Carbon Monoxide Inhalation Reduces Pulmonary Inflammatory Response during Cardiopulmonary Bypass in Pigs

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Background: Cardiopulmonary bypass (CPB) is associated with pulmonary inflammation and dysfunction. This may lead to acute lung injury and acute respiratory distress syndrome with increased morbidity and mortality. The authors hypothesized that inhaled carbon monoxide before initiation of CPB would reduce inflammatory response in the lungs.

Methods: In a porcine model, a beating-heart CPB was used. The animals were either randomized to a control group, to standard CPB, or to CPB plus carbon monoxide. In the latter group, lungs were ventilated with 250 ppm inhaled carbon monoxide in addition to standard ventilation before CPB. Lung tissue samples were obtained at various time points, and pulmonary cytokine levels were determined.

Results: Hemodynamic parameters were largely unaffected by CPB or carbon monoxide inhalation. There were no significant differences in cytokine expression in mononuclear cells between the groups throughout the experimental time course. Compared with standard CPB animals, carbon monoxide significantly suppresses tumor necrosis factor-α and interleukin-1β levels ($P <$ 0.05) and induced the antiinflammatory cytokine interleukin 10 ($P <$ 0.001). Carbon monoxide inhalation modulates effector caspase activity in lung tissue during CPB.

Conclusions: The results demonstrate that inhaled carbon monoxide significantly reduces CPB-induced inflammation *via* suppression of tumor necrosis factor α, and interleukin-1β expression and elevation of interleukin 10. Apoptosis induced by CPB was associated with caspase-3 activation and was significantly attenuated by carbon monoxide treatment. Based on the observations of this study, inhaled carbon monoxide could represent a potential new therapeutic modality for counteracting CPB-induced lung injury.

◇ This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

◆ This article is accompanied by an Editorial View. Please see: Kevin LG, Laffey JG: Carbon monoxide: From poison to therapy for cardiopulmonary bypass–induced lung injury? ANESTHESIOLOGY 2008; 108:977–8.

SYSTEMIC and pulmonary inflammatory responses arising in patients after cardiac surgery with the use of cardiopulmonary bypass (CPB) significantly contribute to CPB-associated morbidity and mortality.[1–3] Postoperative systemic inflammatory response syndrome with severe morbidity is uncommon (approximately 1–2%).[4] Pulmonary impairment and dysfunction range in severity from subclinical functional changes and prolonged postoperative ventilation to acute lung injury and acute respiratory distress syndrome.[5–7] Acute respiratory distress syndrome occurs in approximately 2% of all cases after CPB with an associated mortality of greater than 60%.[8–12] Causative factors for the inflammatory response are, among others, the pulmonary ischemia during bypass and reperfusion injury after declamping, blood contact with the surface of the extracorporeal circulation unit, endotoxemia, surgical trauma, and blood loss.[13,14] The cytokines responsible for the inflammatory response induced by CPB include interleukin (IL)-1β and tumor necrosis factor (TNF)-α.[15,16] Elevated expression of these cytokines aggravate the inflammation and contribute to pulmonary dysfunction.[17–20]

The gaseous molecule carbon monoxide, known as a vasodilative agent, has antiinflammatory properties.[21–25] Although carbon monoxide may be toxic at high doses, low concentrations, such as 250 ppm, have been demonstrated to suppress inflammation and reduce apoptosis in a multitude of *in vitro* and *in vivo* studies.[21,22,26–32] The limited toxicity of carbon monoxide at low doses is underscored by the observation that carbon monoxide is also generated endogenously *via* the inducible catabolism of heme by the heme oxygenase type I.[33] Carbon monoxide by itself is able to activate the soluble guanylate cyclase and the p38 mitogen-activated protein kinase, both leading to a decrease in inflammatory gene expression.[34]

We hypothesized that inhaled carbon monoxide would provide antiinflammatory and antiapoptotic effects in the lungs during CPB, thereby reducing proinflammatory cytokine levels.

Materials and Methods

Animals

German Landrace hybrid pigs weighing 28–32 kg received human care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Insti-

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tute of Laboratory Animal Resources and published by the National Institutes of Health (publication No. 86-23, revised 1985). All procedures were performed in accordance with the German Animal Protection Law after obtaining the ethical permission of the local veterinary office (Regierungspraesidium, Freiburg, Germany).

Experimental Protocol

Healthy pigs were premedicated with intramuscular ketamine (20 mg/kg body weight [BW]) and midazolam (0.5 mg/kg BW). The animals were endotracheally intubated, and anesthesia was maintained with intravenous infusion of fentanyl ($10 \ \mu g \cdot kg \ BW^{-1} \cdot h^{-1}$) and propofol ($4\text{--}6 \ mg \cdot kg \ BW^{-1} \cdot h^{-1}$), and muscle relaxation was performed with cisatracurium ($0.7\text{--}1 \ mg \cdot kg \ BW^{-1} \cdot h^{-1}$). Basal saline volume administration was infused with 350 ml/h. The lungs were ventilated with a frequency of 14/min, a tidal volume of 8–10 ml/kg BW, and a positive end-expiratory pressure of 5 mbar. Inspired oxygen fraction was constantly 0.5. To monitor blood pressures as well as blood gas analysis, an arterial catheter was inserted in the right carotid artery. In addition, a pulmonary thermodilution catheter (7 French; Arrow, Reading, PA) was inserted into the pulmonary artery *via* an 8.5-French sheet (Arrow) in the right internal jugular vein. All animals received sternotomy after complete hemodynamic monitoring, and 300 U/kg BW heparin were administered. Initiation of CPB was achieved using a 24-French venous cannula in the right atrial appendage and a 14-French aortic cannula. The extracorporeal circulation unit (Stoeckert, Munich, Germany) was primed using 1,000 ml isotonic saline solution, 500 ml hydroxyethyl starch (6%), 75 ml mannitol, and 300 U heparin. CPB was maintained for 2 h with completely unloaded beating heart. Continuous positive airway pressure was kept at 5 mbar during this time. After CPB, a 30-s recruiting maneuver was performed, and standard ventilation was reestablished. CPB was removed, protamine matching the heparin dosage was administered, and 2 h of observation time (post-CPB; fig. 1, experimental design) followed. We collected blood gas samples at six defined
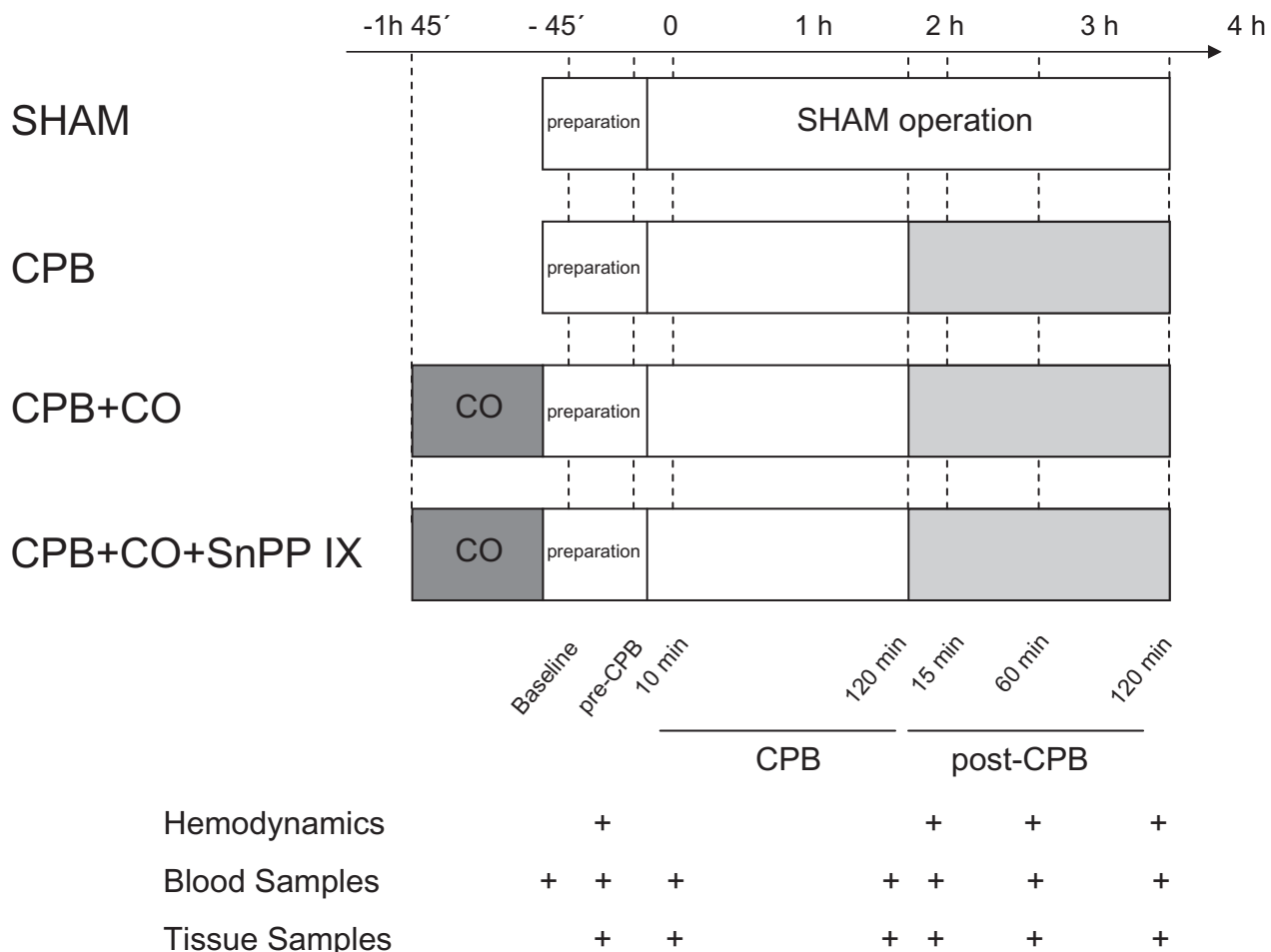
Fig. 1. Experimental protocol. All animals received sternotomy after complete hemodynamic monitoring. Cardiopulmonary bypass (CPB) was maintained for 2 h with completely unloaded beating heart. Continuous positive airway pressure was kept at 5 mbar during this time. CPB was removed, protamine matching the heparin dosage was administered, and 2 h of observation time followed. CO = carbon monoxide; SHAM = control group; SnPP IX = tin protoporphyrin IX.

time points (fig. 1): before CPB (pre-CPB); 10 min after initiation of CPB; at the end of CPB; and 15, 60, and 120 min post-CPB. Blood gas analysis included measurement of hemoglobin concentrations, electrolytes, and acid–base status. Five hundred milligrams of lung tissue was taken for further analysis at each time point. At the same points of time, hemodynamic parameters such as heart rate, mean arterial pressure, mean pulmonary pressure, central venous pressure, and pulmonary capillary wedge pressure were measured, and triplicate measurements of cardiac output (calculated as cardiac index) were performed with the thermodilution technique. Systemic and pulmonary vascular resistances were calculated.

The lung tissue was collected from three lung sites, *i.e.*, cranial portion of the apical lobe, caudal portion of the apical lobe, and basal lobe. Samples were minced and stored at $-80°C$ for protein and messenger RNA (mRNA) analysis. Animals were randomly assigned to three different intervention protocols and one *post hoc* group (fig. 1). The SHAM group (n = 3), serving as negative control, received sternotomy only. In the CBP group CPB (n = 7), serving as positive control, CPB was initiated and maintained for 2 h followed by 2-h observation period. Animals of the CPB + CO group (n = 7) were given carbon monoxide immediately after induction of anesthesia at a concentration of 250 ppm for 1 h.[32,35] Animals of the *post hoc* CPB + CO + SnPP IX group (n = 7) were given the heme oxygenase inhibitor tin protoporphyrin IX (SnPP IX; 50 μmol/kg BW) immediately after induction of anesthesia for suppression of endogenous-derived carbon monoxide production. End-expiratory carbon monoxide concentration was monitored using a carbon monoxide analyzer (MicroSmokerlyzer; Breath CO Monitor, Bedfont, United Kingdom), and the content of carbon monoxide–saturated hemoglobin was measured photometrically during regular blood gas analysis. Inhalation of carbon monoxide was kept at 250 ppm until initiation of the CPB. The animals were killed 2 h after the end of CPB by myocardial potassium injection.

Enzyme-linked Immunoabsorbent Assay

Protein was extracted and enzyme-linked immunoabsorbent assay was performed following the manufacturer's instruction (Quantikine; R&D, Minneapolis, MN). Protein concentration was determined using the Bradford Assay (Bio-Rad Laboratories, Munich, Germany).

Northern Blotting

Lung tissue was homogenized and total RNA was extracted using a one-step guanidinium thiocyanate-phenol-chloroform extraction reagent (Trizol®; Invitrogen, Carlsbad, CA). We separated 10 μg total RNA electrophoretically on 1% agarose gel and transferred it on a nylon membrane (Amersham, Braunschweig, Germany). Northern blotting was performed as previously described.[36]

cDNA and Oligonucleotide Probes

A full-length complementary DNA (cDNA) was constructed using a reverse-transcriptase polymerase chain reaction first strand cDNA kit (Fermentas, St. Leon-Rot, Germany). Polymerase chain reaction was performed using commercially synthesized specific oligonucleotides for TNF-α, IL-1β, and IL-10 (BigBiotech, Freiburg, Germany). The following specific primer sequences were used: TNF-α (478 bp): sense (3'-5') TGC CTA CTG CAC TTC GAG GTT ATC, antisense (5'-3') TGA GTC GAT CAT CCT TCT CCA GCT; IL-1β (385 bp): sense (3'-5') ACA GAA GTG AAG ATG GCC AAA GTC, antisense (5'-3') TCA TGT TGC TCT GGA AGC TGT ATG; IL-10 (446 bp): sense (3'- 5') GCA TCC ACT TCC CAA CCA, antisense (5'-3') CTT CCT CAT CTT CAT CGT CAT.

Polymerase chain reaction products were purified with Qiagen® Gel Extraction Kit and transformed into *Escherichia coli* bacteria *via* plasmid vector (pCR®2.1 TOPO, TOPO TA Cloning; Invitrogen). Cloned plasmids were extracted according to the manufacturer's instructions, and an *Eco*RI digestion was performed to isolate the cDNA insert. The probe was labeled with [α^{32}P]dCTP (Amersham) using the Prime it II Random Primer Labeling kit® (Stratagene, La Jolla, CA).

Preparation of Mononuclear Blood Cells

Blood samples were placed on top of Ficoll buffer and centrifuged at 1,800 rpm for 45 min. Mononuclear cells accumulated above the Ficoll, whereas erythrocytes formed a solid mass at the bottom of the tube. Mononuclear cells were gathered using a Pasteur pipette, washed in CliniMacs® (Miltenyi Biotec, Bergisch Gladbach, Germany) three times, and recentrifuged. RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction, and total RNA was stored at $-80°C$ until used again.

RNase Protection Assay

The ribonuclease protection assay was performed according to the manufacturer's recommendation (MultiProbe RPA; Becton Dickinson, Heidelberg, Germany). Using a customer requested template (supported by Becton Dickinson, Franklin Lakes, NJ), the assay was performed with 2 μg total RNA, extracted from peripheral mononuclear blood cells. The template components were IL-1β (257 bp); IL-6 (191 bp); IL-10 (316 bp); RANTES (regulated upon activation, normal T-cell expressed, and secreted) 172 bp); L32 (140 bp); and glyceraldehyde 3-phosphate dehydrogenase (125 bp).

Fluorogenic Caspase Activity Assay

Fluorogenic caspase activity assay was performed as previously described.[37]

Lung Histopathology

Lung histopathology evaluation was performed in pigs after lung tissues were removed from the animals and were placed in 10% neutral buffered formaldehyde overnight for fixation at room temperature. The tissues were processed into paraffin blocks, and 4-μm-thin microscopic sections were obtained using a microtome and placed on slides. Routine hematoxylin and eosin staining was performed. Histologic and morphologic changes induced by the interventions were evaluated by microscopy.

Quantitative and Statistical Analysis

Data were analyzed by a computerized statistical program (SigmaStat® for Windows Version 3.1; Systat Software Inc., San Jose, CA). The results are expressed as mean ± SD after normality was approved. Two-way analysis of variance for repeated measurements was used to compare parameters within one group. Two-way analysis of variance was used to compare the different groups. A *P* value less than 0.05 was considered statistically significant. Autoradiographs of Northern blots were evaluated by volume quantification and local median of gene expression and normalization against background using two-dimensional scanning (Personal Densitometer; Amersham).

Results

All animals used in this experimental project survived. Seventeen animals were randomly allocated to three groups, SHAM (n = 3), CPB (n = 7), and CPB + CO (n = 7), and one *post hoc* group, CPB + CO + SnPP IX (n = 7), added after the initial experiments. No animals were excluded at any time.

Blood Gas Analysis

At various time points before, during, and after CPB a complete arterial blood gas analysis was conducted, including acid–base status, electrolytes, hemoglobin, carboxyhemoglobin, and lactate concentration. Baseline levels of all parameters were not statistically different between all groups. Hemoglobin concentration in both CPB groups dropped by 20% at the onset of CPB and leveled off by the end without group differences, whereas in the SHAM group hemoglobin levels declined throughout the observation time by 10% because of dilution (data not shown). In both CPB groups, we detected a respiratory alkalosis during CPB with normalization after CPB (data not shown). Arterial oxygen pressure (Pa$_{O_2}$) was significantly lower in the CPB + CO group and the CPB + CO + SnPP IX group at 10 and 120 min CPB compared with the SHAM group (fig. 2A). In the post-CPB observation period, Pa$_{O_2}$ was not further affected by carbon monoxide inhalation (fig. 2A). As expected, carboxyhemoglobin levels in the SHAM group and the CPB group did not change significantly. Carbon monoxide inhalation (250 ppm) increased carboxyhe-
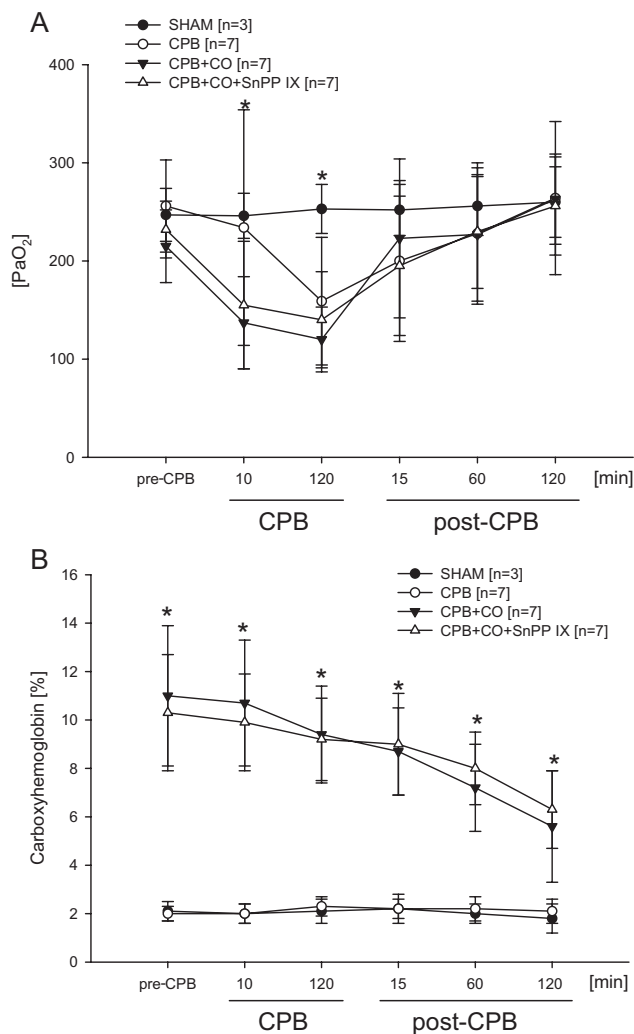
Fig. 2. Arterial oxygen pressure (Pa$_{O_2}$; *A*) and carboxyhemoglobin levels (*B*) in control group (SHAM) (*closed circles*) and groups treated with cardiopulmonary bypass (CPB) (*open circles*), CPB + carbon monoxide (CO) (*closed triangles*), and CPB + CO + tin protoporphyrin IX (SnPP IX) (*open triangles*). Values are mean ± SD. * *P* < 0.05, CPB + CO *versus* SHAM and CPB.

moglobin levels fivefold to sixfold up to peak values of 11.0 ± 2.9% after 1 h of inhalation (fig. 2B). After CPB, carboxyhemoglobin levels in the CPB + CO group and the CPB + CO + SnPP IX group decreased, reaching a value of 5.6 ± 2.3% at 120 min of the post-CBP observation period (fig. 2B). Other arterial blood gas parameters did not differ between groups.

Systemic Hemodynamics

Hemodynamic parameters are presented in table 1 and were largely unaffected by carbon monoxide inhalation. Heart rate significantly increased in the CPB group and the CPB + CO group at 15 and 60 min post-CPB compared with the SHAM group (table 1). Central venous pressure and pulmonary artery occlusion pressure remained unchanged throughout the study in all groups (table 1).

Table 1. Comparison of Hemodynamic Parameters in the Four Groups Studied During the Experimental Protocol

| Time	Group	HR, min^{-1}	MAP, mmHg	mPAP, mmHg	CVP, mmHg	PCWP, mmHg	CI, l · min · m^{-2}	SVR, dyn · s · cm^{-5}	PVR, dyn · s · cm^{-5}
Pre-CPB	SHAM	74 ± 16	95 ± 14	15 ± 1	5 ± 1	6 ± 1	2.7 ± 0.2	34 ± 6	3 ± 1
	CPB	86 ± 16	85 ± 15	11 ± 1	4 ± 2	5 ± 1	2.5 ± 0.5	34 ± 13	3 ± 1
	CPB + CO	93 ± 15	93 ± 13	13 ± 2	2 ± 2	6 ± 1	2.6 ± 0.5	34 ± 7	2 ± 1
	CPB + CO + SnPP IX	87 ± 13	91 ± 16	12 ± 2	5 ± 3	6 ± 2	2.5 ± 0.4	33 ± 9	3 ± 1
Post-CPB 15 min	SHAM	67 ± 12	91 ± 7	15 ± 2	5 ± 1	7 ± 2	2.6 ± 0.2	34 ± 4	3 ± 0
	CPB	106 ± 11*†‡	79 ± 10	16 ± 5	4 ± 2	6 ± 2	2.8 ± 0.5	28 ± 8	4 ± 1
	CPB + CO	94 ± 13‡	87 ± 7	15 ± 3	4 ± 3	7 ± 1	2.8 ± 0.6	34 ± 8	4 ± 2
	CPB + CO + SnPP IX	103 ± 11†‡	94 ± 12	14 ± 3	5 ± 3	5 ± 3	2.5 ± 0.6	31 ± 7	4 ± 2
Post-CPB 60 min	SHAM	70 ± 16	88 ± 7	14 ± 2	6 ± 1	7 ± 2	2.5 ± 0.1	34 ± 3	3 ± 0
	CPB	95 ± 19‡	73 ± 14	12 ± 1	3 ± 2	6 ± 2	2.4 ± 0.4	36 ± 7	3 ± 1
	CPB + CO	89 ± 10‡	80 ± 11	14 ± 2	4 ± 2	7 ± 1	2.5 ± 0.6	33 ± 8	3 ± 1
	CPB + CO + SnPP IX	87 ± 11	88 ± 14	14 ± 4	4 ± 4	6 ± 2	2.3 ± 0.5	37 ± 6	4 ± 2
Post-CPB 120 min	SHAM	70 ± 17	84 ± 8	14 ± 2	7 ± 1	7 ± 2	2.5 ± 0.2	32 ± 4	3 ± 0
	CPB	93 ± 13	76 ± 13	12 ± 1	3 ± 2	6 ± 2	2.2 ± 0.4	39 ± 7	3 ± 1
	CPB + CO	92 ± 12	85 ± 11	13 ± 2	4 ± 2	7 ± 1	2.3 ± 0.5	40 ± 11	3 ± 2
	CPB + CO + SnPP IX	86 ± 14	87 ± 17	12 ± 3	6 ± 3	7 ± 3	2.2 ± 0.6	37 ± 4	3 ± 2

Values are mean ± SD.

* $P < 0.05$ *vs.* pre-cardiopulmonary bypass (CPB).   † $P < 0.05$ *vs.* 120 min post-CPB.   ‡ $P < 0.05$ *vs.* control group (SHAM) at the same time.

CI = cardiac index; CO = carbon monoxide; CVP = central venous pressure; HR = heart rate; MAP = mean arterial pressure; mPAP = mean pulmonary artery pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; SnPP IX = tin protoporphyrin IX; SVR = systemic vascular resistance.

Systemic Effects of Inhaled Carbon Monoxide during CPB

The systemic effects of carbon monoxide on the mononuclear component of the systemic inflammatory response were evaluated by determining cytokine mRNA levels in peripheral mononuclear blood cells using an RNase Protection Assay. There were no significant differences in cytokine expression in mononuclear cells between the SHAM, CPB, or CPB + CO animals throughout the experimental time course (fig. 3, lanes 5–16). Lung tissue of a representative animal served as a positive control (fig. 3, lane 4). Housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase) demonstrated equal amounts of loaded RNA (fig. 3, all lanes).
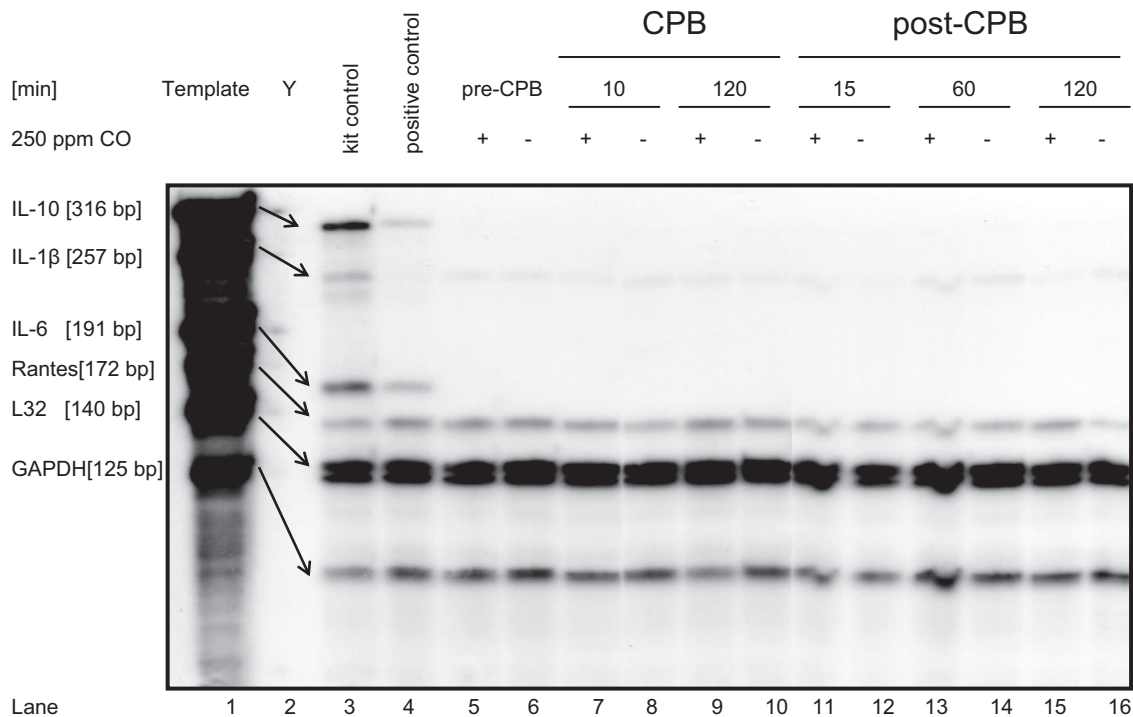
Fig. 3. Representative autoradiograph of RNase protection assay showing the time course of cytokine gene expression in mononuclear blood cells. A total of 10 μg RNA was used in each lane. CPB = cardiopulmonary bypass; CO = carbon monoxide; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL = interleukin; RANTES = regulated upon activation, normal T-cell expressed, and secreted; Y = yeast transfer RNA.
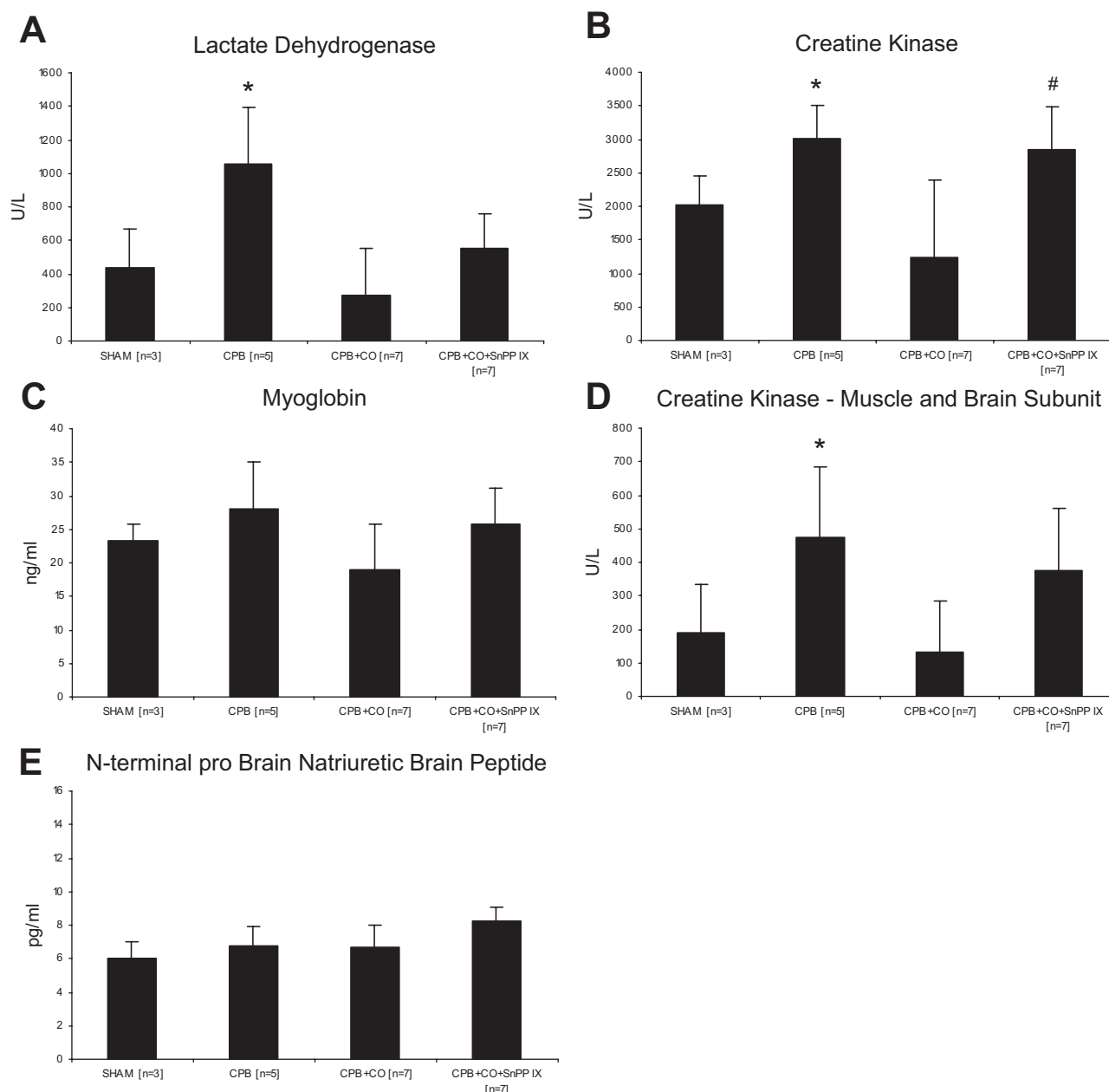
Fig. 4. Activity of lactate dehydrogenase (*A*), creatine kinase (*B*), myoglobin (*C*), creatine kinase–muscle and brain subunit (*D*), and N-terminal pro brain natriuretic brain peptide (*E*) in plasma obtained from pigs after cardiopulmonary bypass (CPB), CPB + carbon monoxide (CO), and CPB + CO + tin protoporphyrin IX (SnPP IX) and time-matched control group (SHAM) animals. Data represent mean ± SD. * *P* < 0.05, CPB *versus* SHAM and *versus* CPB + CO. # *P* < 0.001, CPB + CO + SNPP IX *versus* CPB + CO.

To evaluate the systemic toxic or beneficial effects of carbon monoxide on organ function, we determined plasma activity of lactate dehydrogenase (fig. 4A), creatine kinase (fig. 4B), myoglobin (fig. 4C), creatine kinase–muscle and brain subunit (fig. 4D), and N-terminal pro brain natriuretic peptide (fig. 4E). To evaluate extrapulmonary effects of carbon monoxide, we determined plasma activity of hepatic enzymes (figs. 5A–E) and plasma levels of creatinine (fig. 5F). Our results demonstrate lack of systemic carbon monoxide toxicity and the beneficial effects on organ function.

Carbon Monoxide Inhalation Modulates Cytokine mRNAs and Protein Levels and Effector Caspase Activity in Lung Tissue during CPB

Cytokine mRNAs and protein levels in lung tissue were barely detectable in the SHAM group (data not shown). As shown in figure 6A, transcription of the TNF-α mRNA was also barely detectable at baseline in animals treated with CPB and with CPB + CO (fig. 6A, lanes 1 and 2). TNF-α mRNA expression increased significantly in the CPB group (fig. 6A, upper panel; 10 and 120 min; *P* < 0.05), both during and after CPB (fig. 6A, upper panel;
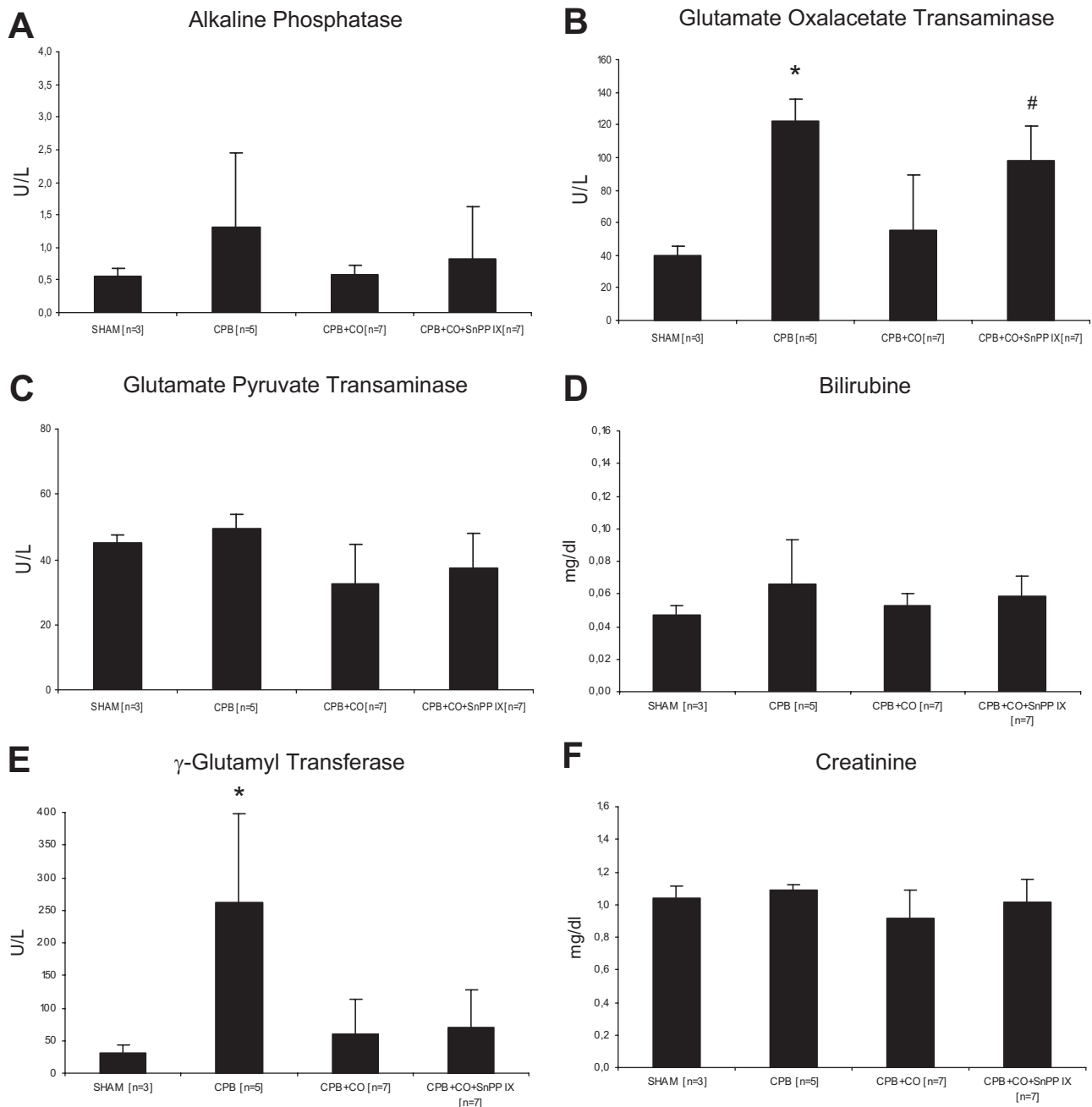
Fig. 5. Activity of alkaline phosphatase (A), glutamate oxalacetate transaminase (B), glutamate pyruvate transaminase (C), bilirubine (D), γ-glutamyl transferase (E), and creatinine (F) in plasma obtained from pigs after cardiopulmonary bypass (CPB), CPB + carbon monoxide (CO), and CPB + CO + tin protoporphyrin IX (SnPP IX) and time-matched control group (SHAM) animals. Data are mean ± SD. * $P < 0.05$, CPB versus SHAM and versus CPB + CO. # $P < 0.001$, CPB + CO + SNPP IX versus CPB + CO.

15, 60, and 120 min; $P < 0.05$). Animals of the CPB + CO group showed no significant differences to baseline.

Interleukin-1β mRNA transcription was significantly increased in the CPB animals (fig. 6B; 120 min CPB; $P < 0.05$ and 15, 60, and 120 min post-CPB; $P < 0.001$).

Pulmonary TNF-α protein levels remained unchanged during the experiments in SHAM animals (fig. 6C). In contrast, CPB significantly increased TNF-α protein expression, reaching a maximum at the end of the observation time (fig. 6C). In the CPB + CO group, TNF-α protein expres-

sion was significantly lower at 10 min CPB (fig. 6C; $P < 0.05$) and during the post-CPB interval (fig. 6C; 60 and 120 min post-CPB; $P < 0.05$). After blocking of the endogenous carbon monoxide production by SnPP IX, TNF-α protein expression remained comparable to carbon monoxide–treated animals (fig. 6C; CPB + CO + SnPP IX).

Likewise, IL-1β protein expression increased significantly in the CPB group at 120 min post-CPB (1,591 pg/ml) compared with the SHAM group, whereas animals of the CPB + CO group showed protein expression
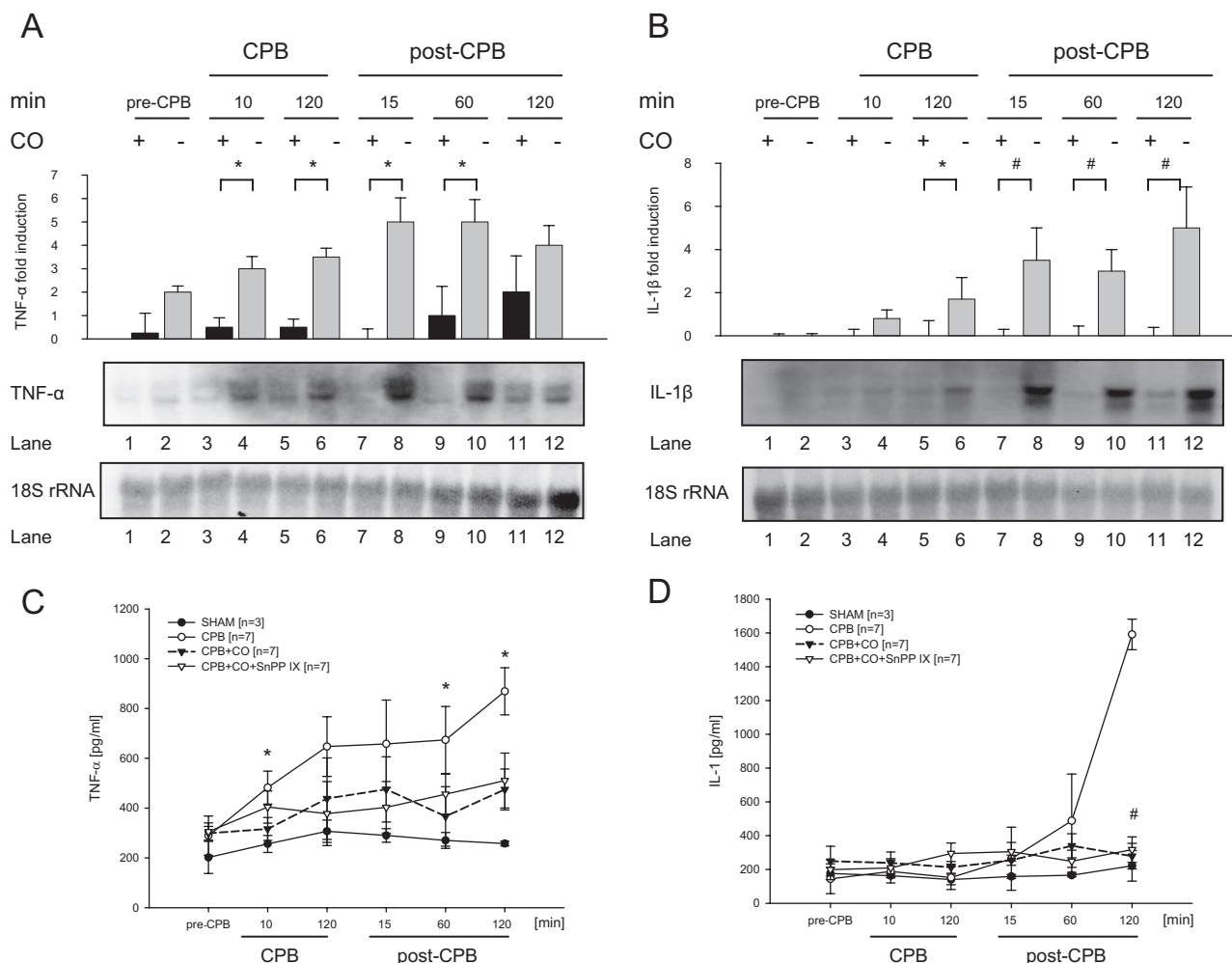
Fig. 6. Proinflammatory cytokine levels in the lung. Values are mean ± SD. (*A*) Tumor necrosis factor (TNF)-*α* messenger RNA (mRNA) levels in animals treated with cardiopulmonary bypass (CPB) (*gray column*) and CPB + carbon monoxide (CO) (*black column*). * *P* < 0.05, CPB + CO *versus* CPB. (*B*) Interleukin (IL)-1*β* mRNA levels in animals treated with CPB (*gray column*) and CPB + CO (*black column*). * *P* < 0.05, CPB + CO *versus* CPB. # *P* < 0.001, CPB + CO *versus* CPB. (*C*) TNF-*α* protein levels in control group (SHAM) animals (*closed circles*) and animals treated with CPB (*open circles*), CPB + CO (*closed triangles*), and CPB + CO + tin protoporphyrin IX (SnPP IX) (*open triangles*). * *P* < 0.05, CPB + CO and CPB + CO + SnPP IX *versus* CPB. (*D*) IL-1 protein levels in SHAM animals (*closed circles*) and animals treated with CPB (*open circles*), CPB + CO (*closed triangles*), and CPB + CO + SnPP IX (*open triangles*). # *P* < 0.001, CPB + CO and CPB + CO + SnPP IX *versus* CPB. rRNA = ribosomal RNA.

comparable to that of SHAM animals (278 *vs.* 192 pg/ml; *P* < 0.001; fig. 6D). After blocking of the endogenous carbon monoxide production by SnPP IX, IL-1 protein expression remained comparable to carbon monoxide–treated animals (fig. 6D; CPB + CO + SnPP IX).

Interleukin-10 mRNA expression was unchanged during CPB (fig. 7A). In contrast, IL-10 mRNA expression was significantly induced by inhaled carbon monoxide treatment. At 120 min CPB and during the whole post-CPB period, IL-10 mRNA expression was 20-fold higher in the CPB + CO group compared with the CPB group (fig. 7A; 120 min post-CPB; *P* < 0.001).

To investigate whether apoptotic effects of the CPB-treated animals were associated with the activation of the crucial effector caspase-3 in the lungs, we performed a caspase-3 activity assay of the cytosols of these tissue samples by using a specific fluorogenic caspase-3 sub-

strate (7-amino-4-methylcoumarin). Likewise, animals exposed to CPB showed a significant increase in caspase-3 activity during post-CPB compared with SHAM animals (fig. 7B; *P* < 0.05 *vs.* CPB). In contrast, animals treated with CPB + CO and with CPB + CO + SnPP IX had a caspase-3 activity not statistically different from SHAM animals (fig. 7B). Therefore, cell death induced by CPB was associated with caspase-3 activation and significantly attenuated by inhaled carbon monoxide treatment.

In addition, IL-10 protein levels in the SHAM and CPB-treated animals were unchanged during the whole experiment (fig. 7C). In contrast, levels of the antiinflammatory cytokine IL-10 were significantly higher in the CPB + CO group (602 *vs.* 70 pg/ml compared with CPB alone; fig. 7C; 60 min post-CPB [*P* < 0.05] and 120 min post-CPB [*P* < 0.001]). In addition, IL-10 protein expression was significantly higher in the CPB + CO + SnPP IX group (469
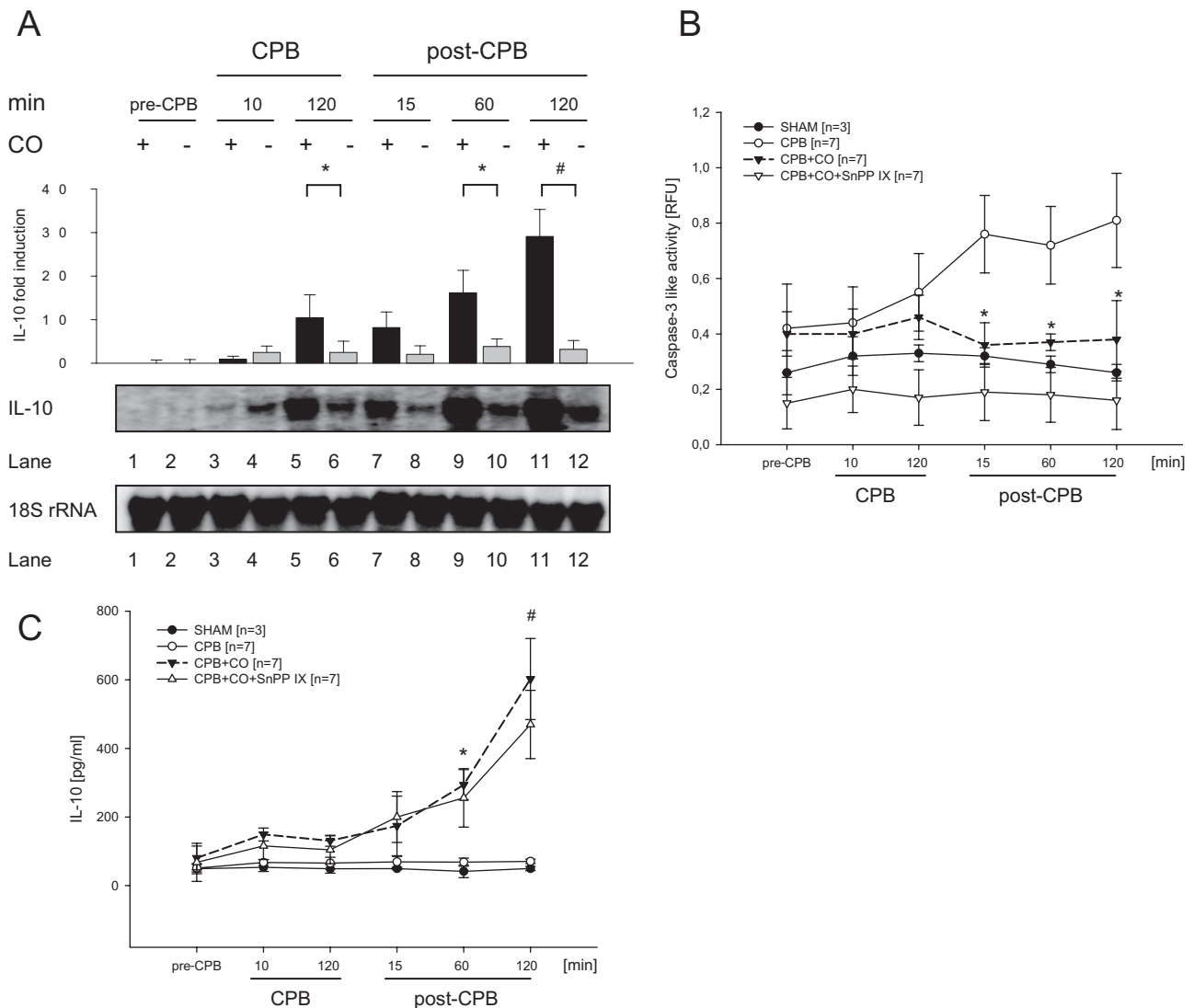
Fig. 7. Interleukin (IL)-10 cytokine levels and caspase-3 like activation in the lung. Values are mean ± SD. (*A*) IL-10 mRNA levels in animals treated with cardiopulmonary bypass (CPB) (*gray column*) and CPB + carbon monoxide (CO) (*black column*). # *P* < 0.001, CPB + CO *versus* CPB. (*B*) Fluorogenic caspase-3 assay of the cytosols. Results are given in arbitrary fluorescent units (RFUs). * *P* < 0.05, CPB + CO *versus* CPB. (*C*) IL-10 protein levels in control group (SHAM) animals (*closed circles*) and animals treated with CPB (*open circles*), CPB + CO (*triangles*), and CPB + CO + tin protoporphyrin IX (SnPP IX) (*open triangles*). * *P* < 0.05, CPB + CO and CPB + CO + SnPP IX *versus* CPB. # *P* < 0.001, CPB + CO and CPB + CO + SnPP IX *versus* CPB. * *P* < 0.05, CPB + CO *versus* CPB. rRNA = ribosomal RNA.

vs. 70 pg/ml compared with CPB alone; fig. 7C; 60 min post-CPB [*P* < 0.05] and 120 min post-CPB [*P* < 0.001]) and comparable to the levels of the CPB + CO animals.

Carbon Monoxide Inhalation Protects Lung Tissue during CPB

Hematoxylin and eosin–stained lung tissue revealed no histopathology 120 min post-CPB in the SHAM animals (fig. 8A). CPB treatment caused atelectasis, edema, and infiltration of macrophages in the alveolar septa. Crucial destruction of the lung architecture was obvious at 120 min post-CPB (fig. 8B). Preoperative inhalation of 250 ppm carbon monoxide inhibited lung damage (fig. 8C). Suppression of endogenous carbon monoxide produc-

tion *via* SnPP IX slightly altered the effect of inhaled carbon monoxide (fig. 8D).

Discussion

The use of CPB in cardiac surgery is often associated with pulmonary inflammation and dysfunction.[1-3] The conditions of CPB, *e.g.*, aortic cross clamping, excluded circulation of heart and lungs, the nutritive blood supply to the lungs continuous only *via* the bronchial arteries, contribute concurrently to the humoral and cellular processes leading to pulmonary dysfunction.[4] After CPB, reperfusion leads to an inflammatory response.[38-40] The incidence of significant pulmonary injury (*e.g.*, acute respiratory distress
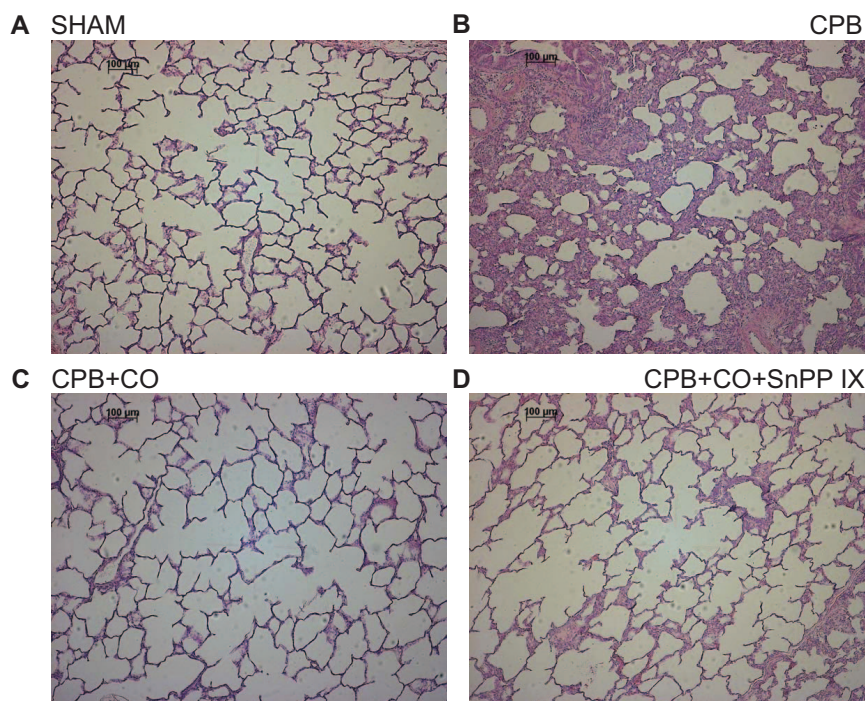
Fig. 8. Microscopic images of the pigs' lung at 120 min after cardiopulmonary bypass (CPB). Representative lung sections of control group (SHAM) animals (*A*) and animals treated with CPB (*B*), CPB + carbon monoxide (CO) (*C*), and CPB + CO + tin protoporphyrin IX (SnPP IX) (*D*) after hematoxylin and eosin staining. Original magnification ×40 (*A–D*).

syndrome) after cardiopulmonary bypass is rare (1.7%).[39] The incidence of a lesser degree of pulmonary dysfunction, defined as a Pa_{O_2}/fraction of inspired oxygen ratio of 150 mmHg or less and chest radiography consistent with pulmonary edema, was 12%.[10] Various interventions failed to blunt the bypass-induced inflammatory response and to decrease lung injury. Carbon monoxide, a powerful antiinflammatory drug, has been demonstrated to suppress the inflammation in a multitude of *in vivo* and transplantation studies.[21–25] Therefore, the hypothesis of this study was that carbon monoxide may potentially serve as a novel intervention in reducing CPB-induced pulmonary inflammation.

Our findings support that inhaled carbon monoxide provides pulmonary antiinflammatory and antiapoptotic effects during CPB. In addition, and of greater importance, these effects occurred when carbon monoxide was administered only as a pretreatment, with the advantage of short exposure time, which results in a limited binding to hemoglobin, because the extent of carbon monoxide hemoglobin formation depends on the dose and the time of application.[41] The effect had the following characteristics: (1) Systemic inflammation and adverse effects on extrapulmonary organs seem to be not important, because cytokine mRNA expression in mononuclear blood cells was not detectable and serum markers of hepatic, cardiac, or renal injury were also not detectable. (2) Carbon monoxide inhalation suppressed the CPB-induced pulmonary expression of the proinflammatory cytokines TNF-α and IL-1β. (3) Carbon monoxide inhalation significantly induced pulmonary IL-10 protein levels during the whole experiment. (4) Analysis of mRNA cytokine transcripts suggests a transcriptional regulation due to carbon monoxide–mediated effects. (5) Apoptosis in the lung is attenuated, because carbon monoxide inhalation inhibited effector caspase activity in lung tissue during CPB. These data suggest that the antiinflammatory and antiapoptotic properties of carbon monoxide may confer cytoprotection in a pig model of CPB-induced pulmonary inflammation and dysfunction.

In the lung, several studies have previously provided evidence that carbon monoxide mediates antiinflammatory and antiapoptotic effects during lung injury. For example, Choi *et al.*[42] demonstrated that the same low concentration of inhaled carbon monoxide (250 ppm) as used in the current study significantly reduced TNF-α levels in lavage fluid in a rat model of ventilator-induced lung injury. In addition, the same group demonstrated that rats exposed to carbon monoxide exhibit a marked tolerance to lethal concentrations of hyperoxia.[35] This increased survival was associated with highly significant attenuation of hyperoxia-induced lung injury as assessed by the volume of pleural effusion, protein accumulation in the airways, and histologic analysis.[35] Otterbein *et al.*[21] further demonstrated that carbon monoxide also exerts antiinflammatory effects in an *in vivo* model of lipopolysaccharide-induced inflammation. Carbon monoxide, even at low concentrations, selectively modulated the proinflammatory/antiinflammatory cascade of cytokines: Carbon monoxide inhibited the lipopolysaccharide-induced production of TNF-α, IL-1β, and macrophage inflammatory protein 1β while increasing IL-10 production.[21] These data are in sharp contrast to similar experiments performed in humans, where carbon monoxide inhalation (10–500 ppm for 1 and 2 h after lipopolysaccharide infusion) did not

attenuate transiently increased plasma concentrations of inflammatory mediators (TNF-α, IL-6, IL-8, IL-10, IL-1).[43]

The pulmonary ischemia during bypass and the reperfusion injury after declamping in our setting may also be considered as an ischemia and reperfusion injury. In a model of renal ischemia and reperfusion in the rat, inhalation of carbon monoxide has been shown to normalize the inflammatory parameters and significantly reduced ischemia and reperfusion injury.[44] Taken together, these data suggest that carbon monoxide is a selective and specific antiinflammatory modulator, and raises the question of which mechanisms are responsible for mediating these effects of carbon monoxide.

The suppression of cell apoptosis by carbon monoxide inhalation may represent a mechanism by which carbon monoxide provides protection against induced organ injury.[22,23,45] Although the precise physiologic mechanism of antiapoptotic effects mediated by carbon monoxide has not yet been established, it is possible that this effect may be related to the powerful antioxidative and antiinflammatory functions of carbon monoxide. Inhaled carbon monoxide might limit the generation of reactive oxygen species, decrease the presence of free metal irons,[46] and down-regulate proinflammatory cytokines.[47] In addition, recent evidence suggested that carbon monoxide–treated animals showed an early up-regulation of the antiapoptotic gene Bcl-2 and down-regulation of proapoptotic genes (e.g., Fax and Bax).[27,48] The up-regulation of antiapoptotic genes and down-regulation of proapoptotic genes can suppress apoptosis of pulmonary cells. Our study showed that pulmonary effector caspase activity decreased after carbon monoxide inhalation. Cell apoptosis serves as a useful marker of lung injury in response to oxidative stress and inflammation such as ischemia and reperfusion injury.[46]

Hyperoxia may significantly alter normal cellular lung physiology, promoting formation of reactive oxygen species, activation of programmed cell death pathways, and expression of inflammatory cytokines.[49] For the most part, up-regulated cytokines confer a degree of tolerance to hyperoxia via attenuation of cell death signaling pathways or augmentation of antioxidant activity.[50] In our experiments, we used moderate hyperoxic levels with Pao$_2$ values below 250 mmHg for a short period to obtain normoxia in the CPB + CO groups.

On the basis of the results presented here from animal studies, we cannot propose that carbon monoxide might possibly be used as an antiinflammatory agent in human cardiac surgery with CPB, because controlled human studies correlating carboxyhemoglobin to organ function do not exist. The use of a gas normally considered toxic must be carefully weighed. In this study, we used lower concentrations compared with the few human studies that examined the effects of continuous carbon monoxide inhalation on carboxyhemoglobin levels.[51,52]

For example, volunteers breathed carbon monoxide concentrations of 400–1,000 ppm until their carboxyhemoglobin levels reached 10–12% and were then assigned to hyperbaric oxygen therapy.[51] A recently published clinical study by Mayr et al.[43] showed no clinical signs of carbon monoxide toxicity after exposure of 250 and 500 ppm. Modest increases in carboxyhemoglobin levels equivalent to that resulting from cigarette smoking do not have any appreciable acute sympathetic and hemodynamic effects in healthy humans.[52] Furthermore, the concentrations used here are comparable to the levels used in humans (0.03%) during measurement of lung diffusion capacity for carbon monoxide, a standard pulmonary function test.[53] However, this study involved continuous carbon monoxide exposure, but it could be that these carbon monoxide concentrations might not be tolerated.

In conclusion, our findings support the theories that inhaled carbon monoxide provides pulmonary antiinflammatory effects during CPB and lead to a better understanding of the complex regulatory function of carbon monoxide in lung injury. Based on the observations of this study, it is tempting to speculate that inhaled carbon monoxide could represent a potential new therapeutic modality for counteracting CPB-induced lung injury.

References

1. Grover FL: The Society of Thoracic Surgeons National Database: Current status and future directions. Ann Thorac Surg 1999; 68:367–73
2. Gao D, Grunwald GK, Rumsfeld JS, Mackenzie T, Grover FL, Perlin JB, McDonald GO, Shroyer AL: Variation in mortality risk factors with time after coronary artery bypass graft operation. Ann Thorac Surg 2003; 75:74–81
3. Laffey JG, Boylan JF, Cheng DC: The systemic inflammatory response to cardiac surgery: Implications for the anesthesiologist. ANESTHESIOLOGY 2002; 97: 215–52
4. Hall RI, Smith MS, Rocker G: The systemic inflammatory response to cardiopulmonary bypass: Pathophysiological, therapeutic, and pharmacological considerations. Anesth Analg 1997; 85:766–82
5. Sinclair DG, Haslam PL, Quinlan GJ, Pepper JR, Evans TW: The effect of cardiopulmonary bypass on intestinal and pulmonary endothelial permeability. Chest 1995; 108:718–24
6. Weissman C: Pulmonary function after cardiac and thoracic surgery. Anesth Analg 1999; 88:1272–9
7. Shroyer AL, Coombs LP, Peterson ED, Eiken MC, DeLong ER, Chen A, Ferguson TB Jr, Grover FL, Edwards FH: The Society of Thoracic Surgeons: 30-day operative mortality and morbidity risk models. Ann Thorac Surg 2003; 75: 1856–64
8. Messent M, Sullivan K, Keogh BF, Morgan CJ, Evans TW: Adult respiratory distress syndrome following cardiopulmonary bypass: Incidence and prediction. Anaesthesia 1992; 47:267–8
9. Christenson JT, Aeberhard JM, Badel P, Pepcak F, Maurice J, Simonet F, Velebit V, Schmuziger M: Adult respiratory distress syndrome after cardiac surgery. Cardiovasc Surg 1996; 4:15–21
10. Rady MY, Ryan T, Starr NJ: Early onset of acute pulmonary dysfunction after cardiovascular surgery: Risk factors and clinical outcome. Crit Care Med 1997; 25:1831–9
11. Kaul TK, Fields BL, Riggins LS, Wyatt DA, Jones CR, Nagle D: Adult respiratory distress syndrome following cardiopulmonary bypass: Incidence, prophylaxis and management. J Cardiovasc Surg 1998; 39:777–81
12. Milot J, Perron J, Lacasse Y, Letourneau L, Cartier PC, Maltais F: Incidence and predictors of ARDS after cardiac surgery. Chest 2001; 119:884–8

13. Butler J, Rocker GM, Westaby S: Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 1993; 55:552–9

14. Schlensak C, Doenst T, Preusser S, Wunderlich M, Kleinschmidt M, Beyersdorf F: Cardiopulmonary bypass reduction of bronchial blood flow: A potential mechanism for lung injury in a neonatal pig model. J Thorac Cardiovasc Surg 2002; 123:1199–205

15. Hill GE, Snider S, Galbraith TA, Forst S, Robbins RA: Glucocorticoid reduction of bronchial epithelial inflammation during cardiopulmonary bypass. Am J Respir Crit Care Med 1995; 152:1791–5

16. Engelman RM, Rousou JA, Flack JE III, Deaton DW, Kalfin R, Das DK: Influence of steroids on complement and cytokine generation after cardiopulmonary bypass. Ann Thorac Surg 1995; 60:801–4

17. Cremer J, Martin M, Redl H, Bahrami S, Abraham C, Graeter T, Haverich A, Schlag G, Borst HG: Systemic inflammatory response syndrome after cardiac operations. Ann Thorac Surg 1996; 61:1714–20

18. Khabar KS, elBarbary MA, Khouqeer F, Devol E, al Gain S, Al Halees Z: Circulating endotoxin and cytokines after cardiopulmonary bypass: Differential correlation with duration of bypass and systemic inflammatory response/multiple organ dysfunction syndromes. Clin Immunol Immunopathol 1997; 85:97–103

19. Brix-Christensen V, Petersen TK, Ravn HB, Hjortdal VE, Andersen NT, Tonnesen E: Cardiopulmonary bypass elicits a pro- and anti-inflammatory cytokine response and impaired neutrophil chemotaxis in neonatal pigs. Acta Anaesthesiol Scand 2001; 45:407–13

20. Brix-Christensen V: The systemic inflammatory response after cardiac surgery with cardiopulmonary bypass in children. Acta Anaesthesiol Scand 2001; 45:671–9

21. Otterbein LE, Bach FH, Alam J, Soares M, Tao LH, Wysk M, Davis RJ, Flavell RA, Choi AM: Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000; 6:422–8

22. Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, Lee PJ: Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J Biol Chem 2003; 278:1248–58

23. Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR, Choi AM: Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation *via* anti-inflammatory and anti-apoptotic effects. Am J Pathol 2003; 163:231–42

24. Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, Choi AM: Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. J Biol Chem 2003; 278:36993–8

25. Nakao A, Toyokawa H, Abe M, Kiyomoto T, Nakahira K, Choi AM, Nalesnik MA, Thomson AW, Murase N: Heart allograft protection with low-dose carbon monoxide inhalation: Effects on inflammatory mediators and alloreactive T-cell responses. Transplantation 2006; 81:220–30

26. Liu XM, Chapman GB, Peyton KJ, Schafer AI, Durante W: Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. Cardiovasc Res 2002; 55:396–405

27. Zhang X, Shan P, Alam J, Davis RJ, Flavell RA, Lee PJ: Carbon monoxide modulates Fas/Fas ligand, caspases, and Bcl-2 family proteins *via* the p38α mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury. J Biol Chem 2003; 278:22061–70

28. Zhang X, Shan P, Alam J, Fu XY, Lee PJ: Carbon monoxide differentially modulates STAT1 and STAT3 and inhibits apoptosis *via* a phosphatidylinositol 3-kinase/Akt and p38 kinase-dependent STAT3 pathway during anoxia-reoxygenation injury. J Biol Chem 2005; 280:8714–21

29. Sarady JK, Zuckerbraun BS, Bilban M, Wagner O, Usheva A, Liu F, Ifedigbo E, Zamora R, Choi AM, Otterbein LE: Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. FASEB J 2004; 18:854–6

30. Lavitrano M, Smolenski RT, Musumeci A, Maccherini M, Slominska E, Di Florio E, Bracco A, Mancini A, Stassi G, Patti M, Giovannoni R, Froio A, Simeone F, Forni M, Bacci ML, D'Alise G, Cozzi E, Otterbein LE, Yacoub MH, Bach F, Calise F: Carbon monoxide improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass in pigs. FASEB J 2004; 18: 1093–5

31. Uemura K, Adachi-Akahane S, Shintani-Ishida K, Yoshida K: Carbon monoxide protects cardiomyogenic cells against ischemic death through L-type Ca2+ channel inhibition. Biochem Biophys Res Commun 2005; 334:661–8

32. Mazzola S, Forni M, Albertini M, Bacci ML, Zannoni A, Gentilini F, Lavitrano M, Bach FH, Otterbein LE, Clement MG: Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. FASEB J 2005; 19:2045–7

33. Ryter SW, Alam J, Choi AM: Heme oxygenase-1/carbon monoxide: From basic science to therapeutic applications. Physiol Rev 2006; 86:583–650

34. Wu L, Wang R: Carbon monoxide: Endogenous production, physiological functions, and pharmacological applications. Pharmacol Rev 2005; 57:585–630

35. Otterbein LE, Mantell LL, Choi AM: Carbon monoxide provides protection against hyperoxic lung injury. Am J Physiol 1999; 276:L688–94

36. Hoetzel A, Geiger S, Loop T, Welle A, Schmidt R, Humar M, Pahl HL, Geiger KK, Pannen BHJ: Differential effects of volatile anesthetics on hepatic heme oxygenase-1 expression in the rat. ANESTHESIOLOGY 2002; 97:1318–21

37. Loop T, Dovi-Akue D, Frick M, Roesslein M, Egger L, Humar M, Hoetzel A, Schmidt R, Borner C, Pahl HL, Geiger KK, Pannen BHJ: Volatile anesthetics induce caspase-dependent, mitochondria-mediated apoptosis in human T lymphocytes *in vitro*. ANESTHESIOLOGY 2005; 102:1147–57

38. Hill GE: Cardiopulmonary bypass-induced inflammation: Is it important? J Cardiothorac Vasc Anesth 1998; 12:21–5

39. Asimakopoulos G: Systemic inflammation and cardiac surgery: An update. Perfusion 2001; 16:353–60

40. Paparella D, Yau TM, Young E: Cardiopulmonary bypass induced inflammation: Pathophysiology and treatment. An update. Eur J Cardiothorac Surg 2002; 21:232–4

41. Hoetzel A, Dolinay T, Schmidt R, Choi AM, Ryter SW: Carbon monoxide in sepsis. Antioxid Redox Signal 2007; 9:2013–26

42. 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; 170:613–20

43. Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, Wagner O, Jilma B: Effects of carbon monoxide inhalation during experimental endotoxemia in humans. Am J Respir Crit Care Med 2005; 171:354–60

44. Neto JS, Nakao A, Kimizuka K, Romanosky AJ, Stolz DB, Uchiyama T, Nalesnik MA, Otterbein LE, Murase N: Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide. Am J Physiol 2004; 287: F979–89

45. Soares MP, Usheva A, Brouard S, Berberat PO, Gunther L, Tobiasch E, Bach FH: Modulation of endothelial cell apoptosis by heme oxygenase-1-derived carbon monoxide. Antioxid Redox Signal 2002; 4:321–9

46. Ng CS, Wan S, Yim AP: Pulmonary ischaemia-reperfusion injury: Role of apoptosis. Eur Respir J 2005; 25:356–63

47. Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A: Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995; 146:1220–34

48. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP: Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 2000; 192:1015–26

49. Lee PJ, Choi AM: Pathways of cell signaling in hyperoxia. Free Radic Biol Med 2003; 35:341–50

50. Bhandari V, Elias JA: Cytokines in tolerance to hyperoxia-induced injury in the developing and adult lung. Free Radic Biol Med 2006; 41:4–18

51. Takeuchi A, Vesely A, Rucker J, Sommer LZ, Tesler J, Lavine E, Slutsky AS, Maleck WH, Volgyesi G, Fedorko L, Iscoe S, Fisher JA: A simple and new method to accelerate clearance of carbon monoxide. Am J Respir Crit Care Med 2000; 161:1816–9

52. Hausberg M, Somers VK: Neural circulatory responses to carbon monoxide in healthy humans. Hypertension 1997; 29:1114–8

53. Graham BL, Mink JT, Cotton DJ: Effects of increasing carboxyhemoglobin on the single breath carbon monoxide diffusing capacity. Am J Respir Crit Care Med 2002; 165:1504–10