role of peroxisome proliferator activated receptor (PPAR)-γ in CO-mediated effects. Lung samples and bronchoalveolar lavage (BAL) fluid were taken from mice without pretreatment (*solid bars*) or pretreated with GW9662 (*open bars*), a specific PPAR-γ inhibitor, and subjected to ventilation for 8 hours ± CO (250 ppm). Nonventilated animals served as controls. (*A*) Representative Western blot of ealry growth response (Egr)-1 (*A, upper panel*). Membrane was stripped and reprobed with β-actin (*A, lower panel*). (*B*) Densitometric analysis of all samples were normalized to β-actin and expressed as fold-induction for Egr-1. (*C*) Total cell count and (*D*) neutrophil fraction of the total cell count were measured in the BAL fluid. Ventilation led to a more pronounced increase in total cell and neutrophil count as compared with non-pretreated animals, which could not be prevented by the simultaneous application of CO. Data presentation and statistical analysis: n = 4–5/group; mean + SEM; *t* test and rank sum test, *P* value as indicated.

vide an antiinflammatory transcriptional control mechanism (reviewed in References 41 and 42). For instance, PPAR-γ plays a major antiinflammatory role by inhibiting Egr-1 expression after ischemia/reperfusion (43).

Furthermore, a recent study revealed that activation of PPAR-γ after a transient increase in reactive oxygen species production accounted for the antiinflammatory effect of CO in cultured murine RAW 264.7 macrophages (27). Because the role of PPAR-γ in VILI is unknown, we tested the hypothesis that CO mediates its effects via PPAR-γ. First, we confirmed that CO induces PPAR-γ in macrophages in a time-dependent manner, and that this effect was absent in cultured endothelial or epithelial cell lines. To investigate the functional role of PPAR-γ in CO-mediated lung protection, mice were pretreated with GW9662 and ventilated in the presence or absence of CO. GW9662 represents an irreversible inhibitor of PPAR-γ that is 10- to 600-fold less potent in binding PPAR-α or PPAR-δ. As our results demonstrate, the protective effects of CO in our VILI model were abolished when PPAR-γ was inhibited. These results establish an antiinflammatory role for PPAR-γ in VILI and indicate that CO-dependent tissue protection was mediated by PPAR-γ. Most interesting, agonistic pharmacologic approaches that increase PPAR-γ activity have been shown to ameliorate lung injury in various models, such as ischemia/reperfusion (43), systemic inflammation (44, 45), or hyperoxia (46). For example, PPAR-γ agonists 15-deoxy-$\Delta^{-12,14}$-prostaglandin-J_2 and rosiglitazone can suppress Egr-1 protein expression and DNA binding activity. Whether the pharamacologic activation of PPAR-γ with such compounds would be effective at reducing VILI remains unknown and warrants further investigation.

Many of the cytoprotective effects of CO have been shown to depend on activation of p38 mitogen-activated protein kinase (p38 MAPK) (17). Previously, we have shown that the protective effect of CO in LPS-mediated VILI was associated with p38 MAPK activation (14). We have also recently shown that p38 and other MAPKs may play a functional role in the initiation and propagation of VILI (47). Although we did not test this directly, we speculate that p38 MAPK may regulate PPAR-γ induction by CO, because p38 MAPK has been previously shown to regulate PPAR-γ in lung cell lines (48, 49).

On the basis of our current findings, we conclude that the application of CO during mechanical ventilation protects against the development of VILI through the down-regulation

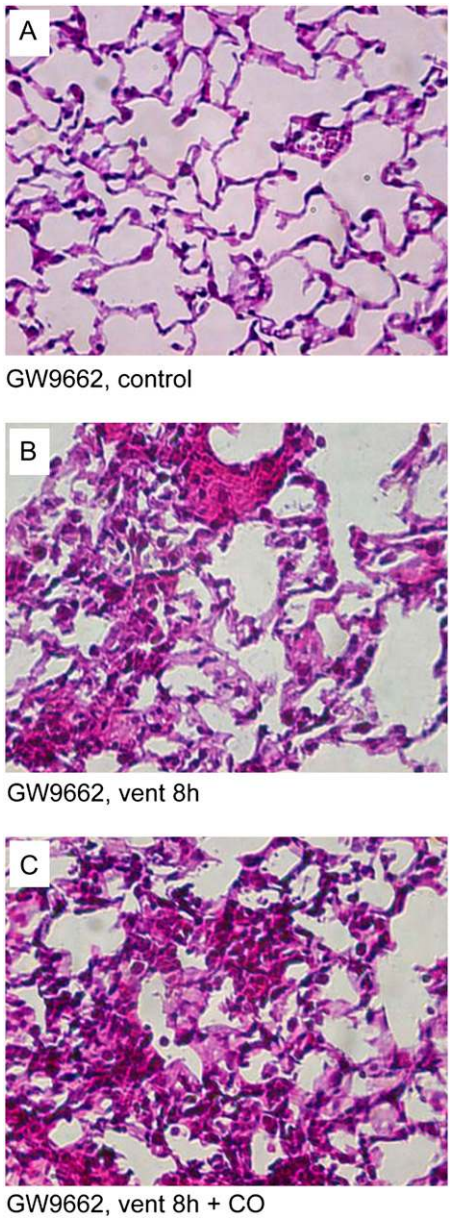

Figure 9. Effect of ventilation and CO on lung injury in GW9662-treated mice. Left lung lobes were taken from animals without ventilation (*A*), with ventilation for 8 hours (*B*), and with ventilation + CO (250 ppm) for 8 hours (*C*). Lung sections were stained with hematoxylin and eosin and presented in ×400 original magnification. As compared with control (*A*), ventilation alone (*B*) as well as in the presence of CO (*C*) led to significant lung injury, including thickening of alveolar walls, hemorrhage, and cellular infiltration.

of inflammatory responses. This protection afforded by CO is manifest in the reduction of cytokine/chemokine production and infiltration of macrophages as well as of neutrophils into the lung. Furthermore, the antiinflammatory effects of CO during ventilation are mediated via induction of PPAR-γ, thus preventing the up-regulation of the proinflammatory transcriptional regulator Egr-1. Thus, inhaled CO at low concentrations may represent a promising future therapeutic option for the reduction of VILI. Whether these and other protective effects of CO application observed in animal models of lung injury can be extrapolated to the treatment of patients is the subject of ongoing clinical trials (www.clinicaltrials.gov).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. The Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000;342:1301–1308.
2. Meade MO, Cook DJ, Guyatt GH, Slutsky AS, Arabi YM, Cooper DJ, Davies AR, Hand LE, Zhou Q, Thabane L, *et al.*; Lung Open Ventilation Study Investigators. Ventilation strategy using low tidal volumes, recruitment maneuvers, and high positive end-expiratory pressure for acute lung injury and acute respiratory distress syndrome: a randomized controlled trial. *JAMA* 2008;299:637–645.
3. Villar J, Kacmarek RM, Pérez-Méndez L, Aguirre-Jaime A. A high positive end-expiratory pressure, low tidal volume ventilatory strategy improves outcome in persistent acute respiratory distress syndrome: a randomized, controlled trial. *Crit Care Med* 2006;34:1311–1318.
4. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM. Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002;110:1703–1716.
5. Hoetzel A, Dolinay T, Schmidt R, Choi AMK, Ryter SW. Carbon monoxide in sepsis. *Antioxid Redox Signal* 2007;9:2013–2026.
6. Zegdi R, Perrin D, Burdin M, Boiteau R, Tenaillon A. Increased endogenous carbon monoxide production in severe sepsis. *Intensive Care Med* 2002;28:793–796.
7. Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase: characterization of the enzyme. *J Biol Chem* 1969;244:6388–6394.
8. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;86: 583–650.
9. Falk DJ, Deruisseau KC, Van Gammeren DL, Deering MA, Kavazis AN, Powers SK. Mechanical ventilation promotes redox status alterations in the diaphragm. *J Appl Physiol* 2006;101:1017–1024.
10. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 1989;86:99–103.
11. Akamatsu Y, Haga M, Tyagi S, Yamashita K, Graca-Souza AV, Ollinger R, Czismadia E, May GA, Ifedigbo E, Otterbein LE, *et al.* Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. *FASEB J* 2004;18: 771–772.
12. Motterlini R, Mann BE, Foresti R, Motterlini R, Mann BE, Foresti R. Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Investig Drugs* 2005;14:1305–1318.
13. Otterbein LE, Mantell LL, Choi AM. Carbon monoxide provides protection against hyperoxic lung injury. *Am J Physiol* 1999;276:L688–L694.
14. Dolinay T, Szilasi M, Liu M, Choi AM. Inhaled carbon monoxide confers antiinflammatory effects against ventilator-induced lung injury. *Am J Respir Crit Care Med* 2004;170:613–620.
15. Hoetzel A, Zhang Y, Choi AM. Carbon monoxide attenuates ventilator induced lung injury [abstract]. *Am J Respir Crit Care Med* 2008;175: A434.
16. Hoetzel A, Choi AM. The protective role of carbon monoxide in ventilator induced lung injury. Presented at the 2007 Annual Meeting of the European Society of Anesthesiology, Munich.
17. Otterbein LE, Bach FH, Alam J, Soares M, Lu HT, Wysk M, Davis RJ, Flavell RA, Choi AMK. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000;6:422–428.
18. Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X, *et al.* Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am J Respir Cell Mol Biol* 2007;36:409–417.
19. Wang X, Wang Y, Kim HP, Nakahira K, Ryter SW, Choi AM. Carbon monoxide protects against hyperoxia-induced endothelial cell apoptosis by inhibiting reactive oxygen species formation. *J Biol Chem* 2007;282:1718–1726.
20. Furchgott RF, Jothianandan D. Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* 1991;28:52–61.

21. Durante W, Johnson FK, Johnson RA. Role of carbon monoxide in cardiovascular function. *J Cell Mol Med* 2006;10:672–686.
22. Cardell LO, Lou YP, Takeyama K, Ueki IF, Lausier J, Nadel JA. Carbon monoxide, a cyclic GMP-related messenger, involved in hypoxic bronchodilation in vivo. *Pulm Pharmacol Ther* 1998;11:309–315.
23. Ameredes BT, Otterbein LE, Kohut LK, Gligonic AL, Calhoun WJ, Choi AM. Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. *Am J Physiol* 2003;285:L1270–L1276.
24. Copland IB, Kavanagh BP, Engelberts D, McKerlie C, Belik J, Post M. Early changes in lung gene expression due to high tidal volume. *Am J Respir Crit Care Med* 2003;168:1051–1059.
25. Kim HP, Wang X, Zhang J, Suh GY, Benjamin IJ, Ryter SW, Choi AM. Heat shock protein-70 mediates the cytoprotective effect of carbon monoxide: involvement of p38beta MAPK and heat shock factor-1. *J Immunol* 2005;175:2622–2629.
26. Yan SF, Fujita T, Lu J, Okada K, Shan ZY, Mackman N, Pinsky DJ, Stern DM. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat Med* 2000;6:1355–1361.
27. Bilban M, Bach FH, Otterbein SL, Ifedigbo E, de Costa DJ, Esterbauer H, Chin BY, Usheva A, Robson SC, Wagner O, *et al.* Carbon monoxide orchestrates a protective response through PPARgamma. *Immunity* 2006;24:601–610.
28. van Bel F, Latour V, Vreman HJ, Wong RJ, Stevenson DK, Steendijk P, Egberts J, Krediet TG. Is carbon monoxide-mediated cyclic guanosine monophosphate production responsible for low blood pressure in neonatal respiratory distress syndrome? *J Appl Physiol* 2005;98:1044–1049.
29. Hashiba T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Tsuburai T, Matsuse T, Ishigatubo Y. Adenovirus-mediated transfer of heme oxygenase-1 cDNA attenuates severe lung injury induced by the influenza virus in mice. *Gene Ther* 2001;8:1499–1507.
30. Hayashi Y, Sawa Y, Fukuyama N, Nakazawa H, Matsuda H. Preoperative glutamine administration induces heat-shock protein 70 expression and attenuates cardiopulmonary bypass-induced inflammatory response by regulating nitric oxide synthase activity. *Circulation* 2002;106:2601–2607.
31. Singleton KD, Wischmeyer PE. Glutamine's protection against sepsis and lung injury is dependent on heat shock protein 70 expression. *Am J Physiol* 2007;292:R1839–R1845.
32. Zuckerbraun BS, Billiar TR, Otterbein SL, Kim PK, Liu F, Choi AM, Bach FH, Otterbein LE. Carbon monoxide protects against liver failure through nitric oxide-induced heme oxygenase 1. *J Exp Med* 2003;198:1707–1716.
33. Uhlig U, Fehrenbach H, Lachmann RA, Goldmann T, Lachmann B, Vollmer E, Uhlig S. Phosphoinositide 3-OH kinase inhibition prevents ventilation-induced lung cell activation. *Am J Respir Crit Care Med* 2004;169:201–208.
34. Reutershan J, Ley K. Bench-to-bedside review: acute respiratory distress syndrome—how neutrophils migrate into the lung. *Crit Care* 2004;8:453–461.
35. Held HD, Boettcher S, Hamann L, Uhlig S. Ventilation-induced chemokine and cytokine release is associated with activation of nuclear factor-κB and is blocked by steroids. *Am J Respir Crit Care Med* 2001;163:711–716.
36. Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS. Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model. *J Clin Invest* 1997;99:944–952.
37. Copland IB, Post M. Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. *J Cell Physiol* 2007;210:133–143.
38. Mishra S, Fujita T, Lama VN, Nam D, Liao H, Okada M, Minamoto K, Yoshikawa Y, Harada H, Pinsky DJ. Carbon monoxide rescues ischemic lungs by interrupting MAPK-driven expression of early growth response 1 gene and its downstream target genes. *Proc Natl Acad Sci USA* 2006;103:5191–5196.
39. Friedman AD. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* 2002;21:3377–3390.
40. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, Gantner BN, Dinner AR, Singh H. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 2006;126:755–766.
41. Standiford TJ, Keshamouni VG, Reddy RC. Peroxisome proliferator-activated receptor-γ as a regulator of lung inflammation and repair. *Proc Am Thorac Soc* 2005;2:226–231.
42. Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* 2001;169:453–459.
43. Okada M, Yan SF, Pinsky DJ. Peroxisome proliferator-activated receptor-gamma (PPAR-gamma) activation suppresses ischemic induction of Egr-1 and its inflammatory gene targets. *FASEB J* 2002;16:1861–1868.
44. Zingarelli B, Sheehan M, Hake PW, O'Connor M, Denenberg A, Cook JA. Peroxisome proliferator activator receptor-gamma ligands, 15-deoxy-Delta(12,14)-prostaglandin J2 and ciglitazone, reduce systemic inflammation in polymicrobial sepsis by modulation of signal transduction pathways. *J Immunol* 2003;171:6827–6837.
45. Liu D, Zeng BX, Zhang SH, Wang YL, Zeng L, Geng ZL, Zhang SF. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, reduces acute lung injury in endotoxemic rats. *Crit Care Med* 2005;33:2309–2316.
46. Rehan VK, Wang Y, Patel S, Santos J, Torday JS. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, prevents hyperoxia-induced neonatal rat lung injury in vivo. *Pediatr Pulmonol* 2006;41:558–569.
47. Dolinay T, Wu W, Kaminski N, Ifedigbo E, Kaynar AM, Szilasi M, Watkins SC, Ryter SW, Hoetzel A, Choi AMK. Mitogen-activated protein kinases regulate susceptibility to ventilator-induced lung injury. *PloS ONE* 2008;3:e1601.
48. Li M, Lee TW, Mok TS, Warner TD, Yim AP, Chen GG. Activation of peroxisome proliferator-activated receptor-gamma by troglitazone (TGZ) inhibits human lung cell growth. *J Cell Biochem* 2005;96:760–774.
49. Azuma Y, Watanabe K, Date M, Daito M, Ohura K. Induction of proliferation by 15-deoxy-delta12,14-prostaglandin J2 and the precursors in monocytic leukemia U937. *Pharmacology* 2004;71:181–191.