Kinetic effects of carbon monoxide inhalation on tissue protection in ventilator-induced lung injury

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Mechanical ventilation causes ventilator-induced lung injury (VILI), and contributes to acute lung injury/acute respiratory distress syndrome (ALI/ARDS), a disease with high morbidity and mortality among critically ill patients. Carbon monoxide (CO) can confer lung protective effects during mechanical ventilation. This study investigates the time dependency of CO therapy with respect to lung protection in animals subjected to mechanical ventilation. For this purpose, mice were ventilated with a tidal volume of 12 ml/kg body weight for 6 h with air in the absence or presence of CO (250 parts per million). Histological analysis of lung tissue sections was used to determine alveolar wall thickening and the degree of lung damage by VILI score. Bronchoalveolar lavage fluid was analyzed for total cellular influx, neutrophil accumulation, and interleukin-1 β release. As the main results, mechanical ventilation induced pulmonary edema, cytokine release, and neutrophil recruitment. In contrast, application of CO for 6 h prevented VILI. Although CO application for 3 h followed by 3-h air ventilation failed to prevent lung injury, a further reduction of CO application time to 1 h in this setting provided sufficient protection. Pre-treatment of animals with inhaled CO for 1 h before ventilation showed no beneficial effect. Delayed application of CO beginning at 3 or 5 h after initiation of ventilation, reduced lung damage, total cell influx, and neutrophil accumulation. In conclusion, administration of CO for 6 h protected against VILI. Identical protective effects were achieved by limiting the administration of CO to the first hour of ventilation. Pre-treatment with CO had no impact on VILI. In contrast, delayed application of CO led to anti-inflammatory effects with time-dependent reduction in tissue protection.

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Mechanical ventilation is commonly used as a supportive strategy in critical care medicine. Unfortunately, mechanical ventilation can contribute to the development of acute lung injury/acute respiratory distress syndrome (ALI/ARDS). Modification of ventilator settings have improved the clinical outcome of patients suffering from ARDS.^{1,2} Unfortunately and despite the implementation of protective ventilatory strategies over the last decade, mechanical ventilation still may lead to ventilator-induced lung injury (VILI) or aggravate pre-existing lung injury. Hence, the current high morbidity and mortality among ALI/ARDS patients still urgently requires the development of additional strategies that would minimize the risk for VILI.

VILI is characterized by a series of biotraumatic events. Depending on the local force in the lung as well as any pre-existing vulnerability of this organ, mechanical stretching of

lung tissue caused by mechanical ventilation can lead to the disruption of the alveolar–capillary barrier, pulmonary edema formation, increased oxidative stress, and significant inflammatory responses. Key features of inflammation subsequent to mechanical ventilation include the influx of immune-competent cells, in particular neutrophils, and the release of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) or macrophage inflammatory protein-1.^{3,4} Subsequently, local lung inflammation may also affect other organs, leading to the development of multiple organ failure.²

The development of strategies to limit or even prevent the inflammatory response to mechanical ventilation, including experimental therapies using small gaseous molecules such as carbon monoxide (CO), have gained considerable interest. CO is an odorless, low-molecular-weight gas, which is well known for its toxic properties at high concentrations.^{5,6} However, CO

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can evolve endogenously, mainly as the product of heme degradation catalyzed by heme oxygenase (HO; E.C. 1:14:99:3) enzymes.^{7–9} Upregulation of the inducible isoform of HO, HO-1, limits pro-inflammatory processes, in part through the generation of CO.^{10,11} In fact, exogenous application of CO can mimic the protective effects of HO-1 upregulation, even when this enzyme activity was chemically blocked.^{12,13} Moreover, treatment with inhaled CO or CO-releasing molecules (CORMs) prevented ischemia–reperfusion injury,^{14–16} hyperoxia-induced injury,^{17,18} sepsis,^{19,20} or organ transplant rejection^{21–24} in several *in vivo* and *in vitro* models. Recently, we and others could demonstrate that inhalation of CO at low concentration limits the inflammatory response upon mechanical ventilation, thus preventing VILI in these animals.^{25–27} With respect to the mechanisms underlying organ protection by CO, multiple signaling pathways have been described, including mitogen-activated protein kinases (MAPKs), caveolin-1, early growth response gene-1 (Egr-1), peroxisome proliferator-activated receptor- γ , heat shock proteins, and others.^{26,27}

Based on the fact that many laboratory reports demonstrated dose–response relationships in the mechanisms of CO action, it is somewhat surprising that currently little is known about the time dependency of CO administration. Regarding potential future clinical implications and potential side effects of CO application, it is rather important to define the kinetic aspects of CO-dependent cytoprotection, its efficacy as a pre-treatment option, and its usefulness as a post-injury therapeutic. In the current studies, we determine the essential role of timing for CO-mediated lung protection in a mouse model of VILI.

MATERIALS AND METHODS

Animals

Male C57BL/6N mice were obtained from Charles River Laboratories (Sulzburg, Germany) and used at a body weight of 22–25 g. All animal experiments were performed in accordance with the guidelines of the local animal care commission (ethics committee of the University of Freiburg, permission No. G-07/25). All mice were anesthetized with ketamine (90 mg/kg, intraperitoneal (i.p.)), and acepromazine (0.9 mg/kg, i.p.) and placed on a 36.5 °C warm heating pad to maintain normal body temperature (measured with a rectal thermometer). A polyethylene catheter was inserted into the left carotid artery for direct blood pressure monitoring as well as for blood gas sampling, and a tracheotomy was established using a 20-gauge catheter. While non-ventilated control mice were subjected to the instrumentation after 6 h spontaneous breathing room air and being sacrificed immediately after, mice randomized to receive mechanical ventilation, were connected to a rodent ventilator (Voltek Enterprises, Toronto, Canada) via a tracheal cannula and ventilated with synthetic air. In the case of CO supplementation (Air Liquide, Kornwestheim, Germany), a concentration of 250 parts per million (p.p.m.) was used and continuously monitored during ventilation. The ventilator was set to a tidal volume of

12 ml/kg body weight, frequency 80–90/min, and positive end-expiratory pressure of 2 cmH₂O. Muscular relaxation was achieved by applying pancuronium (2 mg/kg, i.p.). Anesthesia was maintained by continuous administration of ketamine, acepromazine, and pancuronium i.p. as needed. A 0.7-ml saline bolus was injected i.p. to compensate for evaporation during ventilation. Blood samples were withdrawn from ventilated animals after 30–45 min and pH, PaCO₂, PaO₂, and COHb were measured using an automated blood gas analyzer (ABL600/800, Radiometer, Copenhagen, Denmark) to ensure normal ventilation and without showing any differences between treatment groups regarding pH, PaO₂, and PaCO₂ (data not shown). Another blood sample was taken after 6 h at the end of ventilation and blood gases and COHb were again analyzed. Recruitment maneuvers (inspiratory hold on 30 cmH₂O for 5 s) were performed every 60 min during ventilation to prevent atelectasis. Body temperature (T_b (°C)), arterial blood pressure (mmHg), peak airway pressure and plateau airway pressure (cmH₂O), were continuously monitored (PowerLab 3/80, ADInstruments, Spechbach, Germany) and recorded every 30 min. Total respiratory system compliance was calculated as described previously.²⁸ Tidal volume was divided by the difference between the end-inspiratory and end-expiratory plateau pressures for each time point recorded.

Experimental Groups

The study consisted of four independent sets of experiments, $n = 6–7$ /group (Figure 1). (I) First set of experiments (Figure 1a): non-ventilated control mice were allowed to breathe air spontaneously. Mice receiving mechanical ventilation were randomized to ventilation with either synthetic air or synthetic air supplemented with 250 p.p.m. CO for 6 h. (II) Second set of experiments (Figure 1b): non-ventilated control mice were allowed to breathe air spontaneously. Mechanically ventilated mice were randomized to either ventilation with synthetic air for 6 h; ventilation with CO for the first hour, followed by ventilation with air for 5 h, ventilation with CO for 3 h followed by ventilation with air for 3 h; or ventilation with CO for 6 h. (III) Third set of experiments (Figure 1c): non-ventilated control mice were allowed to breathe air spontaneously. Mechanically ventilated mice were randomized to ventilation with synthetic air for 6 h with spontaneous breathing of air or 250 p.p.m. CO in a sealed chamber for 1 h before ventilation; or ventilation with CO for 6 h. (IV) Fourth set of experiments (Figure 1d): non-ventilated control mice were allowed to breathe air spontaneously. Mechanically ventilated mice were randomized to ventilation with synthetic air for 6 h; ventilation with air for 5 h followed by 1-h ventilation with CO; ventilation with air for 3 h followed by 3-h ventilation with CO; or ventilation with CO for 6 h. In addition, ventilation was performed in another five groups. Here, CO treatment was started as late as 5 h after the onset of ventilation, and total experimental time was extended to 8 or 10 h (Supplementary Figure 1).

Figure 1 Experimental setting and groups. Mice were instrumented but not ventilated (control), ventilated with 12 ml/kg for 6 h with either synthetic air (vent) or synthetic air + 250 p.p.m. carbon monoxide (vent + CO). (a–d) The individual experimental series are depicted. The start and end of CO application for each series is indicated.

Tissue Sampling and Bronchoalveolar Lavage

At the end of each experiment, mice were sacrificed. Blood was withdrawn from the left carotid artery and serum samples were frozen and stored at -80°C . Tissue samples were snap frozen and stored at -80°C for subsequent analysis. First, the left lung lobe was ligated and a bronchoalveolar lavage (BAL) was performed via the tracheal catheter in the right lung lobe using 0.8 ml phosphate-buffered saline. The recovered volume was centrifuged and the supernatant was snap frozen and stored at -80°C until further use. The pellet was re-dissolved, total cells were counted under the light microscope, and the relative amount of neutrophils was determined from methanol-fixed cytospin slides, stained with fast green, eosin and thiazine (Diff-Quick, Medion Diagnostics AG, Düringen, Switzerland). Following the BAL, the right lobes were removed, snap frozen and kept until further use at -80°C . Afterwards, the left bronchus was re-opened and optimal cutting temperature compound (Tissue-Tek, Sikura Fintek GmbH, Staufen, Germany) was mixed with phosphate-buffered saline in a 1:3 ratio, and administered with a constant pressure of 20 cmH₂O via the tracheal catheter to unfold the lung lobe. Then, the lobe was extracted and embedded into

optimal cutting temperature compound, frozen in liquid nitrogen and kept at -80°C until further use.

Measurements of Cytokines and Myeloperoxidase

BAL aliquots were analyzed using IL-1 β ELISA kits (R&D Systems GmbH, Wiesbaden, Germany) according to the manufacturer's instructions. Serum samples were tested for both IL-1 β and myeloperoxidase (MPO) glycoprotein (HK210 ELISA, Hycult Biotech GmbH, Beutelsbach, Germany) according to the manufacturer's instructions.

Histological Examination

Cryosections (12 μm) of the left lung were subjected to hematoxylin and eosin (H + E) staining. From each lung, four representative photos were taken (magnification $\times 400$). Five high power fields were randomly assigned to each photo and alveolar wall thickness was analyzed by Axiovision software (AxioVS40LE, Zeiss, Jena, Germany). In each high power field, the degree of lung damage was determined by a modified VILI score:²⁹ (a) thickness of the alveolar walls, (b) infiltration or aggregation of inflammatory cells, and (c) hemorrhage. Each item was graded in a blinded fashion according to the following five-point scale: 0: minimal damage, 1: mild damage, 2: moderate damage, 3: severe damage, 4: maximal damage. The degree of lung damage was assessed by the sum of scores ranging from 0 to 12 for each high power field. The average of the sum of each field score per lung was compared among groups.

Statistical Analysis

Graphs represent mean values + standard error of means (s.e.m.). Data were further analyzed using the one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls *post hoc* test. In case of two group comparisons, the Student's *t*-test or the Mann–Whitney Rank Sum test was used (Sigmastat statistical software, Synstat, Erkrath, Germany). $P < 0.05$ was considered significant. In case of an unpaired Student's *t*-test or ANOVA followed by a Bonferroni-correction, $P < 0.007$ was considered significant.

RESULTS

Effect of Ventilation and CO on Lung Function

Since lung function during ventilation is critical for the development of VILI, we first analyzed physiological and blood gas parameters during ventilation (Figure 2). No differences could be detected between mice ventilated with air or with CO for 6 h with respect to body temperature, blood pressure, inspiratory peak and plateau pressure, pH, or PaCO₂ (Figure 2a). Although PaO₂ was alike in both groups at the start of ventilation (data not shown) and remained stable in air-ventilated mice, PaO₂ slightly decreased in CO-ventilated mice at the end of the ventilation period. COHb levels were increased in mice receiving CO treatment (Figure 2a). Total respiratory system compliance was slightly lower in CO-ventilated mice without reaching statistical significance (Figure 2b).

Figure 2 Effect of ventilation and carbon monoxide on lung function. Mice were ventilated with 12 ml/kg for 6 h with either synthetic air (air) or synthetic air + 250 p.p.m. carbon monoxide (CO) as indicated. Body temperature (T_b), mean arterial blood pressure (BP), peak airway pressure (P_{peak}), and plateau airway pressure (P_{plateau}) were continuously monitored and recorded every 30 min. Blood gas analysis and COHb measurements were performed at the end of the experiment (**a**). Total respiratory compliance was calculated every 30 min (**b**). Data represent mean values ± s.e.m. for *n* = 7/group. Student's *t*-test and Mann-Whitney Rank Sum test were used for statistical analyses, **P* < 0.05 vs 6-h air.

Effect of Ventilation and CO Inhalation on Lung Injury

Next, we examined the local effects of inhaled CO on the development of lung injury in mechanically ventilated mice. Hematoxylin and eosin-stained sections revealed that non-ventilated control mice displayed no histological signs of lung injury (Figure 3a). Ventilation with air for 6 h markedly increased alveolar wall thickness and cellular infiltration (Figure 3b). In contrast, the presence of CO (250 p.p.m.) during mechanical ventilation prevented lung injury (Figure 3c). Quantitative analysis of all three experimental groups showed a significant reduction of alveolar wall thickness (Figure 3d) and VILI score in CO-treated mice in comparison to air-ventilated animals (Figure 3e).

Effect of Ventilation and CO Inhalation on Lung and Systemic Inflammation

Mechanical ventilation elicits a strong pulmonary inflammation, exemplified by transmigration of inflammatory cells (eg, neutrophils), and the release of pro-inflammatory cytokines,

that has an essential role in the progression of VILI.^{3,4,30,31} While cellular infiltration into non-ventilated lungs was minimal, mechanical ventilation markedly increased the total cell number in the BAL fluid (Figure 4a), the relative number of neutrophils (Figure 4b), and IL-1 β release (Figure 4c). In contrast, the presence of CO during mechanical ventilation clearly reduced total cell count, neutrophil count, and IL-1 β content in the BAL fluid as compared with mechanical ventilation with air alone. In a next step, we analyzed the effect of CO inhalation on systemic pro-inflammatory factors. The MPO glycoprotein is known to be released from activated neutrophils (eg, during lung injury in mechanical ventilation).^{32–34} In our setting, mechanical ventilation clearly increased MPO in serum (Figure 4d). However, CO treatment had no effect on MPO levels. We also analyzed the release of the pro-inflammatory cytokine IL-1 β , revealing that neither mechanical ventilation nor CO inhalation had an impact on systemic IL-1 β release (Figure 4e).

Time-Dependent Effect of CO Application on Lung Function

No differences were detected between groups regarding body temperature, blood pressure, inspiratory peak pressure, plateau pressure, or pH (Supplementary Figure 1A). PaCO₂ was increased in mice treated with CO in the first hour of ventilation. PaO₂ values at the end of the experiment were lower in animals that were subjected to CO for 1 h or for 6 h. COHb levels were highest in mice receiving 6 h CO treatment (Supplementary Figure 1A). Total respiratory system compliance did not differ among groups (Supplementary Figure 1B).

Time-Dependent Effect of CO Application on Lung Injury

To determine the relationship between CO exposure time and lung protective effects, CO administration was initiated with the onset of mechanical ventilation, but time dependently discontinued. In contrast to non-ventilated control mice (Figure 5a), ventilation with air led to an increase in alveolar wall thickness and cellular infiltration (Figure 5b). Ventilation with CO for 1 h, followed by 5-h air ventilation, also decreased both histological signs of lung injury (Figure 5c). Surprisingly, ventilation with CO for 3 h and interruption of CO supplementation for the following 3 h, showed no impact on lung damage as compared with air ventilation alone (Figure 5d). Finally, ventilation with CO for 6 h without discontinuation led to a reduction of alveolar wall thickness and cellular infiltration comparable to 1-h CO exposure (Figure 5e). Quantification of alveolar wall thickness (Figure 5f) and assessment of the VILI score (Figure 5g) for all experimental groups confirmed that exposure to CO for 1 h as well as for 6 h led to an almost identical reduction in ventilation-mediated lung injury.

Time-Dependent Effect of CO Application on Lung Inflammation

In order to determine the time-dependent effects of CO inhalation on the inflammatory response to mechanical

Figure 3 Effect of ventilation and carbon monoxide treatment on lung injury. As controls, mice were allowed to spontaneously breathe air (a), or were ventilated with 12 ml/kg for 6 h with either synthetic air (b) or synthetic air + 250 p.p.m. carbon monoxide (CO) (c). Sections from the left lung lobe were stained by hematoxylin and eosin. Representative pictures are shown for each experimental group (magnification = $\times 400$, a–c). High power fields were randomly assigned to measure alveolar wall thickness (d) and ventilator-induced lung injury (VILI) score (e). Data represent mean values $\pm$ s.e.m. for $n = 7$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ vs control, § $P < 0.05$ vs 6-h CO-ventilated group.

ventilation, we analyzed total cell count, neutrophil infiltration, and IL-1 β release in the BAL fluid (Figure 6). Total cell counts of non-ventilated mice were minimal, whereas ventilation with air alone increased total cell counts (Figure 6a). The presence of CO (250 p.p.m.) for 1, 3, or 6 h reduced the number of infiltrating total cells. Regarding neutrophil cell infiltration, relative neutrophil counts were negligible in non-ventilated mice, whereas air ventilation led to a substantial elevation in neutrophil counts (Figure 6b). Both 1 and 6 h of CO treatment inhibited neutrophil influx. However, 3-h CO followed by 3-h air ventilation was insufficient to prevent neutrophil migration into the BAL fluid. The release of the cytokine IL-1 β was increased by air ventilation but inhibited by all other treatments, regardless of whether CO was applied for 1, 3, or 6 h (Figure 6c).

Effect of Pre-Treatment with CO on Lung Function

Body temperature, blood pressure, inspiratory peak pressure, and plateau pressure did not differ among groups (Supplementary Figure 2A). pH was slightly reduced in pre-treated mice, while PaCO₂ was increased, and PaO₂ was decreased in mice treated with CO at the end of ventilation. COHb levels were highest in mice receiving 6 h CO treatment

(Supplementary Figure 2A). Total respiratory system compliance did not differ among groups (Supplementary Figure 2B).

Effect of Pre-Treatment with CO on Lung Injury

Pre-treatment with CO has been previously shown to confer protective effects in several *in vivo* and *in vitro* models.^{20,35–40} Especially, the administration of 250 p.p.m. CO for 1 h before the onset of the deleterious treatment proved to exert beneficial effects.^{36,41–43} Therefore, we subjected mice to breathe 250 p.p.m. CO spontaneously for 1 h before instrumentation and mechanical ventilation for the following 6 h in the absence of CO. Representative histological slides in Figure 7 show normal lung architecture in non-ventilated control mice (Figure 7a) and lung damage in mice ventilated with air for 6 h, characterized by increased thickness of alveolar septa and cellular infiltration (Figure 7b). One hour of spontaneous inhalation of CO (250 p.p.m.) before mechanical ventilation had no impact on lung injury (Figure 7c). As an internal control group, ventilation with CO for 6 h without pre-treatment clearly reduced lung injury (Figure 7d). Quantification of alveolar thickness (Figure 7e) and VILI score (Figure 7f) confirmed the lack of lung protection when mice were pre-treated for 1 h with CO inhalation.

Figure 4 Effect of ventilation and carbon monoxide treatment on lung inflammation. As controls, mice were allowed to spontaneously breathe air, or were ventilated with 12 ml/kg for 6 h with either synthetic air or synthetic air + 250 p.p.m. carbon monoxide (CO). Bronchoalveolar lavage was performed in the right lung. Total cells were counted under a light microscope (a) and the relative amount of neutrophils (b) was determined by cytospin analysis. Interleukin-1 β (IL-1 β) contents were quantified by ELISA (c). Myeloperoxidase (MPO; d) and IL-1 β (e) release was determined in serum samples by ELISA. Graphs represent mean values $\pm$ s.e.m., $n = 7$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ vs control, § $P < 0.05$ vs 6-h CO-ventilated group.

Effect of Pre-Treatment with CO on Lung Inflammation

Similar results were obtained with respect to the effects of CO pre-treatment on the inflammatory response. While mechanical ventilation alone increased cell count (Figure 8a), neutrophil count (Figure 8b), and IL-1 β release (Figure 8c), no alterations in these parameters were observed with 1 h CO pre-treatment. In contrast, CO administration over 6 h reduced the inflammatory response as described above.

Effect of Delayed CO Treatment on Lung Function

There were no differences detectable between groups concerning body temperature, blood pressure, inspiratory peak pressure, plateau pressure, pH, or PaCO₂ (Supplementary Figure 3A). PaO₂ was slightly decreased in the 3-h air followed by 3-h CO-treated group at the end of ventilation. COHb levels were high in all groups receiving CO treatment (Supplementary Figure 3A). Total respiratory system compliance differed at two time points among groups (Supplementary Figure 3B).

Effect of Delayed CO Treatment on Lung Injury

The effects of delayed CO administration on VILI were evaluated (Figure 9). As compared with control animals (Figure 9a), ventilation with air alone for 6 h clearly induced alveolar wall thickening and cellular infiltration as signs of

lung injury (Figure 9b). The onset of CO application after 5 h of air ventilation failed to alter lung injury (Figure 9c). In contrast, administration of CO after 3 h of air ventilation reduced alveolar thickness (Figure 9d). The effect was even more pronounced if CO was administered at the start of mechanical ventilation (Figure 9e). Quantitative analysis of alveolar wall thickness (Figure 9f) as well as analysis of VILI score (Figure 9g) confirmed these results. Extension of total experimental time and extension of the ventilation period (Supplementary Figure 4) provided protective effects by CO application, even when CO was administered as late as 5 h after the onset of mechanical ventilation. This was true for 5-h air/3-h CO as compared with the time-matched air ventilation, and to a lesser extent in the 5-h air/5-h CO group (Supplementary Figure 5A–E). Although we only tested two animals per group, a trend towards reduction of lung damage as assessed by the means of decreased alveolar wall thickening and VILI score (data not shown) became evident for both time points (5-h air/3-h CO and 5-h air/5-h CO), when quantitatively analyzing the cryosections.

Effect of Delayed Treatment with CO on Lung Inflammation

With respect to the inflammatory response, total cell counts in BAL fluid (Figure 10a), neutrophil infiltration

Figure 5 Effect of minimizing carbon monoxide treatment on lung injury. As controls, mice were allowed to spontaneously breathe air (a), or were ventilated with 12 ml/kg for 6 h with synthetic air (b). Additionally, 250 p.p.m. carbon monoxide (CO) was applied for 1 (c), 3 (d), or 6 h (e), followed by ventilation with air alone for the remaining 5, 3, or 0 h of the experiment, as indicated. Sections from the left lung lobe were stained with hematoxylin and eosin. Representative pictures are shown for each experimental group (magnification = $\times 400$, a–e). High power fields were randomly assigned to measure alveolar wall thickness (f) and ventilator-induced lung injury (VILI) score (g). Data represent mean values $\pm$ s.e.m. for $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ vs control, # $P < 0.05$ vs 6-h CO-ventilated group, \$ $P < 0.05$ vs 1-h CO and 5-h air-ventilated group.

(Figure 10b), and IL-1 β content (Figure 10c) were increased in air-ventilated mice. Surprisingly, delaying CO administration (250 p.p.m.) for 5 h and 3 h from the initiation of mechanical ventilation still significantly reduced the inflammatory response. However, the continuous presence of CO for the entire ventilation interval afforded the most pronounced reduction of inflammatory parameters. Interestingly, the delayed start of CO treatment and the simultaneous extension of mechanical ventilation decreased neutrophils and IL-1 β release in the 5-h air/3-h CO group. However, there was only a trend towards reduction of IL-1 β in 5-h air/5-h CO mice and we did not observe an effect on

neutrophils when mice were ventilated for 10 h in the presence or absence of CO (data not shown).

DISCUSSION

Despite the fact that mechanical ventilation represents a life-saving tool in emergency and critical care medicine, cyclic stretching of the lung caused by mechanical ventilation may lead to VILI.² We and others^{25–27} have previously described that application of CO can substantially prevent the development of VILI during mechanical ventilation. However, the kinetics of the protective effects of CO remain incompletely described. For example, it remains unclear for how long CO

Figure 6 Effect of minimizing carbon monoxide treatment on lung inflammation. As controls, mice were allowed to spontaneously breathe air, or were ventilated with 12 ml/kg for 6 h with synthetic air. Additionally, 250 p.p.m. carbon monoxide (CO) was applied for 1, 3, or 6 h, followed by ventilation with air alone for the remaining 5, 3, or 0 h of the experiment, as indicated. Bronchoalveolar lavage was performed in the right lung. Total cells were counted under a light microscope (a). The relative amount of neutrophils was determined by cytospin analysis (b). Interleukin-1 β (IL-1 β) contents were quantified by ELISA (c). Graphs represent mean values $\pm$ s.e.m., $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), $^*P < 0.05$ vs control, $^{\S}P < 0.05$ vs 6-h CO-ventilated group, $^{\#}P < 0.05$ vs 1-h CO and 5-h air-ventilated group, $^{\circ}P < 0.05$ vs 3-h CO and 3-h air-ventilated group.

must be applied, or for how long the application of CO can be delayed in order to achieve the observed therapeutic effects. Therefore, the present study aimed to rigorously define the time dependence of the protective effects of CO treatment. According to the present results, we report that CO inhalation prevents VILI. Importantly, CO inhalation for as little as 1 h was sufficient for a sustained protective effect. Exclusive pre-treatment with CO demonstrated no impact on

the development of VILI. Finally, the delayed administration of CO (ie, up to 3 h after the onset of mechanical ventilation) still conferred protection against VILI.

A ventilation mode using 12 ml/kg was chosen in order to evoke a moderate degree of lung injury and inflammatory responses comparable to what we have previously described.^{26,27} In our setting and as recently demonstrated,^{26,27} inhalation of CO (250 p.p.m.) does not alter or worsen blood gas or lung function parameters. The observed slight decrease of PaO₂ and in some cases an elevated PaCO₂ in CO-ventilated mice at the end of the experiment appears not to have any deleterious effect on lung function and protection against VILI. PaO₂ in all groups analyzed are within physiological ranges for mice. Neither hyperoxemia nor hypoxemia was detected at any time point in the present experimental setting. This is in line with a previous study of our group applying the same experimental setting.²⁷ Moreover, lower PaO₂ values in CO-ventilated animals might result from interaction between oxygen and CO. However, and as depicted in the Supplementary Figures, PaO₂ values were variable in CO groups independent of the duration of CO application and independent of the observed protective effects. Also in agreement with previous reports, administration of CO prevented the development of VILI with respect to observed reduction in alveolar wall thickening, VILI score, total cellular influx, neutrophil transmigration, and cytokine release. With respect to pro-inflammatory cytokine release, we could demonstrate that CO inhalation exerts local rather than systemic effects. Emerging questions include for how long and for what time interval from the onset of injury must CO be administered in order to exert maximal protective effects. Both matters are of major clinical importance, if treatment options with low-dose CO are to be implemented into clinical practice in the future. First, if shorter exposures have the same effect as longer exposure times, the toxic side effects of CO treatment may be minimized. Second, if pre-treatment with CO would exert protective effects, procedures at risk for the development of ALI (eg, cardiopulmonary bypass or transplantation) could be considered for preparatory CO inhalation. Third, if delayed CO treatment would affect lung injury, CO could be considered as a potential therapeutic to be applied after lung injury has been diagnosed.

Regarding the first aspect of the study, we speculated that CO may confer protection against VILI even if the duration of application was minimized. This idea is underscored by one other study on acute pulmonary sepsis induced by intratracheal instillation of hydrochloric acid.¹⁹ While inhalation of 500 p.p.m. CO immediately after instillation for 6 h substantially decreased neutrophil influx and severe lung damage, as well as the upregulation of the adhesion receptor CD11b on blood neutrophils after 6 and 24 h, continuous application of CO for 24 h could not prevent lung injury. The notion that CO preferentially modulates the initiation of inflammatory cascades and the early development of lung injury is clearly supported by our data. CO inhalation at the

Figure 7 Effect of pre-treatment with carbon monoxide on lung injury. As controls, mice were allowed to spontaneously breathe air (a), or were ventilated with 12 ml/kg for 6 h with either synthetic air (b, c) or synthetic air (air) + 250 p.p.m. carbon monoxide (CO) (d). In addition, mice were allowed to spontaneously breathe air (b) or 250 p.p.m. CO (c) for 1 h before 6-h ventilation with air. Sections from the left lung lobe were hematoxylin and eosin stained. Representative pictures are shown for each experimental group (magnification = $\times 400$, a–d). High power fields were randomly assigned to measure alveolar wall thickness (e) and ventilator-induced lung injury (VILI) score (f). Graphs represent mean values $\pm$ s.e.m., $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), $^*P < 0.05$ vs control, $^{\S}P < 0.05$ vs 6-h CO-ventilated group.

onset of mechanical ventilation limited to the first hour reduced inflammatory cell influx, pro-inflammatory cytokine release, as well as alveolar wall thickening and VILI score to a similar extent compared with continuous 6 h CO application. Thus, the first hour during mechanical ventilation seems to have an important role in the initiation of inflammation where CO treatment can provide protection. In this regard, we recently reported that mechanical ventilation can upregulate Egr-1 within the first hour of treatment.²⁶ The essential role of this pro-inflammatory protein in the development of VILI has been further shown by the fact that Egr-1 knockout mice were resistant to VILI during mechanical ventilation, and that CO exerted lung protective effects via modulation of the Egr-1 pathway.²⁶ Surprisingly and in contrast to the 1-h as well as to the 6-h group, CO application for 3 h, followed by 3-h air ventilation had no impact on the development of lung injury. Two potential explanations might apply: First, depending on time and concentration of CO application as well as depending on the subject's condition, CO might exert beneficial as well as deleterious effects. Therefore, it might be reasonable that 3 h CO inhalation leads to some latent injury as compared with 1 h of application. In this case, a recovery by further and continuous administration of CO—acting as an anti-inflammatory agent as in the 6-h group—would be absent. Second, it is possible that the discrepant data are due to experimental variability. Cell counts as well as IL-1 β were not increased in the 3-h CO/3-h air group, supporting the assumption that despite clear anti-inflammatory patterns in this group, the absence of lung protection results from experimental variability.

As the second aim of this study, we investigated whether CO application before mechanical ventilation might exert protective effects in the lung. Pre-treatment or 'pre-conditioning' with CO has been studied extensively over the last few years. Analogous to ischemic or anesthetic preconditioning,^{44–46} inhalation of CO before injurious events elicited substantial protective effects in different models and organs including the lung, kidney, heart, liver, and others.^{14,16,20,35–39,47,48} Mechanistically, CO confers protection during ischemia/reperfusion by limiting the pro-inflammatory and fibrinolytic response, through the modulation of several signal transduction pathways (eg, MAPKs, heat shock proteins, Egr-1, etc.).^{16,36,37} In contrast to these studies, our results indicate that inhalation of CO before mechanical ventilation limits neither the resulting lung injury nor the inflammatory response. In light of the above studies, our findings are surprising and support the notion that preconditioning effects of CO are specific to the organ and/or the injury model employed. Furthermore, pre-treatment with CO may be effective for ischemia–reperfusion injury by specifically limiting the respiratory burst and the production of reactive oxygen species.^{14,16,36,37,39,40,47} However, we speculate that the development of VILI by ventilation associated with mechanical stretch, is based on a different

Figure 8 Effect of pre-treatment with carbon monoxide on lung inflammation. As controls, mice were allowed to spontaneously breathe air, or were ventilated with 12 ml/kg for 6 h with either synthetic air or synthetic air + 250 p.p.m. carbon monoxide (CO) as indicated. In addition, mice were allowed to spontaneously breathe air or 250 p.p.m. CO (COpre) for 1 h before 6-h ventilation with air. Bronchoalveolar lavage was performed in the right lung. Total cell counts were determined under the light microscope (a). The relative amount of neutrophils was determined by cytospin analysis (b). Interleukin-1 β (IL-1 β) contents were quantified by ELISA (c). Graphs represent mean values $\pm$ s.e.m., $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ vs control, § $P < 0.05$ vs 6-h CO-ventilated group; n.d., not detectable.

Figure 9 Effect of therapeutic treatment with carbon monoxide treatment on lung injury. As controls, mice were allowed to spontaneously breathe air (a), or were ventilated with 12 ml/kg for 6 h with synthetic air (b). In addition, mice were ventilated with air for 5 h (c), 3 h (d), or 0 h (e), followed by ventilation with 250 p.p.m. carbon monoxide (CO) for 1, 3, or 6 h as indicated. Sections from the left lung lobe were hematoxylin and eosin stained. Representative pictures are shown for each experimental group (magnification = $\times 400$, a–e). High power fields were randomly assigned to measure alveolar wall thickness (f) and ventilator-induced lung injury (VILI) score (g). Graphs represent mean values $\pm$ s.e.m., $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), $^*P < 0.05$ vs control, $^{\S}P < 0.05$ vs 6-h CO-ventilated group, $^{\circ}P < 0.05$ vs 3-h air 3-h CO-ventilated group.

pathological mechanism, that is apparently not affected by CO pre-treatment. Therefore, and with respect to the positive results obtained by minimizing CO application during ventilation, it appears to be important to administer CO simultaneously rather than before mechanical ventilation.

Finally, we intended to define whether delayed CO application during mechanical ventilation could exert therapeutic effects. Interestingly, only a very limited number of studies have focused on the therapeutic potential of CO application in terms of organ protection when CO is applied after the insult has been set. As mentioned above, lessons might be learned from ischemia–reperfusion models that consist of two

subsequent injurious events (ie, ischemia followed by reperfusion). Ischemia itself leads to cellular dysfunction and injury.^{49–51} The application of the CORM-3 after the ischemic period and with the onset of reperfusion substantially reduced heart infarct size in mice.⁵² Likewise, inhalation of 125 or 250 p.p.m. CO with the onset of reperfusion inhibited edema formation and infarct volume in a mouse cerebral occlusion model.⁵³ CO-mediated protection appears not to be restricted to local effects. As demonstrated in remote limb ischemia–reperfusion models, exposure of animals to 250 p.p.m. CO or to CORMs at the time of reperfusion reduced the intestinal and pulmonary inflammatory response.^{54,55} However, it is

Figure 10 Effect of therapeutic treatment with carbon monoxide treatment on lung inflammation. As controls, mice were allowed to spontaneously breathe air or were ventilated with 12 ml/kg for 6 h with synthetic air. In addition, mice were ventilated with air for 5, 3, or 0 h, followed by ventilation with 250 p.p.m. CO for 1, 3, or 6 h as indicated. Bronchoalveolar lavage was performed in the right lung. Total cell counts were determined under the light microscope (a). The relative amount of neutrophils was determined by cytospin analysis (b). Interleukin-1 β (IL-1 β) contents were quantified by ELISA (c). Graphs represent mean values $\pm$ s.e.m., $n = 6$ /group. ANOVA (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ vs control, § $P < 0.05$ vs 6-h CO-ventilated group, # $P < 0.05$ vs 5-h air 1-h CO-ventilated group, ° $P < 0.05$ vs 3-h air 3-h CO-ventilated group; n.d., not detectable.

well known that reperfusion represents the most important event for ischemia–reperfusion injury, and therefore, application of CO at the beginning of reperfusion cannot be considered as a post-treatment relative to ischemia. Two reports investigated post-treatment with CO in models closer to the clinical situation in which the injurious event occurs

before a therapeutic strategy can be initiated. One hour after lipopolysaccharide instillation, Liu *et al*⁵⁶ treated rats with 250 p.p.m. CO and demonstrated that the resulting intestinal injury was prevented. The authors found upregulation of p38 MAPK, reduced neutrophil activity, and decreased expression of intracellular adhesion molecule-1, indicating that CO limited inflammation even when the application was postponed.⁵⁶ In another study, Tsui *et al*⁵⁷ induced hepatitis in mice and subjected the animals to breathe CO at 500 p.p.m. for 1 h at various time points after the onset of liver injury. In all cases, CO-treated mice displayed improved survival rates, underscoring the potent therapeutic effects of low-dose CO exposure. Moreover, these studies suggested time-dependent effects of CO treatment: the later the application, the worse the outcome. Both studies are in agreement with the results of our present study. Based on our histological results and evaluation of VILI score, we could demonstrate that a delay of CO inhalation for 3 h after onset of mechanical ventilation still provides protective effects. It is important to note that the beneficial effects disappear over time (ie, starting CO inhalation after 5 h showed no impact on the development of VILI). Surprisingly, significant reduction in cell count, neutrophil fraction, and IL-1 β release clearly indicate that CO still exerts anti-inflammatory action, even when the gas was inhaled as late as 5 h after the onset of mechanical ventilation. The fact that the observed anti-inflammatory effects in this experiment did not translate into organ protection may have several explanations. First, it might be speculated that delayed CO application on one hand directly inhibits transmigration of pro-inflammatory cells into the lung that would subsequently limit cytokine release at a time point when the inflammatory response is still in process. On the other hand, delayed CO application might fail to impact the early initiation of other mechanisms independent of pro-inflammatory cascades, for example, stretch-induced signaling. In this case, with the assumption that multiple pathological mechanisms orchestrate VILI, the net effect of delayed CO application would lead to reduction of the inflammatory response without affecting lung injury. Second, these experiments were completed after 6 h (ie, 1 h after administration of CO). The time from initiation of CO inhalation to analysis might have been too short to record relevant effects at the organ level. In our experience, neutrophil infiltration in this model is postponed for several hours after the start of mechanical ventilation with moderate tidal volumes. Given the essential role of neutrophils for the progression of VILI and the time-dependent effectiveness of CO, reduction of neutrophils would need to be sustained at least several hours in order to reduce organ injury. Thus, an inhibitory effect on neutrophil transmigration may be detected by neutrophil counts but not necessarily by reduction of organ injury at the time of analysis. Supplementary data provided in this study point in this direction. Extension of CO inhalation time to 3 or 5 h starting 5 h after air ventilation, clearly decreased signs of lung damage as compared with time-matched air-ventilated animals, for

example, alveolar wall thickening and VILI score. However, there was a tendency for the anti-inflammatory function of CO application to decline at later time points.

In conclusion, exposure to low-dose CO exerts profound protective effects in VILI. In the present study, we provide clear evidence that short-term exposure to CO beginning at the onset of mechanical ventilation protects against VILI as effectively as long-term inhalation. In contrast to other organs and experimental models, CO pre-treatment alone does not confer protective or anti-inflammatory effects. However, delayed application of CO still provides lung protection when inhalation is initiated within 3 h of mechanical ventilation. Anti-inflammatory effects are conferred even when CO administration is postponed for 5 h after the onset of mechanical ventilation.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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