Inhalation of carbon monoxide following resuscitation ameliorates hemorrhagic shock-induced lung injury

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Abstract. Even after successful resuscitation, hemorrhagic shock frequently causes pulmonary inflammation that induces acute lung injury (ALI). We previously demonstrated that when CO is inhaled at a low concentration both prior to and following hemorrhagic shock and resuscitation (HSR) it ameliorates HSR-induced ALI in rats due to its anti-inflammatory effects. In the present study, we administered CO to the same model of ALI only after resuscitation and examined whether it exerted a therapeutic effect without adverse events on HSR-induced ALI, since treatment of animals with CO prior to HSR did not prevent lung injury. HSR were induced by bleeding animals to achieve a mean arterial pressure of 30 mmHg for 1 h followed by resuscitation with the removed blood. HSR resulted in the upregulation of inflammatory gene expression and increased the rate of apoptotic cell death in the lungs. This was determined from an observed increase in the number of cells positive for transferase-mediated dUTP-fluorescein isothiocyanate (FITC), nick-end labeling staining and activated caspase-3. HSR also resulted in prominent histopathological damage, including congestion, edema, cellular infiltration and hemorrhage. By contrast, CO inhalation for 3 h following resuscitation significantly ameliorated these inflammatory events, demonstrated by reduced histological damage, inflammatory mediators and apoptotic cell death. The protective effects of CO against lung injury were notably associated with an increase in the protein expression level of peroxisome proliferator-activated receptor (PPAR)-γ, an anti-inflammatory transcriptional regulator in the lung. Moreover, CO inhalation did not affect the hemodynamic status or tissue oxygenation during HSR. These findings suggest that inhalation of CO at a low concentration exerts a potent therapeutic effect against HSR-induced ALI and attenuates the inflammatory cascade by increasing PPAR-γ protein expression.

Introduction

Hemorrhagic shock (HS), which is typically observed following traumatic and surgical injuries, leads to organ hypoperfusion, ischemia and additional reperfusion injuries during resuscitation. HS and resuscitation (HSR) frequently induces systemic inflammatory responses that result in lung inflammation (the lung is the first targeted organ). Advanced phase HSR leads to acute lung injury (ALI), which is responsible for the substantial morbidity and mortality in critically ill patients (1,2). Pathogenesis is characterized by increased production of pro-inflammatory mediators and the activation of pulmonary inflammatory cells, which leads to development of interstitial edema and impaired lung function (3,4). Therefore, modulation of these inflammatory responses may provide an important avenue for therapeutic intervention when treating lung injuries, including HSR-induced ALI.

Carbon monoxide (CO) is commonly considered poisonous in high concentrations due to its ability to interfere with oxygen delivery. However, CO is produced by the enzymatic reaction of heme oxygenase-1 (a stress-inducible protein) in the body and previous evidence indicates that endogenous CO is important in the physiological functioning of organs and possesses anti-inflammatory properties that have applications

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in a variety of biomedical fields (5). Moreover, administration of CO at low concentrations has been demonstrated to mediate potent cytoprotective and anti-inflammatory effects during hyperoxic lung injuries (6), endotoxemia (7), ischemic/reperfusion injuries (8) and HSR-induced multiple organ failure (MOF) (9). We previously demonstrated that inhalation of CO at low concentrations both prior to and following HSR exerts a potent protective effect against ALI in rats, as determined by improvements in histological findings as well as the suppression of the inflammatory signaling cascade (10). However, to evaluate the possible clinical applications of CO as a therapeutic modality, we must determine the protective effects of CO at various stages, including prior to or following resuscitation, as well as clarify the molecular mechanisms behind the anti-inflammatory effects of CO.

In the present study, we have demonstrated that CO inhalation at a low concentration following resuscitation ameliorates HSR-induced ALI by attenuating apoptotic cell death in association with upregulation of peroxisome proliferator-activated receptor (PPAR)-γ, an anti-inflammatory transcriptional regulator. Present findings are likely to aid the expansion of new therapeutic avenues for CO gas in the field of therapeutic medical gas and critical care.

Materials and methods

Animals. The studies was approved by the Animal Use and Care Committee of the Okayama University Medical School (Okayama, Japan) in accordance with the guidelines for the care and use of laboratory animals that follows the ARRIVE reporting guidelines (11). Male Sprague-Dawley rats weighing 380-420 g (Clea Japan, Inc., Tokyo, Japan) were housed in a temperature-controlled (25°C) room with a 12-h light/dark cycle and free access to food and water until the start of the experiments.

HS protocol. All rats were subjected to sham or HS under pentobarbital anesthesia (sodium pentobarbital: 50 mg/kg, i.p.) as previously described (12). Briefly, HS was initiated by withdrawing blood from the left femoral vein to achieve a mean arterial blood pressure of 30±5 mmHg which was maintained for 1 h. Arterial pressure was measured via catheters inserted into the left femoral artery. Rats were resuscitated with shed blood. The sham group underwent the same instrumentation procedures, with the exception of bleeding.

Experimental design. Rats were randomly assigned to the following three groups: sham-operated control (Sham), air-treated HSR (HSR/AIR) and CO-treated HSR (HSR/CO). CO exposure was performed at 250 ppm for 3 h following the completion of resuscitation, as previously described (10). Rats in the Sham and HSR groups were maintained in room air in laminar flow cages. Some HSR rats were exposed to CO at 250 ppm for 1 h prior to the onset of HS (CO/HSR). At specific time points following resuscitation, rats were sacrificed by decapitation under light anesthesia with ethyl ether. The lungs were excised and frozen immediately in liquid nitrogen and stored at -80°C until further use.

In an additional set of experiments, HSR/AIR and HSR/CO rats were subjected to a median laparotomy under ether anesthesia for 3 h following the onset of resuscitation, in order to examine the effect of CO exposure on the CO content of lung tissues. Blood was collected from the abdominal aorta and the lungs were perfused *in situ* with physiological saline until the venous effluent became clear. The lungs were then removed for preparation of tissue homogenates.

Histological study. Tissues fixed in 10% neutral-buffered formalin were embedded in paraffin and cut in 4-6 μm-thick sections. After deparaffinization, the sections were stained with hematoxylin and eosin followed by a light microscope examination by a blinded observer. Grading of lung injury severity was performed according to previously described methods with modifications for five independent experiments (13-15).

Lung sections adjacent to those used for histopathological study were subjected to staining for neutrophils with a naphthol AS-D chloroacetate esterase-staining kit (Sigma Diagnostics, St. Louis, MO, USA) as described previously (16). The number of positively stained cells was counted in five nonconsecutive sections per rat at a x400 magnification by a blinded observer.

Lung wet weight to dry weight ratio. Lung tissue samples were blotted and weighed (wet weight) before drying for 24 h at 110°C (dry weight). The wet weight/dry weight ratio was calculated by dividing the wet weight by the dry weight as an index of pulmonary edema (17).

RNA isolation and northern blot analysis. Total RNA was isolated from the frozen rat tissues with Tri-Reagent™ (Sigma) according to the manufacturer's instructions. Northern blotting was performed as described previously (17). Briefly, 20 μg of total RNA was separated on a 1.2% (w/v) agarose gel containing 6.5% (v/v) formaldehyde and transferred on Bio-Rad Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA, USA). The blotted membrane was hybridized with [α-^{32}P]dCTP-labeled cDNA probes for tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS), respectively, followed by washing at high stringency. The membrane was exposed to X-ray films at -70°C, and autoradiographs and 18S ribosomal RNA were quantified with an image scanner (GelPrintTM 2000i, Genomic Solutions, Ann Arbor, MI, USA) and computerized image analysis software (Basic Quantifier™ version 3.0, Genomic Solutions). The relative amounts of radiolabeled cDNA that hybridized to the blots were normalized to 18S ribosomal RNA levels for correction of loading errors.

Preparation of nuclear extracts and western blot analysis. Nuclear extracts were isolated from the lung tissues using Nuclear and Cytoplasmic Extraction Reagents (NE-PER™; Thermo Fisher Scientific, Inc., Rockford, IL, USA), as previously described (18). The nuclear protein content was determined using the BCA Protein Assay kit™ (Thermo Fisher Scientific, Inc.). Samples were analyzed on a 12.5% polyacrylamide gel (e-PAGEL™; ATTO, Tokyo, Japan), according to the manufacturer's instructions. Following electrophoresis, proteins were transferred to a Hybond-P PVDF membrane (GE Healthcare, Little Chalfont, UK) and blocked with 4% (w/v) BlockAce™ (DS Pharma Biomedical Co., Ltd.,

Osaka, Japan). Blots were incubated with rabbit anti-PPAR-γ polyclonal antibody (1:1,200; Abcam, Cambridge, UK) and then treated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The antigen-antibody complexes were visualized using an ECL™ chemiluminescence system (GE Healthcare).

Real-time reverse transcription (RT)-polymerase chain reaction (PCR). Real-time RT-PCR was performed using SYBR Premix Ex Taq™ (Takara Bio, Shiga, Japan) and a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany), as previously described (16). The interleukin-10 (IL-10) messenger RNA (mRNA) level was normalized to the β-actin mRNA level.

Histological detection of apoptotic cell death. Detection and quantification of apoptotic cells were performed using the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein isothiocyanate (FITC) nick-end labeling (TUNEL) method with the MEBSTAIN Apoptosis kit II (MBL, Nagoya, Japan), according to the manufacturer's instructions. Sections were incubated with FITC-labeled avidin and then counterstained with 0.5 μg/ml propidium iodide to visualize the nuclei.

Immunostaining of activated caspase-3 was performed using rabbit polyclonal primary antibody (1:250; Asp 175, no. 9661; Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions. Sections were incubated with diluted Cyanine 3-conjugated donkey anti-rabbit secondary antibody (1:100; Chemicon International, Temecula, CA, USA).

Apoptotic and activated caspase-3-positive cells were counted using a confocal laser scanning microscope (model LSM510; ZEISS, Jena, Germany).

Measurement of carboxyhemoglobin (COHb) and tissue CO content. Blood COHb levels in the arterial blood were measured using the OSM3 Hemoximeter (Radiometer Copenhagen, Copenhagen, Denmark). The tissue CO content was measured as previously described (10).

Detection of tissue hypoxia by pimonidazole adducts. Levels of tissue hypoxia were detected by pimonidazole hydrochloride staining on lung (Hypoxyprobe™-1, Natural Pharmacia International, Inc., Burlington, MA, USA) as described previously (19). Slides were stained with a primary mouse monoclonal antibody for pimonidazole adducts, followed by a secondary FITC donkey anti-mouse IgG (Chemicon International). Images were captured as described earlier. Hypoxic rats breathed 6% oxygen continuously for 90 min as positive control. The atmosphere in the cages was sampled continuously from the chamber exhaust outlet and the O_2 concentration was monitored with a gas analyzer (CAPNOMAC™, Datex-Ohmeda, Finland).

Statistical analysis. Statistical analysis was undertaken using the Student's t-test or analysis of variance followed by Scheffé's F-test, as appropriate. P<0.05 was considered to indicate a statistically significant result. Data are shown as the mean ± SEM. We used statistical package Statview (Abacus Concepts, Berkeley, CA, USA).

Results

Therapeutic effect of CO inhalation on HSR-induced lung injury. While sections obtained from the Sham rats appeared normal, sections from HSR rats developed interstitial edema, as demonstrated by pronounced alveolar septal thickening and inflammatory cell infiltration 12 h after HSR (Fig. 1). CO exposure for 3 h following HSR markedly mitigated these pathological changes, including congestion, edema, inflammation and hemorrhage; however, CO exposure for 1 h prior to HSR did not affect the pathological findings (Fig. 1A). The significant effects of CO exposure following HSR were also confirmed by the scoring system based on histopathological changes reviewed by a blinded independent researcher. Only CO inhalation following HSR suppressed lung injuries, as revealed by histological damage that was confirmed by a decrease in the total histological score (Fig. 1B). By contrast, CO inhalation prior to HSR did not affect the histopathological scores (Fig. 1C).

Effect of CO inhalation on neutrophil sequestration in lungs following HSR. The number of infiltrating neutrophils was markedly increased in the HSR/AIR group at 12 h following resuscitation compared with that in the Sham group (Fig. 2A and B). By contrast, neutrophil recruitment was identified as significantly reduced in the lungs of the CO-treated rats (Fig. 2A and B).

CO inhalation reduced lung edema following HSR. Lung wet/dry ratio, a parameter of lung edema, was identified as significantly increased within 12 h in the HSR group, compared with that of the Sham group (Fig. 2C). However, CO inhalation following HSR significantly attenuated HSR-induced lung edema (Fig. 2C).

Effect of CO treatment on the gene expression of HSR-induced inflammatory mediators, including TNF-α and iNOS. Although mRNA levels of TNF-α and iNOS were barely detectable in the Sham group, these genes were significantly upregulated in the HSR/AIR group at 3 h after resuscitation (Fig. 3). CO inhalation following HSR significantly decreased mRNA expression levels of TNF-α and iNOS to almost the same levels as those measured in the Sham group (Fig. 3).

Effect of CO treatment on the expression of IL-10 and PPAR-γ in the lungs following HSR. Pulmonary IL-10 mRNA was barely detectable in the Sham group (Fig. 4A). The HSR procedure slightly increased the IL-10 mRNA level in the HSR/AIR group compared with that in the Sham group (Fig. 4A). Notably, CO treatment resulted in further upregulation of IL-10 mRNA (Fig. 4A). While PPAR-γ protein was expressed at high levels in the Sham group, levels in the HSR/AIR group was almost the same as that in the Sham group at 3 h following resuscitation (Fig. 4B and C). However, CO treatment significantly increased this level in comparison to that measured in the HSR/AIR group (Fig. 4B and C). The level measured in the HSR/CO group was almost twice as large as that measured in the HSR/AIR group (Fig. 4B and C).
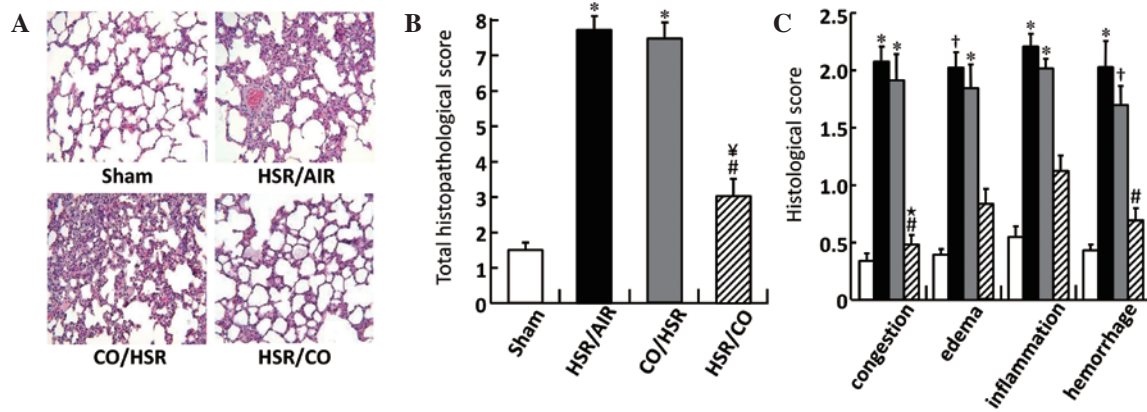
Figure 1. Comparative effects of carbon monoxide (CO) exposure at various times on histological changes in the lungs following hemorrhagic shock and resuscitation (HSR). (A) Rats subjected to HSR were exposed to CO for 1 h prior to onset of hemorrhagic shock or for 3 h following resuscitation or were maintained in air. Lungs from HSR rats following these various treatments were excised 12 h after resuscitation and subjected to histological examination. Representative images from five independent experiments (hematoxylin-eosin staining, x200 original magnification). (B) Severity of histopathological changes in the lungs were graded according to criteria for congestion, edema, inflammation and hemorrhage. Ten areas of the lung parenchyma from each rat were graded as 0 (no findings or normal), 1 (mild), 2 (moderate) or 3 (severe) for each of the four parameters. (C) Sum of histological scores for each of the four parameters were calculated (n=5, for each group). *P<0.01 vs. Sham; †P<0.05 vs. Sham; #P<0.01 vs. HSR/AIR; ¥P<0.01 vs. CO/HSR. Sham, sham-operated control rats; HSR/AIR, HSR rats maintained in air; CO/HSR, HSR rats exposed to CO for 1 h prior to hemorrhagic shock; HSR/CO, HSR rats exposed to CO for 3 h following resuscitation
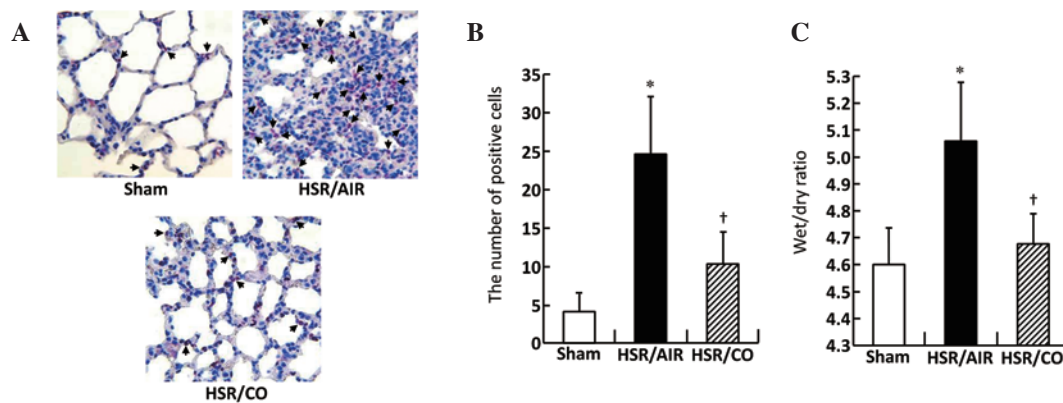

Figure 2. Effect of carbon monoxide (CO) inhalation on neutrophil accumulation in the lungs and wet/dry-weight (W/D) ratio of the lungs following hemorrhagic shock and resuscitation (HSR). Rats subjected to HSR were exposed to CO for 3 h following resuscitation or maintained in air. Lungs were excised 12 h after resuscitation and were used for the staining of neutrophils using the naphthol AS-D chloroacetate method and the determination of the W/D ratio. (A) Representative images of five independent experiments. Arrows indicate positively stained neutrophils (x400 original magnification). (B) Number of neutrophils in the lung sections of five nonconsecutive sections/rat at x400 magnification (n=5 for each group). *P<0.01 vs. Sham; †P<0.01 vs. HSR/AIR. (C) W/D ratio as an index of pulmonary edema (n=5, for each group). *P<0.01 vs. Sham; †P<0.05 vs. HSR/AIR. Sham, sham-operated control rats; HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats.
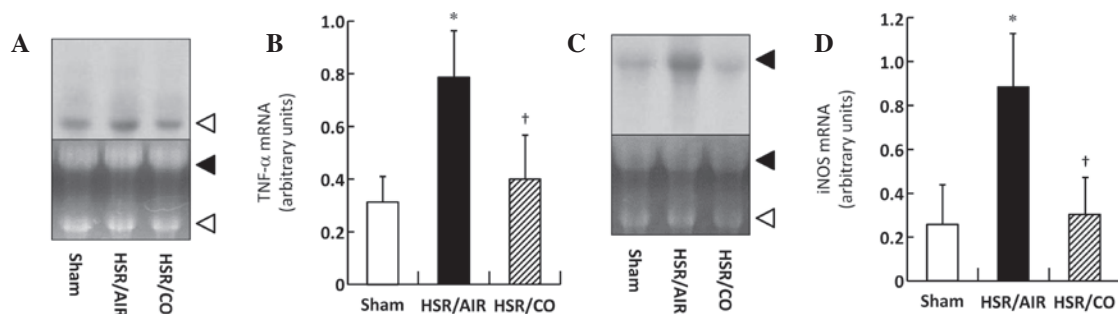

Figure 3. Effect of carbon monoxide (CO) inhalation on gene expression of tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) in the lungs following hemorrhagic shock and resuscitation (HSR). Lungs from HSR rats exposed to CO for 3 h following resuscitation or maintained in air were excised 3 h after resuscitation and expression levels of TNF-α and iNOS mRNA were determined by northern blot analysis. Autoradiographic signals of RNA blot hybridized with [α-32P] dCTP-labeled (A) TNF-α or (C) iNOS cDNA. Closed and open arrowheads indicate 28S and 18S ribosomal RNA, respectively. Densitometric analysis of (B) TNF-α and (D) iNOS mRNA, normalized to 18S ribosomal RNA. Concentrations are expressed in arbitrary units. Data are presented as the mean ± SEM (n=5, for each group). *P<0.01 vs. Sham; †P<0.05 vs. HSR/AIR. Sham, sham-operated control rats; HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats.

Table I. Effect of inhaled carbon monoxide (CO) on central hemodynamics following HSR.

| Rat treatment | Mean arterial pressure (mmHg) | | Heart rate (beats/min) | | Arterial blood gas analysis (3 h after HSR) | | |
	End of resuscitation	3 h after HSR	End of resuscitation	3 h after HSR	pH	PO_2 (mmHg)	PCO_2 (mmHg)
HSR/AIR	100.0±3.03	70.0±5.76	315.2±4.08	344.0±21.76	7.412±0.013	101.4±5.96	44.8±1.80
HSR/CO	100.4±10.97	73.2±8.38	284.8±18.00	297.6±5.31	7.412±0.013	117.0±12.95	40.7±1.36

Rats subjected to HSR were exposed to CO for 3 h following resuscitation or maintained in air. Mean arterial pressure and heart rate were measured at the completion of resuscitation and 3 h after resuscitation. Arterial blood gas analysis was performed 3 h after resuscitation. Data are presented as the mean ± SEM (n=5 for each group). HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats. HSR, hemorrhagic shock and resuscitation; PO_2, partial pressure of oxygen; PCO_2, partial pressure of carbon dioxide.
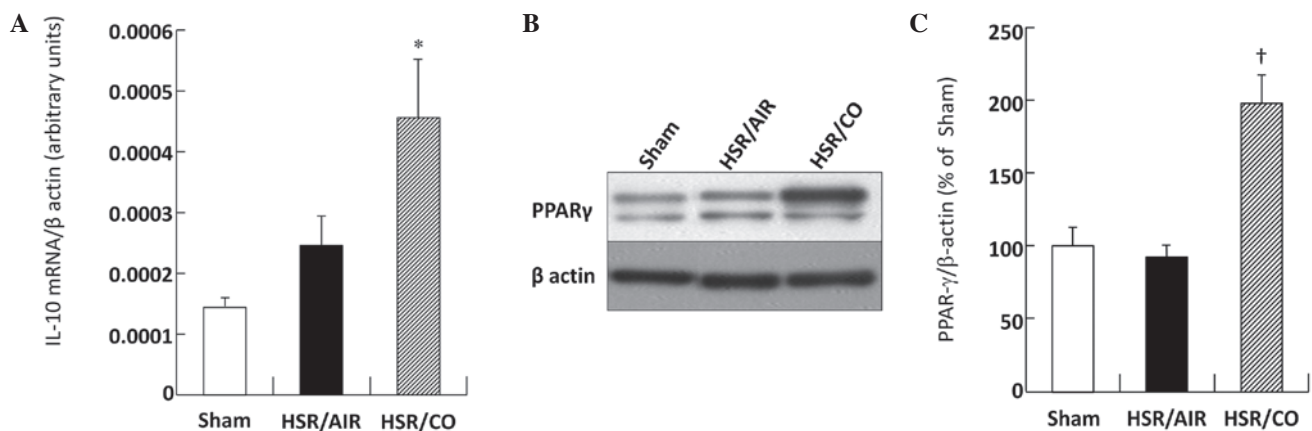

Figure 4. Effect of carbon monoxide (CO) inhalation on IL-10 gene expression and PPAR-γ protein expression in the lungs following hemorrhagic shock and resuscitation (HSR). Lungs from HSR rats exposed to CO for 3 h following resuscitation or maintained in air were excised 3 h after resuscitation and levels of IL-10 mRNA and PPAR-γ protein were determined by real-time RT-PCR and western blot analysis, respectively. (A) IL-10 mRNA level was normalized to β-actin and is expressed in arbitrary units. Data are presented as the mean ± SEM (n=12 for each group). *P<0.05 vs. HSR/AIR. (B) Western blot analysis using anti-rat PPAR-γ and anti-β-actin as the loading control. (C) Densitometric analysis of blot normalized to the density of β-actin, PPAR-γ is expressed as percentage concentration of the Sham group. Data are presented as the mean ± SEM (n=10 for each group). †P<0.01 vs. HSR/AIR. Sham, sham-operated control rats; HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats.

Effect of CO inhalation on apoptotic cell death in the lungs. While TUNEL-positive cells were barely detected in the Sham group, their numbers were significantly increased at 12 h following HSR in the lungs of the experimental rats (Fig. 5A and B). By contrast, CO inhalation significantly decreased the number by ~30% of the level measured in the HSR/AIR group (Fig. 5A and B). Activated caspase-3-positive cells were barely detected in the lungs of the Sham group, while their number was significantly increased at 12 h following resuscitation in the HSR/AIR rats (Fig. 5C and D). By contrast, the number of activated caspase-3-positive cells in the CO-treated HSR rats was low low (P<0.027; HSR/AIR vs. HSR/CO) and at almost the same level as that measured in the Sham group (Fig. 5C and D).

Effect of CO inhalation on blood COHb levels and CO tissue content in the lungs. Blood COHb levels in the HSR/CO group were markedly increased (19.40±0.66%) compared with those in the HSR/AIR group (3.08±0.06%; Fig. 6A). CO content in the lungs was markedly increased to 5.43±0.89 pmol/mg tissue

following CO exposure 3 h after resuscitation, while CO content in the lungs of the HSR/AIR group was 0.49±0.02 pmol/mg tissue (Fig. 6B).

CO did not affect the hemodynamic status during HSR. Mean arterial pressure and heart rate in the HSR/CO group were almost identical to those in the HSR/AIR group, even at 3 h after resuscitation (Table I). Moreover, CO exposure did not exert a significant effect on pH, partial pressure of oxygen (PO_2) and partial pressure of carbon dioxide (PCO_2) levels in the arterial blood (Table I).

CO did not induce tissue hypoxia. While there was no pimonidazole-binding in sham-operated control rats, sections from unoperated animals exposed to hypoxia, used as positive controls, revealed significantly enhanced pimonidazole-binding compared with sham-operated control rats (Fig. 7). By contrast, sections from HSR rats 3 h after resuscitation revealed no pimonidazole-binding, regardless of CO inhalation (Fig. 7).
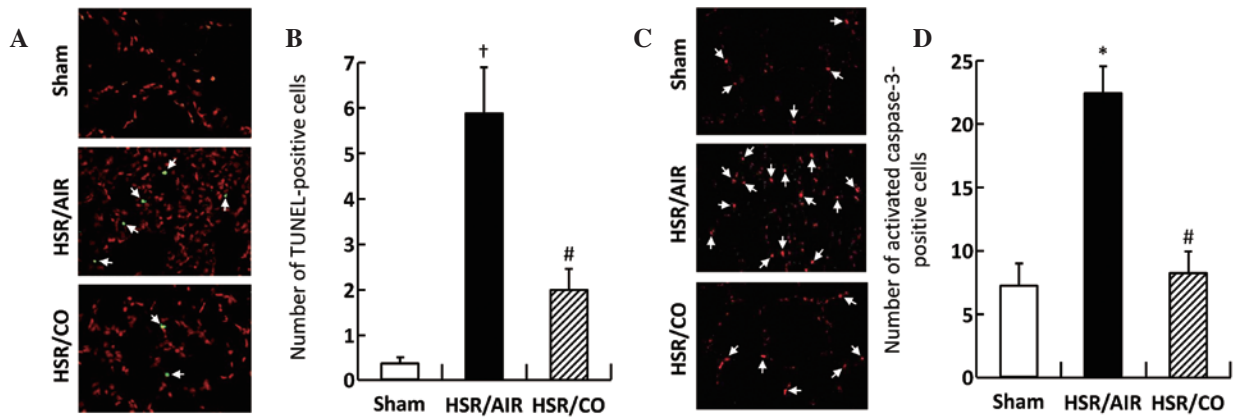
Figure 5. Effect of carbon monoxide (CO) inhalation on apoptotic cell death in the lungs following hemorrhagic shock and resuscitation (HSR). Lungs from HSR rats exposed to CO for 3 h following resuscitation or maintained in air were excised 12 h after resuscitation. (A) Sections were stained by TUNEL method (representative images of five independent experiments) and (C) immunohistochemical analysis of activated caspase-3. The arrows indicate TUNEL- or activated caspase-3-positive cells (x400 original magnification). Quantification of (B) TUNEL- and (D) activated caspase-3-positive cells (n=5 for each group). *P<0.05 vs. Sham; †P<0.01 vs. Sham; #P<0.05 vs. HSR/AIR. Sham, sham-operated control rats; HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick-end labeling.
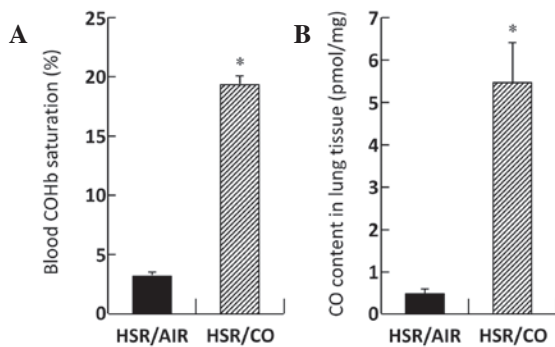

Figure 6. Effect of carbon monoxide (CO) inhalation on blood CO-hemoglobin (COHb) levels and CO tissue content in the lungs following hemorrhagic shock and resuscitation (HSR). HSR rats were exposed to CO for 3 h following resuscitation or maintained in air. Three hours after resuscitation, (A) COHb levels in the arterial blood and (B) CO content in the lungs were measured. Data are presented as the mean ± SEM (n=5 for each group). *p<0.01 vs. HSR/AIR. HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats.
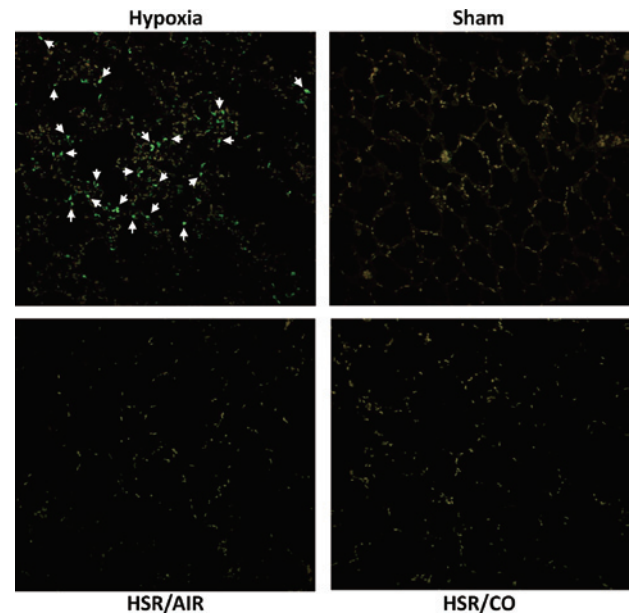

Figure 7. Detection of tissue hypoxia with hypoxia marker pimonidazole. Rats pretreated with hypoxia marker pimonidazole were subjected to HSR. Following resuscitation, HSR rats were exposed to CO for 3 h or maintained in air. Lungs were excised 3 h after HSR and pimonidazole binding cells were detected. Representative images from five independent experiments are shown. Arrows indicate hypoxic cells binding with pimonidazole (x200, original magnification). Hypoxia, rats exposed to 6% oxygen for 1.5 h used as hypoxic control; Sham, air-treated rats with sham surgery; HSR/AIR, air-treated HSR rats; HSR/CO, CO-treated HSR rats.

Discussion

The present study demonstrates that inhalation of CO at a low concentration following HSR exerts potent therapeutic effects against HSR-induced ALI. The findings of this study indicate that CO treatment suppresses molecular activation of HSR-induced inflammatory events. CO exerted anti-inflammatory effects that are, at least in part, mediated through induction of IL-10, an anti-inflammatory cytokine and activation of PPAR-γ, an anti-inflammatory transcriptional regulator. HS leads to exaggerated systemic inflammatory responses, which may be important in the development of MOF. MOF is associated with high mortality rates, reported in specific cases to exceed 50% (1). ALI is the first clinical manifestation of organ failure and a major factor leading to MOF, which is characterized by acute lung inflammation involving the local recruitment and activation of polymorphonuclear leukocytes and the release of pro-inflammatory mediators (1,20). Therefore, a novel anti-inflammatory modality that attenuates the overwhelming lung inflammation that develops following HS-induced lung injury may hold important therapeutic potential.

We previously demonstrated that combined treatment of CO prior to and following HSR exerts potent anti-inflammatory effects, resulting in reduced inflammatory cell influx into the lungs and marked attenuation in the expression of pro-inflammatory cytokines (10). The present study demonstrates that exposure to CO alone following HSR confers cytoprotective effects on HSR-induced lung injuries, primarily through

its anti-inflammatory effects. This finding suggests that post-treatment use of CO may have potential as a therapeutic approach against lung injuries and inflammation that result from multiple causes. Prevention of lung injuries by treatment with CO prior to noxious stimuli, including lipopolysaccharide (LPS), hyperoxia and ischemia/reperfusion, have been described by other investigators using experimental animal models (6,7,21). The results of these studies are consistent with our findings, but treatment with CO following harmful insult also has been reported to provide protection against lung injuries that are induced by various oxidative stimuli (22-25). Novel observations of the present study provide further evidence that post-treatment use of CO represents a potent therapeutic modality for treatment of ALI, which may contribute to clinical benefits in the treatment of lung injuries and inflammation.

CO possesses various anti-inflammatory properties that contribute to the reduction of oxidative stress and apoptotic cell death and are, at least in part, mediated through the p38 mitogen-activated protein kinase signaling pathway (26). However, the exact molecular mechanisms of anti-inflammatory actions of CO remain elusive. Previous studies, including ours, indicate that CO inhibits the activation of nuclear factor κB and activator protein-1, leading to amelioration of inflammatory lung disease by various causes, including LPS and HSR (10,27,28). The beneficial effects of CO have also been reported in association with the upregulation of IL-10 expression (26). Moreover, a previous study indicated that the anti-inflammatory effects of CO involve the inhibition of upstream toll-like receptor signaling pathways (29). Thus, multiple mechanistic actions of CO have been postulated. Previous observations have suggested another mechanism of CO that is exerted on the inflammatory response, which is mediated by PPAR-γ (30,31). In accordance with these reports, upregulation of PPAR-γ was accompanied by the protective effects of CO against ALI following HSR in the present study. Previous observations clearly demonstrate that the endogenous ligands of PPAR-γ reduce organ injury and dysfunction, including detrimental effects to the liver, kidneys, intestines and lungs, which are caused by HS (32). More recently, augmented PPAR-γ expression by exogenous agonists was demonstrated to contribute to the modulation of systemic and pulmonary inflammation in HSR (33). These investigators also indicated that PPAR-γ agonists exert anti-apoptotic effects in the lungs following severe hemorrhage (34). Further studies using PPAR-γ-neutralizing antibodies or PPAR-γ-knockout animals are likely to be important in clarification of the function of PPAR-γ in HSR.

Although it is important to establish a clinically applicable strategy for using CO in treatment of ALI following HSR, CO exposure is associated with serious health problems. CO is well known for its high affinity for hemoglobin, which is more than 200 times greater than that of oxygen, as well as its ability to block the binding of oxygen (35), thereby leading to impaired delivery of oxygen to tissues and ultimately resulting in hypoxic tissue damage and death (36). In the present study, rats exposed to 250 ppm CO for 3 h exhibited considerably high blood COHb levels (19.40±0.66%). However, arterial blood gas analysis revealed that there was no difference in PaO_2 levels between CO and AIR treated-rats in the HSR groups (Table I). Moreover, as shown in Fig. 7, CO inhalation did not induce lung tissue hypoxia as determined by the pomonidazole hydrochloride staining, which is known as an *in vivo* hypoxia marker (19). Instead, CO treatment of rats markedly improved the clinical presentation of HSR-induced ALI.

There are two concerns regarding species differences, particularly between small and large animals, prior to clinical application of CO. One is the differences is rate of CO uptake into the body. Longer reaction time is required to form COHb in large animals, including humans, than in small animals, including mice and rats, which is attributable to the lower minute volume of ventilation/unit of body weight in large animals (37). The other is differences in affinity of CO to hemoglobin. The affinity of CO to hemoglobin is approximately 4 times higher than that of oxygen in rodents (38). The magnitude of the affinity differences between two gases is significantly smaller in rodents than in humans. Thus, humans may inhale higher concentration of CO than mice to obtain the same level of CO in the tissue which is released from COHb. However, it was recently reported that CO inhalation only at 100 ppm suppresses LPS-induced pulmonary inflammation in mice (39). This allows us to hypothesize that CO exposure below the lethal toxicity dose may exert a protective effect on lung injury and inflammation in the clinical setting.

In conclusion, the present study demonstrates that HSR causes significant pulmonary inflammation, as shown by the increase in the gene expression levels of inflammatory mediators, neutrophil emigration and pulmonary edema, which leads to apoptotic cell death. CO inhalation following HSR significantly reduces these indices with additional increases in gene expression of IL-10, an anti-inflammatory cytokine and protein expression of PPAR-γ, an anti-inflammatory transcriptional regulator, resulting in amelioration of HSR-induced injury. These findings indicate that CO inhalation possesses a potent therapeutic effect on HSR-induced lung injury, at least in part, through the attenuation of the inflammatory signaling pathway. Although the application of CO to therapeutic modalities are likely to require additional preclinical and clinical studies on its safety and efficacy for human use, strategies for the attenuation of the HSR-induced inflammatory signaling pathway, as shown in the present study, may expand the currently limited therapeutic options.

Acknowledgements

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