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Original Article

Effects of inhaled carbon monoxide and glucocorticoids in porcine endotoxin sepsis

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Abstract: Background: Recent animal studies have demonstrated that pre-treatment with inhaled carbon monoxide (iCO) exert anti-inflammatory effects in various septic models. In all these models, there is no information whether iCO might act therapeutically after the onset of septic damage. The objective of this study was to investigate the potential anti-inflammatory effects of iCO to treat established injury in a model of porcine endotoxin sepsis. Methods: Five groups of pigs (n=6 in each group), were studied under anesthesia and mechanical ventilation: healthy control group (HC); lipopolysaccharide (LPS) groups, animals received continuous IV infusion of LPS for 6 hours; 2.5 hours after of LPS infusion treated groups received either: 250 ppm of iCO for 3.5 h, (LPS+CO group); 3 mg/Kg hydrocortisone bolus [Steroid (ST)], (LPS+ST group); or both steroid and iCO, (LPS+CO+ST group). Measurements of haemodynamics, blood gases, respiratory mechanics and biochemistry of organ function, were made. At the end of the experiment lung tissue was taken for analysis of histology and inflammatory markers: tumor necrosis factor-alpha (TNF-α), nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) and glucocorticoid receptor (GR). Results: LPS administration induced a dramatic inflammatory injury in lungs, increased expression of TNF-α, NF-κB, AP-1, down regulation of GR, pulmonary hypertension and severe deterioration of respiratory mechanics, oxygenation and organ function. Treatment with steroids and to greater extent with iCO significantly improved the microscopic appearance of the lung but had no effect on inflammatory markers. iCO significantly decreased pulmonary hypertension induced by LPS, without an obvious protective effect on organ function. Conclusion: Using this porcine sepsis model we find that treatment with iCO after the septic damage decreases pulmonary hypertension and partially protects the lung tissue from the inflammatory destruction induced by LPS but has no beneficial effects on organ function.

Keywords: Carbon monoxide, steroids, porcine sepsis model, acute lung injury, organ function

Introduction

Sepsis remains a major cause of morbidity and mortality, and is a major cause of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. Several therapies targeting the upregulated inflammatory pathways of sepsis have been studied to improve survival, but despite continuing research few effective therapies have been identified. In the last few years animal studies indicate that carbon monoxide (CO) inhalation might modulate the inflammatory response to lipopolysaccharide (LPS). The exact mechanisms by which CO acts at the molecular level remain incompletely understood. CO affects several intracellular signal pathways, including chemokines, macrophage inflammatory protein (MIP)-1β, mitogen-activated protein kinase pathway (MAPK), nuclear factor kappa B (NF-κB), adhesion molecules, tumor necrosis factor alpha (TNF-α), interleukins, and others [2]. Many animal studies in rodents/mice have demonstrated that inhaled CO (iCO) at low concentrations can serve as a potent systemic anti-inflammatory molecule [2]. Thus, pre-treatment with inhaled CO in a porcine model of endotoxic shock significantly ameliorated several of the acute pathological changes induced by LPS [3]. However, the effectiveness of CO treatment is not a universal finding. Negative results were seen from the first clinical study in humans during experimental endotoxemia and iCO [4]. Similarly, no effects of iCO were found in a mouse model of ALI induced by LPS or oleic

acid [5].

Other groups of anti-inflammatory agents include the glucocorticoids (GCs), non-steroid anti-inflammatory drugs, as well as nitric oxide (NO). Steroids (ST) have been used in the treatment of acute lung injury and ARDS, sepsis, as well as in a broad spectrum of other diseases. However, no clear benefit has been documented in ALI/ARDS, although short course of "physiologic" doses of hydrocortisone, may increase survival rate and shock reversal in patients with vasopressor dependent septic shock [6]. In a previous study from our group we found that there is an interaction between inhaled NO and GCs. Inhaled NO up-regulated the expression of glucocorticoid receptors (GR), and simultaneous administration of glucocorticoids enhanced the anti-inflammatory effect in a porcine endotoxin, "sepsis" model [7]. NO and CO have many similar functions, and since CO also acts as a potential anti-inflammatory substance, we hypothesized that a combination of CO and steroid also has an enhanced anti-inflammatory effect.

Pre-treatment with iCO before the septic insult is far from the clinical reality and we considered that the evolution of sepsis before iCO administration is more clinically relevant. We therefore investigated the effects of low dose inhaled CO on hemodynamics, respiratory parameters, organ function, as well as the anti-inflammatory effects on the lung in a porcine model of endotoxin induced model of sepsis and ALI. We chose an LPS model because sepsis is a major cause of mortality in intensive care units and frequently associated with the development of indirect, extrapulmonary ALI. LPS challenge was done as an infusion rather than as a triggering bolus event, because this procedure produces a reliable model of an experimental sepsis response without immediate overwhelming pulmonary hypertension resulting in cardiovascular failure [8]. In addition, we also studied if the combination of iCO and steroids can have synergistic effects.

Material and methods

Animal preparation and monitoring

The Animal Research Ethics Committee of Uppsala University approved the study. Thirty pigs of Swedish country breed of either sex 2-3 months old and weighing 26-35 kg (mean 29.1 ±3.17 Kg) were studied. Anesthesia was induced with 0.04 mg/kg atropine i.m., 6 mg/kg tiletamine/zolazepam (Zoletid, Virbac Laboratories), and 2.2 mg/kg xylazine chloride (Rompur, Bayer AG, Germany). Anesthesia was maintained by continuous infusion of ketamine 750 mg/h, midazolam 2.5 mg/h, pancuronium 7 mg/h, and fentanyl, 150 μg/h. 1000 ml of pre-warmed (38°C) isotonic saline was given i.v. prior to baseline measurements to prevent dehydration, and hydration was thereafter maintained by i.v infusion at a rate of 15ml/kg/h. The pig was in the supine position, the trachea was intubated with a 7 mm inner diameter tube and mechanical ventilation was provided (Servo ventilator 300, Servo Screen 390, Maquet Life Support Systems Sweden). The initial settings were as follows: volume-controlled mode (constant inspiratory flow), tidal volume 10 ml/Kg, rate 20 breaths/min, inspiration to expiration ratio of 1:2, positive end expiratory pressure 5 cm H_2O and inspired fraction of oxygen 0.5 to protect the animals from deleterious effects of hypoxemia after the creation of lung damage. Before the baseline measurements the respiratory rate was adjusted to obtain normocapnia ($PaCO_2$ = 35-40 mm Hg) and then the ventilator settings were kept constant throughout the experiment. A catheter was surgically inserted into the carotid artery for continuous arterial pressure monitoring and arterial blood sampling. A triple lumen 7 Fr thermodilution pulmonary artery catheter (CritCath SP5107H-14 Becton Dickinson Utah, USA) and an 18–gauge catheter were surgically placed via the right jugular vein into the pulmonary artery and superior vena cava respectively, verified by pressure wave forms. A balloon catheter was surgically inserted into the bladder after a suprapubic incision.

Protocol and groups

Five groups of 6 pigs each were studied under anesthesia and mechanical ventilation for a six-hour period. One hour after surgical preparation, baseline measurements were made (blood gases, hemodynamic parameters, blood samples) and then the animals were randomly submitted to one of the following groups. The healthy control group (HC) received only anesthesia and mechanical ventilation. In the LPS group (LPS), as well as in the other groups receiving LPS, a sepsis-model with acute lung injury was created thirty minutes (time 0) after the baseline measurements by continuous i.v. infusion of endotoxin (Escherichia coli lipopoly-

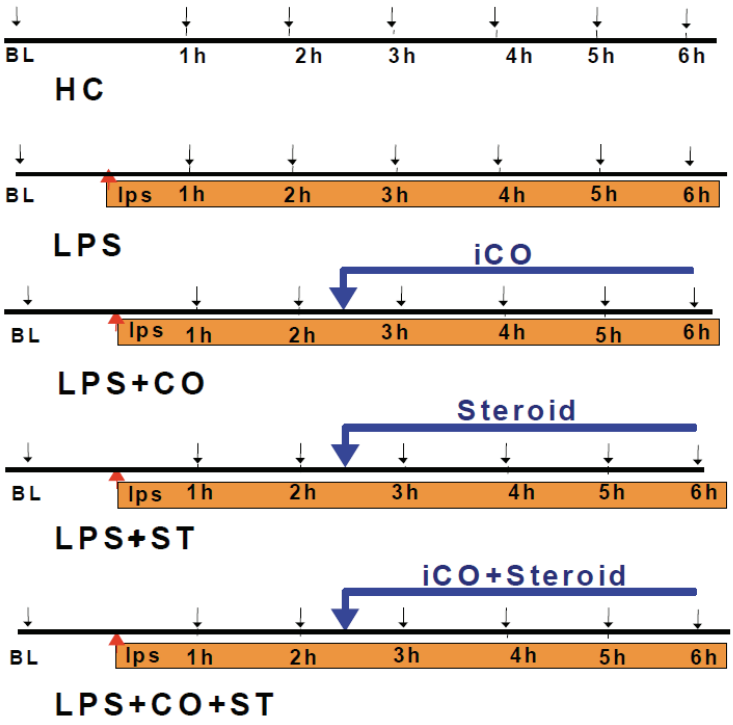

Figure 1 Schematic drawing of the protocol. Five groups of 6 pigs each were studied under anesthesia and mechanical ventilation for 6 hour period. (HC) =Healthy control pigs, (LPS) = continuous iv infusion of lipopolysaccharide (LPS) for 6 hours (20μg/kg/hour for 2.5 hours followed by 10 μg/kg/hour for 3.5 hours), (LPS+CO) = LPS + 250 ppm of CO inhalation continuously for 3.5 hours, (LPS+ST)=LPS+ iv bolus of 3 mg/kg hydrocortisone 2.5 hours after LPS infusion, (LPS+CO+ST)= LPS +250 ppm inhalation of CO continuously for 3.5 hours +iv bolus of 3 mg/kg hydrocortisone. Treatment with CO, ST or CO+ST was initiated 2.5 hours after of LPS infusion. BL=base line.

saccaride, 0111:B4 Sigma-Aldrich, Stockholm, Sweden, dissolved in saline) for 6 hours (20 μg /kg/hour for 2.5 hours, followed by 10 μg/kg/hour for the remaining 3.5 hours). The LPS+steroid group (LPS+ST) received an i.v. bolus of hydrocortisone 3 mg/kg (Solucortef, Pharmacia) after 2.5 hours of endotoxin infusion. The LPS+iCO group (LPS+CO) received continuously 250 ppm inhaled CO after 2.5 hours of endotoxin infusion for the remaining 3.5 hour study period. The LPS+iCO+steroid group (LPS+CO+ST) received both steroid and inhaled CO (as above). The experiments thus lasted for 6 hours and treatment with CO or ST was initiated after 2.5 hours of LPS infusion, when mean pulmonary arterial pressure (MPAP) had stabilized. At the end of the 6- hour period the pigs were killed by an overdose of potassium chloride and lung (left lower lobe) tissues were taken for histological evaluation and measurements of glucocorticoid receptors and inflammatory markers. A schematic drawing of the protocol is shown in **Figure 1**.

Measurements

Heart rate (HR), mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP) and central venous pressure (CVP) were continuously displayed on a monitor (series 7010, Marquette Electronics; Milwaukee, WI) using pressure transducers (Gabarith ™ PMSET 1DT-XX, Becton Dickinson Critical Care Systems Utah USA) zeroed at the midthoracic level. Cardiac output (CO) was measured by thermodilution as the average of three determinations after injection of 10 ml cold isotonic saline randomly distributed over the respiratory cycle. Pulmonary capillary wedge pressure (PCWP) was recorded at the end of expiration. Pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) were calculated by standard formulas.

Arterial blood samples were collected and analyzed immediately for pH, PaO_2, $PaCO_2$, Base Excess (BE), arterial O_2 saturation, hemoglobin and carboxyhemoglobin (COHb) concentration (ABL 500, Radiometer; Copenhagen, Denmark; OSM 3, Hemoximeter; Radiometer). Blood gas data were corrected for pig blood. Inspiratory flow, tidal volume and airway pressures (peak airway pressure, end-inspiratory/end-expiratory pause pressures) were measured in the ventilator. Respiratory system compliance (Crs) was calculated by dividing tidal volume with the dif-

ference between the end-inspiratory and the end expiratory pause pressures. Airways resistance (Raw) was calculated as the difference between the peak airway pressure and the end-inspiratory pause pressure divided by the inspiratory flow.

Blood samples for leucocytes cell count, neutrophils, and biochemical analysis (serum creatinine, urea, bilirubin, conjugated bilirubin, troponine and cortisol) were immediately sent to the laboratory for analysis. After baseline recordings and sampling, the measurements were repeated as follows: hemodynamic, and pulmonary function parameters obtained at 0.5, 1.5, 2.5, 3, 4, 5, 6 hours, blood gases at 2.5, 3, 4, 5, 6 hours, and blood samples at 2.5, 4, 6 hours after the initiation of LPS infusion (time 0).

CO administration

Tank gas with CO at a concentration of 1990 +/- 2‰ parts per million (ppm) in 21% O_2, make up N_2 (AGA AB Linde gas, Lidingö, Sweden) was fed into the air inlet of the ventilator by using a gas/air mixer (961 Servo ventilator Siemens Elema Sweden). The inhaled CO was set to 250 ppm, by means of the gas/air mixer, and the concentration in inspired gas was checked with a high-sensitive CO meter (MIRAN 1A-CVR General purpose Gas analyzer), daily calibrated at zero and 302 ppm CO, (302 ppm +/- 2% CO tank, Linde gas, Lidingö, Sweden). The expired gas from the ventilator was evacuated via a suction channel to outside air and the ambient air at the laboratory was checked for CO with a sensitive portable CO analyser (Interscan 4000 series).

Histopathology

All tissues were immediately put into 10% neutral formalin for fixation. The tissues were embedded in paraffin cut into 5 μm thick slices and conventional haematoxylin and eosin (HE) staining was performed. Morphological changes in lung were studied in five high-power fields (HPFs) in each of two lung sections from each pig and were quantified into five grades (-, +, ++, +++, ++++). The grading was based on number of inflammatory cells, edema formation, and hemorrhage. Inflammatory cells: number of neutrophils per high power field (/HPF) -: <5/HPF; +: <15/HPF; ++: <25/HPF; +++: <35/HPF; ++++: >35/HPF. Edema: - the width of septum between two lung lobules in Healthy Control tissue; +: two times the width of the septum in healthy control; ++: three times the width of the normal septum; +++: four times the width of the normal septum;++++ : five times the width of the normal septum. Hemorrhage: areas of hemorrhage in tissue -: <10%; +: <25%; ++: <50%; +++: <75%; ++++: >75% .

Immunohistochemistry

Immunohistochemical detections of GR, activator protein-1 (AP-1), NF-κB and TNF-α were achieved with standard streptavidin-biotin-peroxidase detection techniques (GR: Santa Cruz Biotechnology, Inc Catalogue No. sc-I004 USA, Rabbit Polyclonal Antibody, dilution 1:200; AP-1: SIGMA, Product No. A5968 Germany, dilution 1.200; NF-κB; SIGMA, Product No. N5823 Germany, dilution 1:100; TNF-α: Santa Cruz Biotechnology, Inc, Catalogue No. sc- 7317 USA, mouse monoclonal Antibody, dilution 1:100). Pilot experiments showed that microwave antigen retrieval and overnight incubation of the primary antibodies yielded the best sensitivity. The antibodies were detected with the peroxidase-antiperoxidase method using 3-amino-9-ethyl-carbazole (AEC, SIGMA Catalogue No. A-6926 Germany) as chromogen. All slides were counterstained with 0.1% Certified Hematoxylin (SIGMA Catalogue No. MHS-16 Germany).

Image analysis of immunohistochemistry

An image-analysis system consisting of a 12-bit cooled charge-coupled device camera (Sensys KAF 1400, Photometrics, Tucson, AZ) mounted on a fully automated Leica DM RXA microscope (Wetzlar, Germany) was used to digitize gray-scale images to a dual-Pentium host computer. Microscope settings were kept constant throughout all measurements (Leica PL Fluotar 40x/0.75). A stabilized 12-V tungsten-halogen lamp (100 W) was used for illumination. Microdensitometry was performed with a custom-designed filter manufactured by Omega Optical (Brattleboro, VT) for absorbency measurement of the Vector red substrate (central wavelength 525 nm, half bandwidth 10 ± 2 nm). The optimal central wavelength was determined by measurement of the substrate in a Leica MPV SP microscope photometer system. The findings of immunochemical staining in lung for GR,

Table 1. Hemodynamic parameters

		HC	LPS	LPS+CO	LPS+ST	LPS+CO+ST
HR	BL	115.2±15.2	108.7±6.3	106.5±13.7	102.2±10	98.3±12.3
b/min	2.5h	99.7±20.3	131.5±17.7	112.3±10.3	112.7±5.5	108.7±16.3
	6 h	103.2±22.2	128.7±14	116.7±6.9	104.0±10.5	112.3±15.8
MAP	BL	80± 13.9	78.2 ±11.2	72.8±11.4	85±9.4	80±12.7
mm Hg	2.5h	78±15.3	65.7±10.4	64±13.5	75.3±8.5	69.7±8.5
	6h	91.2±20.6	71±13.4	71.3±12.8	82.3±18.7	86.2±19
MPAP	BL	15.6±1.2	17.6±2.8	15.6±1.2	14.8±0.4	15.1±0.9
mm Hg	2.5h	16.6±1.3	39±2.19	36±2.4	36.6±3.5	36±4
	6h	17.3±2.3	42.5±3.7	30.6±3.7*	34.5±3.7	35.1±3.5
CVP	BL	4.7±1.4	5.2±1.5	4.8±1.3	4.2±0.4	4.5±1.4
mmHg	2.5h	5.7±1.8	7.2±1.7	7±1.4	5.3±1.4	6±1.4
	6h	5.8±2.5	7.8±2.3	6.7±1.8	6.3±1.4	6.3±1
PCWP	BL	7.7±1.4	7.7±1.2	6.7±0.8	7.3±1.4	7.3±1
mmHg	2.5h	8.7±1.6	10.3±1.5	10±1.4	9.8±2.6	9.5±1.4
	6h	8.3±2	10.7±1.4	8.2±1.2	9.0±0.9	8.8±1
CO	BL	4.0±0.8	3.9±0.5	3.6±0.7	3.7±0.4	3.7±0.7
l/min	2.5h	3.7±1.1	3.8±0.8	2.8±1.1	3±0.8	3.1±0.5
	6h	4.2±1	2.5±0.8	2.8±1.1	2.3±0.5	2.9±0.9
SVR	BL	1525±284	1505±123	1536±180	1751±98	1695±349
dynes/sec/cm^{-5}	2.5h	1602±176	1282±343	1918±1259	1918±462	1642±249
	6h	1624±167	2143±783	2177±1077	2631±733	2358±1003
PVR	BL	161.2±24.8	204.4±17.3	208.4±58.5	161.7±20.9	174.8±38
dynes/sec/cm^{-5}	2.5h	178.9±31	669.7±114	843±405	764±229	691±165
	6h	175.3±30.2	1093±398.8	734.6±314.3	914.9±304	788.5±265

Effects of treatment on hemodynamic parameters during a 6-h endotoxin infusion. 2.5 hours after the onset of endotoxin infusion treated animals received either 250 ppm inhaled CO continuously, or an iv bolus of 3 mg/kg iv hydrocortisone or both inhaled CO and steroids. Data are expressed as means ±SD at baseline, 2.5 and 6 hours after of LPS infusion (before treatment and 3.5 hours of CO or steroid treatment respectively). Comparisons between LPS group vs treated groups (LPS+CO, LPS+ST, LPS+CO+ST) were made by two–way ANOVA for repeated measurements during treatment period (3-6h) *P<0.05 or **P<0.01. With the exception of parameter changes on HC group all parameters from all groups were <0.05 compared with baseline (ns= HR, MAP). BL= baseline, HR=heart rate, MAP= mean arterial pressure, MPAP= mean pulmonary arterial pressure, CVP=central venous pressure, PCWP=pulmonary capillary wedge pressure, CO=cardiac output, SVR=pulmonary vascular resistance, PVR=systemic vascular resistance.

NF-κB, AP-1, and TNF-α were quantified in five different grades, from - to ++++, according to the number of positive cells per high-power field from five HPFs in each of two tissue samples from each pig. The different treatment groups were blinded to the slide reader. Number of positive cells per high power field/HPF. GR; -<10/HPF; +<20/HPF; ++<30/HPF; +++<40/HPF; ++++>40/HPF. NF-κB; -<5/HPF; +<15/HPF; ++<25/HPF; +++<35/HPF; ++++>35/HPF. AP-1; -<10/HPF; +<20; ++<30HPF; +++<40/HPF; ++++ >40HPF. TNF-α;- <15/HPF; +<25/HPF; ++<35/HPF; +++<45/HPF; ++++ >45/HPF.

Statistical analysis

Mean ± standard deviations (SD) were calculated for all variables. Two-way ANOVA for repeated measurements before and during intervention (3h-6h) followed by Tukey's honest test was used for comparisons within and between groups. Statistical significance was defined as *P<0.05.* For quantitative analysis of image data (immunohistochemistry and histology) a five-grade scale was used, from no to abundant findings of a particular phenomenon (-, +. ++, +++, ++++) as described in the relative section. Two tissue samples from each pig were taken for analysis, and in each sample 5 different microscopy fields were analyzed and used for probability tests. Differences between groups were evaluated with Kruskal-Wallis Test, followed by Mann-Whitney U test where statistically significant differences were found.

Results

Hemodynamics, gas exchange and biochemistry of organ function at baseline, during the evolution of lung damage by LPS infusion and during the treatment period are shown in

Table 2. Blood gases, respiratory parameters and carboxyhemoglobin levels

		HC	LPS	LPS+CO	LPS+ST	LPS+CO+ST
pH	BL	7.46±0.06	7.47±0.02	7.49±0.03	7.48±0.03	7.47±0.02
	2.5h	7.44±0.03	7.30±0.06	7.35±0.06	7.37±0.06	7.34±0.04
	6 h	7.44±0.04	7.24±0.07	7.29±0.07	7.29±0.09	7.31±0.05
$PaCO_2$	BL	40.0±5.25	39.1±2.2	38±1.3	38.1±2.6	38.8±2.5
mmHg	2.5h	40.1±2.83	39.9±4.88	43.8±5.78	44.5±3.99	45.8±3.51
	6h	38.9±3.6	47.8±5.4	47.7±7.2	50.8±11	48.8±7.4
PaO_2	BL	224.7±37	242±22	240±21	234±10	247±17
mm Hg	2.5h	229.5±21	147±46	156±43	159±48	157±47
	6h	225.5±19	96±24	140±65	118±38	138±60
BE	BL	4.2±1.31	4.6±1.8	5.4±1.2	5.0±0.9	4.1±1.0
	2.5h	3.2±0.83	-3.0±2.45	-1.3±1.6	-0.07±1.2	-0.4±1.62
	6h	2.8±1.4	-6.2±3.3	-3.4±1.7	-2.4±2.2	-1.8±0.9
COHb	BL	0.4±0.2	0.4±0.2	0.3±0.1	0.3±0.1	0.2±0.1
%	2.5h	0.5±0.6	0.2±0.2	0.47±0.2	0.2±0.0	0.3±0,1
	6h	0.3±0.2	0.6±0.8	7.9±1.8**	0.3±0.2	8.2±2.2**
Crs	BL	27.3±3.5	29.5±4.9	26.2±3.4	27.8±1.8	27.7±3.1
mL/cm	2.5h	23.4±1.8	17.8±3.2	18.2±4.4	16.8±1.9	17.8±2.9
H_2O	6h	23.6±1.6	15.2±1.8	18.4±4.5	17±1.9	17.6±3.4
Raw	BL	18.7±2.9	18.2±6.1	20.1±6	19.7±2.9	19.4±2.8
Cm H_2O	2.5h	17.7±1.6	20.8±5.4	24.1±4	22.3±3.8	24.2±1.5
L/sec	6h	19.7±2.1	23.4±4.1	22.1±4.4	21.9±1.9	25.5±5.9

Effects of treatment of 250 ppm inhaled CO, 3 mg/kg iv steroids or both inhaled CO and steroids on blood gases and respiratory parameters during a 6-h endotoxin infusion. Data are expressed as means ±SD at baseline, 2.5 and 6 hours after the onset of LPS infusion (before treatment and 3.5 hours of CO or steroid treatment respectively) *P<0.05 or **P<0.01 compared LPS group vs treated groups (LPS+CO, LPS+ST, LPS+CO+ST) during treatment period (3-6h). With the exception of changes parameter on HC group, all parameters from all groups were <0.05 compared with baseline (ns= COHb in LPS and LPS+ST groups). BL= baseline, pH=arterial pH, PaO_2=arterial partial pressure of oxygen, $PaCO_2$=arterial partial pressure of CO_2, BE= base excess COHb=carboxyhemoglobin levels, Crs=respiratory system compliance, Raw=airways resistance.

Tables 1-3. For clarify, tables show mean values ± SD for baseline, 2.5 (before the initiation of the treatment), and 6 hour data (end of treatment).

Effects of endotoxin damage

Infusion of LPS caused the well known biphasic deterioration in circulatory variables with a first peak in pulmonary arterial pressure 30 min after onset of LPS infusion and then a second peak that plateaued at 2.5-3 hours (**Figure 2a**). Mean arterial pressure, CVP and PCWP showed a similar reaction but with smaller changes. Cardiac output showed a decrease after ½ hour and a slow continuing decrease over the latter 3 hours of the experiment. PaO_2 fell rapidly during the initial phase of endotoxin infusion and then continued to fall to approximately 40% of baseline value during the succeeding study period. $PaCO_2$ and airway resistance showed a slow continuing rise during the whole study period while respiratory compliance gradually decreased. Base Excess and pH fell during the first 2½ hour with a further slight decrease during the following 3 ½ hour study period. See **Table 1** and **2** for statistics on the data reported above and additional data.

Leukocytes and neutrophils in plasma fell dramatically during the initial period of endotoxin exposure and then remained at a low level. Serum urea showed no significant change over the study period. Serum creatinine and liver function variables bilirubin and conjugated bilirubin increased during the latter half of the study period, i.e. after 2 ½ - 3 hours of endotoxin exposure. (See also **Table 3** for statistics on blood cells, biochemistry on liver, kidney, myocardial function and cortisol in plasma).

Effects of CO and steroid

CO prevented some of the increase in PAP with a significant difference compared to LPS alone

Table 3. Blood count, markers of organ function and cortisol levels

		HC	LPS	LPS+CO	LPS+ST	LPS+CO+ST
Leucocytes	BL	14.5±3.4	14.6±1.3	17.5±4.2	18.5±1.9	17±2.8
x10^9/L	2.5h	18.9±5.2	1.7±0.3	2.8±1	2.5±0.4	2.8±0.6
	6 h	17.3±4.8	2.7±0.7	3.8±1.2	3.4±1.1	4.1±1.1
Neutrophiles	BL	7.1±2.4	6.7±0.6	8.3±3.2	9.7±2.7	7.7±1.9
x10^9/L	2.5h	12±4.3	0.3±0.1	0.4±0.3	0.3±0.1	0.5±0.2
	6h	11.9±3.8	1.1±0.4	1.7±0.7	1.5±0.9	1.9±1.1
Creatinine	BL	82±9.9	82.2±7	82.7±2	82±7	79±8.9
µmol/L	2.5h	81.5±9.4	78.2±6.8	79.5±2.6	76±6.8	78.7±9.4
	6h	78.3±8.0	98.2±19	97.8±12	99.3±5.2	90±11
Urea	BL	3.2±0.6	2.7±0.5	3.3±0.9	3.6±0.7	3.1±0.6
mmol/l	2.5h	2.9±0.6	2.4±0.4	2.7±0.6	2.8±0.8	2.6±0.6
	6h	2.9±0.4	3.1±0.6	3.0±0.7	3.2±0.9	2.9±0.8
Bilirubin	BL	4.2±0.4	4.1±0.4	4.5±0.8	4.2±0.4	4.2±0.4
µmol/L	2.5h	4.3±0.5	5.0±0.8	4.8±0.7	4.5±0.5	4.5±0.8
	6h	4.3±0.8	12.3±6	11.9±3.9	12.3±6.0	11.2±4.4
Bil. conj	BL	1.5±1.1	0.5±0.4	0.8±0.6	1.6±1.3	1.3±1.3
µmol/L	2.5h	2.4±1.2	0.9±0.7	1.7±1.3	1.7±1.2	2.2±1.5
	6h	2.0±0.8	4.7±4	6.4±3.8	4.2±5.6	8.2±4.6
Troponine I	BL	0.5±0.3	0.5±0.2	0.4±0.2	0.5±0.3	0.5±0.3
ug/L	2.5h	0.6±0.3	0.6±0.5	0.5±0.2	0.5±0.1	0.3±0.1
	6h	0.44±0.2	1.4±1.4	0.6±0.4	1.5±2.1	0.5±0.1
Cortisol	BL	27.3±18	68.5±66	51.3±53	53.7±64	41.4±75
nmol/L	2.5h	120±34	164±21	191±79	162±32	182±52
	6h	85±3.2	137±53	128±14	334±40**	322±76.6**

Effects of treatment of 250 ppm inhaled CO, 3 mg/kg iv steroids or both inhaled CO and steroids on blood count and biochemistry of organ function during a 6-h endotoxin infusion. Data are expressed as means ±SD at baseline, 2.5 and 6 hours after the onset of LPS infusion (before treatment and 3.5 hours of CO or steroid treatment respectively). *P<0.05 or **P<0.01 compared LPS group vs treated groups (LPS+CO, LPS+ST, LPS+CO+ST) during treatment period (3-6h). With the exception of parameter changes on HC group all parameters from all groups were <0.05 compared with baseline (ns=urea, troponine) BL= baseline, Bil Conj= Bilirubin conjugated.

(p<0.05) (**Figure 2a**, **Table 1**). There was a similar trend for PVR, although no statistical significance was achieved. Cardiac output, CVP, MAP, SVR, PCWP and heart rate were not affected by any of the tested treatments although the steroid groups tended to have higher MAP and the iCO group lower MAP (**Table 1**). PaO_2, $PaCO_2$, pH and Base Excess did not improve with either CO or steroid treatment (**Table 2**). Minor changes were seen in leukocytes and neutrophils during the different treatment modalities. In terms of organ function serum creatinine, urea, bilirubin and conjugated bilirubin did not show any significant differences between treatment groups and LPS group (**Table 3**).

COHb and cortisol

The breathing of CO 250 ppm for 3 ½ hours resulted in an increase in COHb from 0.3% at baseline to 8.05±0.2% in the two groups that received iCO (CO group and CO+ST group) (p<0.01) (**Figure 2b**). Cortisol in plasma rose with a peak value at 4 hours and then decreased to the 6 hour value that was still much higher than at baseline in the two groups that received steroids (ST and CO+ST groups) p<0.01

Immunohistochemistry and morphology of lung tissue

Healthy controls showed some staining of the glucocorticoid receptor (GR) whereas imflammatory markers (NF-κB, AP-1, TNF-α) were minor or absent. LPS infusion caused significant down-regulation (p<0.05) of the GR and a significant increase in all inflammatory markers,

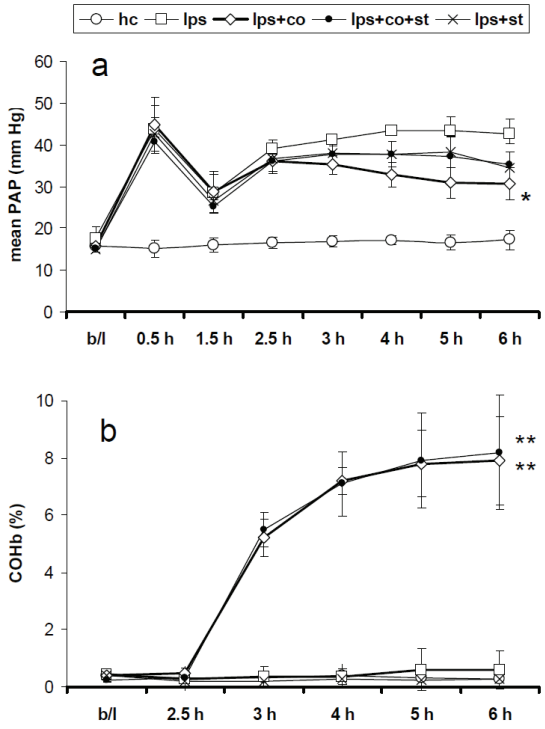

Figure 2. Pulmonary arterial pressures and COHb levels. Effects of treatment with inhaled CO, steroids and both inhaled CO +steroids on (a) mean pulmonary arterial pressure (MPAP) and (b) carboxyhemoglobin levels (COHb) in the five different groups of pigs during 6 hours of LPS infusion. *P<0.05 or **P<0.01 comparing LPS group vs treated groups (LPS+CO, LPS+ST, LPS+CO+ST) during treatment period (3-6h).

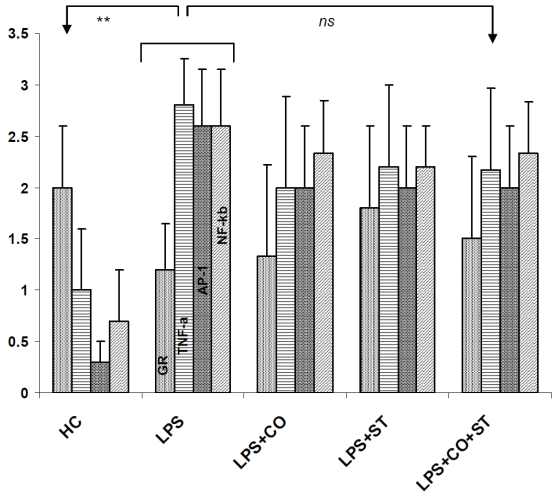

Figure 3. Immunohistochemistry in the lung tissue. Quantitative scoring of immunochemical staining in lung for GR, NF-κB, AP-1 and TNF-α in healthy control pigs (HC), pigs exposed to endotoxin (LPS), and treated pigs with inhaled CO (LPS+CO), steroids (LPS+ST), and both inhaled CO plus steroids (LPS+CO+ST). The LPS group differed significantly (** p<0.01) from the healthy control group in all inflammatory changes. For treated groups (LPS+CO, LPS+ST, LPS+CO+ST) comparing to LPS group p=ns.

p<0.01. Inhaled CO and a combined treatment with both CO and steroid showed significant up-regulation of the GR while steroid treatment alone showed an up-regulation of the GR that approached the level of healthy control. The effects on inflammatory markers (TNF-α, AP-1, NF-κB) were modest and insignificant with either treatment (**Figure 3**, **Figure 4**).

Microscopy of lung tissue showed that LPS caused a marked infiltration of inflammatory cells, edema and hemorrhage compared to healthy control pigs p<0.001. CO treatment blunted all three parameters (inflammatory cells, edema and hemorrhage) to a significant extent and steroid treatment reduced only edema and hemorrhage p<0.05 (**Figure 4**, **Figure 5**). The combined treatment with CO and steroid produced even less edema, number of inflammatory cells and hemorrhage (p<0.01).

Discussion

The major findings in the current study are that inhaled CO decreased pulmonary hypertension and improved histological appearance of the lung after LPS damage, whereas it had no obvious protective effect on organ function and respiratory mechanics.

In recent years it has become apparent that CO confers protection to tissues against oxidative injury. Inhalation of CO in animal studies has been demonstrated to posses anti-inflammatory, antiapoptotic and antiproliferative effects in different experimental models [2, 9-11]. The underlying pathophysiological and antinflammatory mechanisms have been studied in mice, while only few studies have been done in larger animals [9] and have included physiological parameters that are used in the daily clinical practise [3, 12]. In all previous injury models, inhaled CO was administered before the development of injury, and there is

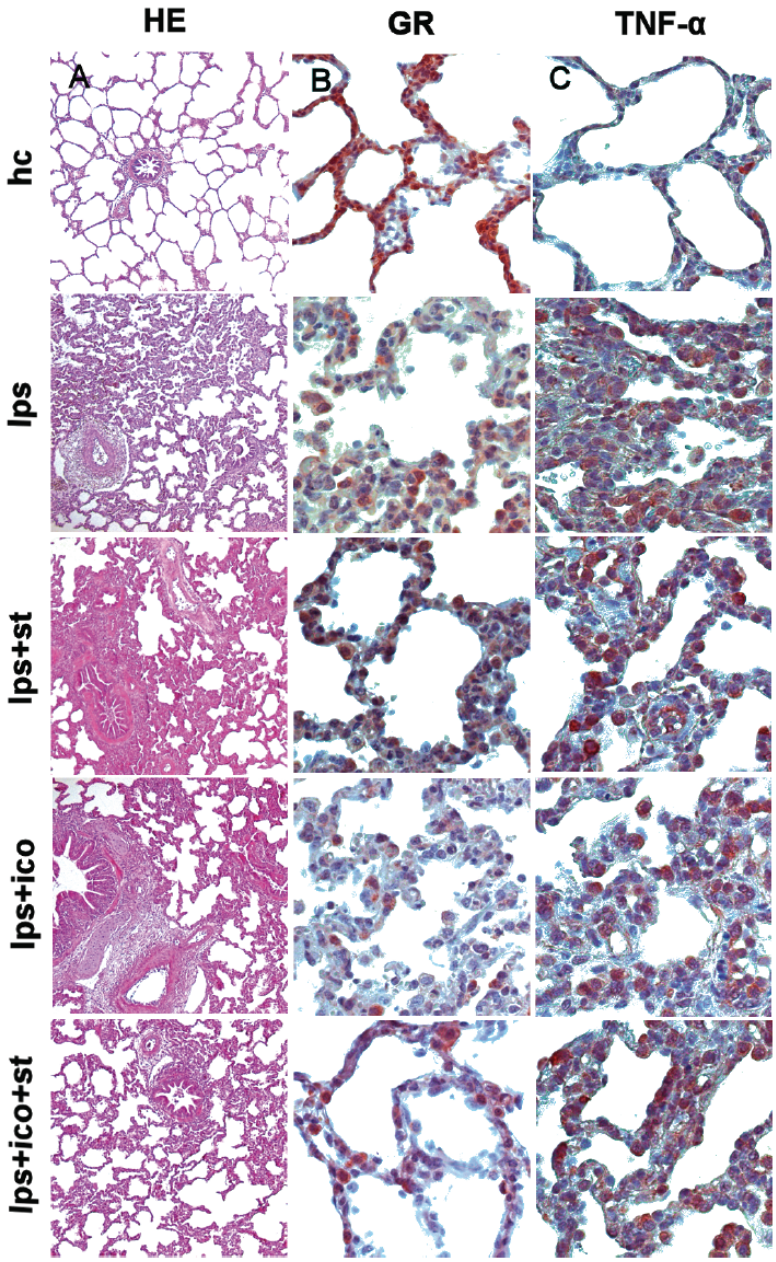

Figure 4. Microphotographs of lung tissue **(column A),** showing normal structure in healthy control pigs (hc) and the effects caused by exposure to endotoxin in the lipopolysaccharide (lps), lps+steroids (lps+st), lps+ inhaled carbon monoxide (lps+ico), and lps+inhaled co+steroids (lps+ ico+st) groups. In the lung tissue of the lps group, note the pulmonary edema (proteinaceous fluid in the alveolar space), thrombosis in the veins, and hemorrhage and inflammatory cell infiltration in the thicker alveolar septa. In the tissues from the lps+st group and lps+ico group, steroid or inhaled co treatment prevents some of the damage in the lung, compared to lps group. In the tissue from the lps+ico+st group, lung tissue show even fewer changes than in the lps group. Expression of glucocorticoid receptor (GR) **(column B)** and TNF-α **(column C)** in lung tissue from the different groups. Lung tissues from the hc group tissue showed some staining of the glucocorticoid receptor while imflammatory marker TNF-α where minor or absent. Note the small number of positively stained cells with GR in the LPS pigs, the slightly larger number in the lps+ico and lps+ico+st pigs, and the large increase in the lps+st pigs. The endotoxin infusion on the lps pigs produced a marked expression of TNF-α in lung tissue, while treatment with steroid, inhaled co or steroid+inhaled co had no effect on TNF-α.

no information whether CO might act therapeutically (i.e. after the disease process has started) to treat established injury. We hypothesized that if CO administration after the endotoxic shock/injury also exerts protective/positive effects it will be of value in the treatment of ALI and ARDS. We therefore investigated, as far as we know for the first time, the potential beneficial effects of inhaled CO after onset of endotoxin "septic" damage in a porcine model because this procedure is close to clinical reality. However, it seems that the strong anti-inflammatory properties of iCO found in many injury animal models were abolished when iCO was administered after the septic insult and the inhaled CO had rather modest beneficial effects on organ function. Although there are no published studies regarding the therapeutic use of CO after the injury damage the results from a recent study seem to agree and strengthen our findings. Tsui et al found that CO inhalation for 1 hour after initiation of

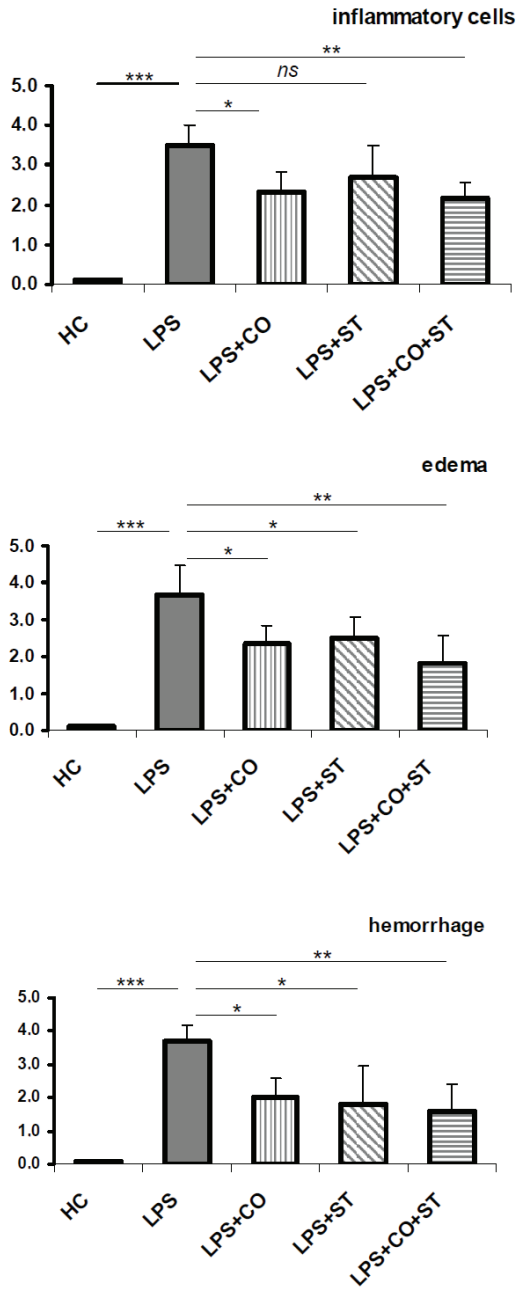

Figure 5. Morphological changes in the lung. Quantitative scoring of morphological changes in lung tissue based on number of inflammatory cells (a), edema formation (b), and hemorrhage (c) in healthy control pigs (HC), pigs exposed to endotoxin (LPS), and treated with inhaled CO (LPS+CO), steroids (LPS+ST), and both inhaled CO plus steroids (LPS+CO+ST) groups. The LPS group differed significantly (*** $p<0.001$) from the healthy control group in all inflammatory changes. For treated groups (LPS+CO, LPS+ST, LPS+CO+ST) comparing to LPS group *$p<0.05$ or **$p<0.01$.

fulminant hepatitis induced by LPS significantly increased the survival of mice, but the survival rate declined progressively from 80% to 20% when the CO was given 1 or 6 hours respectively after initiation of hepatitis. [13] An explanation for the poor results on organ function in our study might thus be that the animals were exposed to endotoxin for a long period before commencement of iCO. This suggests that as early start of treatment as possible may be of importance. It may also be that our inflammatory stimulus was too strong to allow detection of weak anti-inflammatory properties of CO and/or that CO exposure may have prolonged effects on sepsis that were not detected in the current short-term study.

Microscopy and inflammatory markers

LPS administration at the used dose induced a dramatic inflammatory injury in the lungs and a marked expression of AP-1, NF-κB, and TNF-α in lung tissue, while GR receptor expression was down-regulated. Treatment with iCO and to less extent with steroids significantly attenuated the inflammatory response and improved the microscopic appearance of the lung. The effects on inflammatory markers (TNF-α, AP-1, NF-κB) were insignificant with either treatment.

Although treatment with iCO and steroids in our model exerted some protective effect it seems that the underlying protective pathophysiological mechanism does not correlate with the studied nuclear inflammatory markers AP-1 and NF-κB in lung tissue. In vitro studies in RAW 264.7 and endothelial cells showed that HO-1/CO treatment interacts with NF-κB to protect the cells from LPS [14] and TNF-α mediated apoptosis [15], although studies in mice revealed that the anti-inflammatory effect of CO on sepsis was associated with the upregulation of cytoplasmic p38 mitogen-activated protein kinase pathway [2]. To conclude, despite that many studies have demonstrated cellular and tissue protection afforded by CO in animal models of sepsis, the specific signal transduction pathway mediating the anti-inflammatory effects of CO still remains under investigation.

The effects of CO on TNF-α in pigs and humans are very limited and contradictory. Similar to our findings Mazzola [3] and Aberg [16] concluded no effect of CO on TNF-a production after LPS administration in pigs. Additionally in a

study performed in humans inhalation of CO after LPS infusion did not attenuate the increased plasma concentrations of inflammatory mediators (TNF-α, IL-10) [4]. In contrast to these data Goebel et al demonstrate that inhaled CO significantly reduces cardiopulmonary bypass induced inflammationin pigs via suppression of TNF-α and elevation of interleukin-10, [12].

Vascular, pulmonary and organ function effects of CO

An important finding of our study was that inhaled CO significantly decreased the pulmonary hypertension induced by endotoxin without apparent effect on the systemic circulation. Similar to NO, CO relaxes numerous vascular tissues by activating soluble guanylate cyclase and stimulating calcium–activated potassium channels in vascular smooth muscle. Their actual response, however, varies with the vascular bed [17-20], and there are also instances of vessels failing to respond [21-22]. The reasons for the divergent action by CO in different vascular beds are not known.

The vascular effects of CO on the pulmonary circulation are variable. Most studies in this category examined the vasodilatory effect of inhaled CO in hypoxic pulmonary vasoconstriction. Dubuis et al found in rats that continuous inhalation of carbon monoxide attenuates hypoxic pulmonary hypertension development presumably through activation of calcium activated potassium channels [23]. Christou *et al* demonstrated that the induction of HO-1 expression and CO production inhibits the structural remodeling of pulmonary arteries and the development of pulmonary hypertension in response to chronic hypoxia in rats [24]. Similar protection against pulmonary hypertension is noted in transgenic mice overexpressing HO-1 [25]. Another study showed a small decrease in pulmonary vascular resistance in sheep during hypoxemia after inhalation of carbon monoxide [26]. However other studies in healthy pigs [27], rats [28] and fetal lambs [29] during hypoxemia, showed no pulmonary vasodilation by inhaled CO. Interesting findings emerged from a recent study by Zuckerbraun *et al*, who studied, the effect of inhaled CO in the treatment of chronic pulmonary hypertension in three rodent models. The pulmonary arterial hypertension (PAH) was induced either by Monocrotaline sodium, or by chronic hypoxia, or by using the genetically susceptible fawn-hooded hypertensive rat. A 1 hour/day exposure to CO reversed established pulmonary hypertension and right ventricular hypertrophy, as well as restored the pulmonary vascular architecture, to near normal. The ability of CO to reverse PAH requires functional endothelial nitric oxide synthase and NO generation [30]. We were unable to determine a positive effect of CO exposure on respiratory mechanics, blood gases, hemodynamic parameters and organ function. Inhaled CO tended to improve PaO_2, however no additive effect was seen by combined CO and steroid treatment.

Carboxyhemoglobin levels

In our model the inhalation of 250 ppm CO for 3.5 hours produced a time dependent increase in HbCO values to a peak of 8%. These levels are lower than the levels found in mice after inhalation 250 ppm CO for hours but rather similar to studies after inhalation of CO in big animals or human subjects [4]. Affinity constants of haemoglobin for CO, and half-lives of HbCO differ markedly between species. Small animals such as hamsters, have HbCO values exceeding 20% when breathing 250 ppm CO for 1 hour [31], while mice have values of HbCO between 10-14% after inhalation of 200-500 ppm CO [32-34]. In contrast, larger animal such as miniature pigs show HbCO values less than 10% after 1 hour, and pigs HbCO levels of about 12% after inhalation of 250 ppm CO for 2 hours [9]. Published data on COHb levels in pigs after CO exposure are limited. Mazzola et all found in CO treated pigs that COHb rose from a surprisingly high 4.6% at baseline to 14% (net increase 9.4%) after 1 hour of 250 ppm CO inhalation. Goebel et al found in a porcine model of cardiopulmonary bypass that 250 ppm of CO inhalation for 1 hour increased COHb levels from 2% at the baseline to 11% (net increase 9%). In these two positive studies animals were exposed to CO before the injury and then inhalation discontinued and the protective effect of CO gradually declined. Thus, in the first study [3], net COHb levels of 9% decreased to approximately to 4.5% thirty minutes after CO was ceased and returned to baseline after 90 minutes. In the second study [12] the net COHb levels of 7-8% (during injured period of cardiopulmonary by pass) decreased to 3.6% at the end of the experiments. These levels

were enough to protect the pigs from LPS and cardiopulmonary bypass damage, respectively. In our experiment model the net COHb levels increased to 7.75% for duration of 3.5 hours. Even though optimal protective values of COHb and time of exposure to CO have not been determined from current/other studies in pigs it seems that the levels of COHb and the duration of exposure found in our model was at least comparable with the previous mentioned positive studies.

In conclusion using a well known sepsis model in pigs we show for the first time that CO exposure after the creation of septic damage has some important physiologic effects. Firstly, iCO can protect the lung tissue from the inflammatory destruction, especially when combined with steroids. Secondly, the anti-inflammatory properties of CO are too weak to preserve the organ function in our model, in contrast to the results from studies where iCO was given before the endotoxin sepsis. Thirdly, despite the severe lung damage iCO can decrease the pulmonary hypertension induced by LPS. Thus, using this sepsis model we find some evidence that inhaled CO therapy may be beneficial in the treatment of sepsis-induced pulmonary hypertension. Our results suggest that further studies are needed to clarify any potential role of CO or other inhaled substances in modifying the progression of sepsis.

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Abbreviations: ALI= acute lung injury, ARDS= acute respiratory distress syndrome, CO= carbon monoxide, LPS= lipopolysaccharide, MAPK= mitogen activated protein kinase, NF-κB=nuclear factor kappa B, TNF-α = tumor necrosis factor-alpha, iCO= inhaled carbon monoxide, GSs= glucocorticoids, NO= nitric oxide, ST= steroids, GR= glucocorticoid receptors, groups: HC=control group, LPS= lipopolysaccharide, LPS+CO= LPS+iCO, LPS+ST= LPS+steroid, LPS+CO+ST= LPS+iCO+steroid, MPAP= mean pulmonary arterial pressure, HR= heart rate, MAP= mean arterial pressure, CVP= central venous pressure, CO= cardiac output, PCWP= pulmonary capillary wedge pressure, PVR= pulmonary vascular resistance, SVR= Systemic vascular resistance, BE= base excess, COHb= carboxyhemoglobin, Crs= respiratory compliance, HE=heamatoxylin eosin, HPF=high power field, Raw= airway resistance, AP-1= activator protein-1, HO-1= heme oxygenase-1, PAH= pulmonary arterial hypertension.

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References

[1] Angus DC and Wax RS. Epidemiology of sepsis: an update. Crit Care Med 2001; 29: S109-116.

[2] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA and Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000; 6: 422-428.

[3] Mazzola S, Forni M, Albertini M, Bacci ML, Zannoni A, Gentilini F, Lavitrano M, Bach FH, Otterbein LE and Clement MG. Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. Faseb J 2005; 19: 2045-2047.

[4] Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, Wagner O and Jilma B. Effects of carbon monoxide inhalation during experimental endotoxemia in humans. Am J Respir Crit Care Med 2005; 171: 354-360.

[5] Ghosh S, Wilson MR, Choudhury S, Yamamoto H, Goddard ME, Falusi B, Marczin N and Takata M. Effects of inhaled carbon monoxide on acute lung injury in mice. Am J Physiol Lung Cell Mol Physiol 2005; 288: L1003-1009.

[6] Annane D, Bellissant E, Bollaert PE, Briegel J, Confalonieri M, De Gaudio R, Keh D, Kupfer Y, Oppert M and Meduri GU. Corticosteroids in the treatment of severe sepsis and septic shock in adults: a systematic review. JAMA 2009; 301: 2362-2375.

[7] Da J, Chen L and Hedenstierna G. Nitric oxide up-regulates the glucocorticoid receptor and blunts the inflammatory reaction in porcine endotoxin sepsis. Crit Care Med 2007; 35: 26-32.

[8] Schmidhammer R, Wassermann E, Germann P, Redl H and Ullrich R. Infusion of increasing doses of endotoxin induces progressive acute lung injury but prevents early pulmonary hypertension in pigs. Shock 2006; 25: 389-394.

[9] 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 FH

and Calise F. Carbon monoxide improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass in pigs. Faseb J 2004; 18: 1093-1095.
[10] Ryter SW and Otterbein LE. Carbon monoxide in biology and medicine. Bioessays 2004; 26: 270-280.
[11] Ghosh S, Gal J and Marczin N. Carbon monoxide: endogenous mediator, potential diagnostic and therapeutic target. Ann Med 2010; 42: 1-12.
[12] Goebel U, Siepe M, Mecklenburg A, Stein P, Roesslein M, Schwer CI, Schmidt R, Doenst T, Geiger KK, Pahl HL, Schlensak C and Loop T. Carbon monoxide inhalation reduces pulmonary inflammatory response during cardiopulmonary bypass in pigs. Anesthesiology 2008; 108: 1025-1036.
[13] Tsui TY, Obed A, Siu YT, Yet SF, Prantl L, Schlitt HJ and Fan ST. Carbon monoxide inhalation rescues mice from fulminant hepatitis through improving hepatic energy metabolism. Shock 2007; 27: 165-171.
[14] Sarady JK, Otterbein SL, Liu F, Otterbein LE and Choi AM. Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages. Am J Respir Cell Mol Biol 2002; 27: 739-745.
[15] Brouard S, Berberat PO, Tobiasch E, Seldon MP, Bach FH and Soares MP. Heme oxygenase -1-derived carbon monoxide requires the activation of transcription factor NF-kappa B to protect endothelial cells from tumor necrosis factor-alpha-mediated apoptosis. J Biol Chem 2002; 277: 17950-17961.
[16] Aberg AM, Abrahamsson P, Johansson G, Haney M, Winsö O and Larsson JE. Does carbon monoxide treatment alter cytokine levels after endotoxin infusion in pigs? A randomized controlled study. J Inflamm (Lond) 2008; 5: 13.
[17] Coceani F, Kelsey L and Seidlitz E. Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase. Br J Pharmacol 1996; 118: 1689-1696.
[18] Graser T, Vedernikov YP and Li DS. Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. Biomed Biochim Acta 1990; 49: 293-296.
[19] Furchgott RF and Jothianandan D. Endothelium -dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 1991; 28: 52-61.
[20] Achouh PE, Simonet S, Fabiani JN and Verbeuren TJ. Carbon monoxide induces relaxation of human internal thoracic and radial arterial grafts. Interact Cardiovasc Thorac Surg 2008; 7: 959-962.
[21] Johnson FK and Johnson RA. Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. Am J Physiol Regul Integr Comp Physiol 2003; 285: R536-541.
[22] Kozma F, Johnson RA, Zhang F, Yu C, Tong X and Nasjletti A. Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels. Am J Physiol 1999; 276: R1087-1094.
[23] Dubuis E, Potier M, Wang R and Vandier C. Continuous inhalation of carbon monoxide attenuates hypoxic pulmonary hypertension development presumably through activation of BKCa channels. Cardiovasc Res 2005; 65: 751 -761.
[24] Christou H, Morita T, Hsieh CM, Koike H, Arkonac B, Perrella MA and Kourembanas S. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. Circ Res 2000; 86: 1224-1229.
[25] Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA and Kourembanas S. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci U S A 2001; 98: 8798-8803.
[26] Nachar RA, Pastene CM, Herrera EA, Riquelme RA, Sanhueza EM, Troncoso S and Llanos AJ. Low-dose inhaled carbon monoxide reduces pulmonary vascular resistance during acute hypoxemia in adult sheep. High Alt Med Biol 2001; 2: 377-385.
[27] Aberg AM, Hultin M, Abrahamsson P and Larsson JE. Circulatory effects and kinetics following acute administration of carbon monoxide in a porcine model. Life Sci 2004; 75: 1029-1039.
[28] Carraway MS, Ghio AJ, Suliman HB, Carter JD, Whorton AR and Piantadosi CA. Carbon monoxide promotes hypoxic pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol 2002; 282: L693-702.
[29] Grover TR, Rairigh RL, Zenge JP, Abman SH and Kinsella JP. Inhaled carbon monoxide does not cause pulmonary vasodilation in the late-gestation fetal lamb. Am J Physiol Lung Cell Mol Physiol 2000; 278: L779-784.
[30] Zuckerbraun BS, Chin BY, Wegiel B, Billiar TR, Czsimadia E, Rao J, Shimoda L, Ifedigbo E, Kanno S and Otterbein LE. Carbon monoxide reverses established pulmonary hypertension. J Exp Med 2006; 203: 2109-2119.
[31] Klimisch HJ, Chevalier HJ, Harke HP and Dontenwill W. Uptake of carbon monoxide in blood of miniture pigs and other mammals. Toxicology 1975; 3: 301-310.
[32] Otterbein LE, Otterbein SL, Ifedigbo E, Liu F, Morse DE, Fearns C, Ulevitch RJ, Knickelbein R, Flavell RA and Choi AM. MKK3 mitogen-activated protein kinase pathway mediates

carbon monoxide-induced protection against oxidant-induced lung injury. Am J Pathol 2003; 163: 2555-2563.

[33] Clayton CE, Carraway MS, Suliman HB, Thalmann ED, Thalmann KN, Schmechel DE and Piantadosi CA. Inhaled carbon monoxide and hyperoxic lung injury in rats. Am J Physiol Lung Cell Mol Physiol 2001; 281: L949-957.

[34] Otterbein LE, Mantell LL and Choi AM. Carbon monoxide provides protection against hyperoxic lung injury. Am J Physiol 1999; 276: L688-694.