Protective effects of inhaled carbon monoxide in pig lungs during cardiopulmonary bypass are mediated via an induction of the heat shock response

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Background. Cardiopulmonary bypass (CPB) may cause acute lung injury leading to increased morbidity and mortality after cardiac surgery. Preconditioning by inhaled carbon monoxide reduces pulmonary inflammation during CPB. We hypothesized that inhaled carbon monoxide mediates its anti-inflammatory and cytoprotective effects during CPB via induction of pulmonary heat shock proteins (Hsps).

Methods. Pigs were randomized either to a control group, to standard CPB, to carbon monoxide+CPB, or to quercetin (a flavonoid and unspecific inhibitor of the heat shock response)+control, to quercetin+CPB, and to quercetin+carbon monoxide+CPB. In the carbon monoxide groups, lungs were ventilated with 250 ppm carbon monoxide in addition to standard ventilation before CPB. At various time points, lung biopsies were obtained and pulmonary Hsp and cytokine concentrations determined.

Results. Haemodynamic parameters were largely unaffected by CPB, carbon monoxide inhalation, or administration of quercetin. Compared with standard CPB, carbon monoxide inhalation significantly increased the pulmonary expression of the Hsps 70 [27 (SD 3) vs 69 (10) ng ml$^{-1}$ at 120 min post-CPB, $P<0.05$] and 90 [0.3 (0.03) vs 0.52 (0.05) after 120 min CPB, $P<0.05$], induced the DNA binding of heat shock factor-1, reduced interleukin-6 protein expression [936 (75) vs 320 (138) at 120 min post-CPB, $P<0.001$], and decreased CPB-associated lung injury (assessed by lung biopsy). These carbon monoxide-mediated effects were inhibited by quercetin.

Conclusions. As quercetin, a Hsp inhibitor, reversed carbon monoxide-mediated pulmonary effects, we conclude that the anti-inflammatory and protective effects of preconditioning by inhaled carbon monoxide during CPB in pigs are mediated by an activation of the heat shock response.

Br J Anaesth 2009; 103: 173–84

Keywords: model, lung damage; pig; surgery, cardiovascular

Accepted for publication: March 23, 2009

Cardiopulmonary bypass (CPB) may induce a systemic inflammatory response and pulmonary dysfunction by various mechanisms.$^{1,2}$ We previously demonstrated that inhaled carbon monoxide attenuates CPB-induced inflammation via reduced expression of tumour necrosis factor-α and interleukin (IL)-1β, and increased expression of IL-10.$^3$

Carbon monoxide is physiologically generated during the breakdown of haem by the enzyme haem oxygenase, exerts anti-inflammatory and cytoprotective properties, and can ameliorate ischaemia/reperfusion injury, transplant rejection, sepsis, and autoimmunity in animal models.$^{3–10}$ Inhalation of low concentrations of carbon monoxide (250 ppm) suppressed inflammation and reduced apoptosis in numerous in vitro and in vivo studies.$^{3,5,6,11–16}$

†Both authors contributed equally to this work.
The protective effects of carbon monoxide involve an increased heat shock response, demonstrated by expression of the inducible heat shock protein 70 (Hsp-70) in murine lung endothelial cells and fibroblasts. The cytoprotective capacity of Hsps may be partly related to their ability to stabilize intracellular protein structures, which allows cells and organisms facing life-threatening insults resumption of normal cellular and physiological activities.

The aim of this study was to elucidate a mechanism by which inhaled carbon monoxide may exert its protective effects during CPB. We hypothesized that the protective effects of inhaled carbon monoxide are mediated via increased expression of Hsps.

Methods

Animals

German Landrace Hybrid pigs weighing 33–34 kg received humane 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 Institute of Laboratory Animal Resources and published by the National Institute of Health (Publication No. 86-23, revised 1985). All procedures were performed after obtaining the ethical approval of the local veterinary office (Regierungspräsidium, Freiburg, Germany) and in accordance with the German Animal Protection Law.

Experimental protocol

Healthy pigs were premedicated with i.m. ketamine [20 mg kg$^{-1}$ body weight (BW)] and midazolam (0.5 mg kg$^{-1}$ BW). Anaesthesia was induced with i.v. propofol (2 mg kg$^{-1}$ BW) and fentanyl (2 µg kg$^{-1}$ BW) and maintained by i.v. infusion of fentanyl (10 µg kg$^{-1}$ BW h$^{-1}$) and propofol (4–6 mg kg$^{-1}$ BW h$^{-1}$). Muscle relaxation was achieved by cis-atracurium (0.7–1 mg kg$^{-1}$ BW h$^{-1}$). The trachea was intubated and the lungs were ventilated at a rate of 14 litre min$^{-1}$, a tidal volume of 8–10 ml kg$^{-1}$ BW, and a PEEP of 5 cm H$_2$O. Inspired oxygen fraction was maintained at 0.5. Normal saline was infused at 350 ml h$^{-1}$. The right carotid artery was cannulated to monitor arterial pressure and obtain blood samples for analysis. A thermodilution catheter (7 F, Arrow, Reading, PA, USA) was introduced into the pulmonary artery via the right internal jugular vein. After establishment of full haemodynamic monitoring, sternotomy was performed, 300 IU kg$^{-1}$ BW heparin administered, the extracorporeal circuit (Stoeckert, Munich, Germany) primed (with isotonic saline solution 1000 ml, 6% hydroxyl ethyl starch 500 ml, mannitol 75 ml, and heparin 300 IU), and CPB started (via a 24 F venous cannula in the right atrial appendage and a 14 F aortic cannula). CPB was maintained for 2 h with a completely unloaded beating heart. Continuous positive airway pressure was maintained at 5 cm H$_2$O. At the end of CPB, an inspiratory pressure of 25–30 cm H$_2$O was maintained for 30 s to recruit the lungs before ventilation (as described above) was re-established. The extracorporeal circuit was then removed and protamine in a dose matching that of heparin was administered. This was followed by a 2 h observation period (post-CPB). At six defined time points, we obtained blood samples (for determination of haemoglobin and electrolyte concentrations, pH, $P_{a\text{CO}_2}$, $P_{a\text{O}_2}$, and acid–base status) and lung biopsies (0.5 g), and determined and calculated haemodynamic variables (heart rate, mean arterial pressure, mean pulmonary pressure, central venous pressure, pulmonary capillary wedge pressure, thermodilution cardiac output, and systemic and pulmonary vascular resistance): before CPB (pre-CPB), 10 min after the start of CPB, at the end of CPB, and 15, 60, and 120 min post-CPB. Lung biopsies were collected from three sites (the cranial and caudal portions of the apical lobe, and the basal lobe) across groups by the same individual (C.S.). Samples were minced and stored at −80°C for protein and mRNA analysis.

Animals were randomized to one of the six groups by means of a random computer-generated list. Animals of Group SHAM (n=3) underwent sternotomy only and served as control. In Group CPB (n=7), CPB was initiated and maintained for 2 h followed by a 2 h observation period. Animals of Group CO+CPB (n=7) received carbon monoxide before operation immediately after induction of anaesthesia at a concentration of 250 ppm for 1 h. Animals of the remaining three groups were treated identically, except for additional injection of quercetin (Q) 500 mg i.v. (Q0125, Sigma-Aldrich Biochemie, Hamburg, Germany), an inhibitor of the heat shock response, immediately after induction of anaesthesia [Groups Q+SHAM (n=3), Q+CPB (n=7), and Q+CO+CPB (n=7)]. The dose of quercetin was selected on the basis of several porcine studies providing data on bioavailability and target organ concentration.

End-expiratory carbon monoxide concentration was monitored using a carbon monoxide analyzer (MicroSmokerlyzer, Breath CO Monitor, Bedfont, UK). The content of carbon monoxide saturated haemoglobin was measured photometrically during regular blood gas analysis. Plasma concentrations of various indicators of organ function were determined at the end of the experiment (120 min post-CPB). The animals were killed 2 h after the end of CPB by intracardiac potassium injection.

Enzyme-linked immunoabsorbent assay

Protein was extracted and enzyme-linked immunoabsorbent assays were performed according to the manufacturers’ instruction (Quantikine, R&D, Minneapolis, MN, USA, and StressXPress, Biomol, Hamburg, Germany). Protein concentration was determined using the Bradford Assay (Bio-Rad Laboratories, Munich, Germany).
cDNA and oligonucleotide probes
A full-length cDNA was constructed using a reverse transcriptase polymerase chain reaction (PCR) first-strand cDNA kit (Fermentas, St Leon-Rot, Germany). PCR was performed using commercially synthesized oligonucleotides for Hsp-70, Hsp-90, and IL-6 (BigBiotech, Freiburg, Germany). Specific primer sequences were used: Hsp-70 (419 bp): sense (3'-5') CAG CGG CAG GCC ACC AAG GAC; antisense (5'-3') TGC ACC GCC GGG TCT CAA. Hsp-90 (488 bp): sense (3'-5') CTT ATG CAG AAG TGT AGT; antisense (5'-3') GTA CAT CCG CAA AAT CTC TGC; antisense (5'-3') GGT CTG TGA ATG CAG TTT ATC. PCR 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, Karlsruhe, Germany). Cloned plasmids were extracted according to the manufacturer’s instructions and an EcoRI digestion was performed to isolate the cDNA insert. The probe was labelled with [α-32P]dCTP (Amersham, Braunschweig, Germany) using the Prime It II Random Primer Labeling Kit® (Stratagene, La Jolla, CA, USA).

Northern blotting
Lung tissue was homogenized and total RNA was extracted using a one-step guanidinium thiocyanate–phenol–chloroform extraction reagent (Trizol®, Invitrogen). We electrophoretically separated 10 μg of total RNA on agarose gel 1% and transferred it onto a nylon membrane (Amersham). Northern blotting was performed 1% agarose gel 1% and transferred it onto a nylon membrane (Amersham). Northern blotting was performed. Autoradiographies 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).

Electrophoretic mobility shift assay
The mobility shift assay was performed as previously described using the heat shock element consensus sequence 5'-CTA GAA GCT TCT AGA AGC TTC TAG-3'.

Lung histopathology
Lung biopsies were placed overnight in 4% neutral buffered formaldehyde 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 haematoxylin and eosin staining was performed. Histological and morphological changes induced by the interventions were evaluated by microscopy. For immunohistochemical analysis, antibodies against Hsp-70 [mouse monoclonal (C92F3A-5) to Hsp-70, Abcam #ab47455], Hsp-90 (US Biological mouse anti HSP-90, #1834-558), IL-6 (rabbit polyclonal to IL-6, Abcam #ab6672), and macrophage [mouse monoclonal (MAC-387) against the calprotectin molecule expressed by macrophages, Abcam #ab22506] were used (Abcam, Cambridge, UK, and US Biological, Swampscott, MA, USA).

Quantitative and statistical analysis
Data were analysed by a computerized statistical program (SigmaStat® for Windows Version 3.1, Systat Software Inc., San Jose, CA, USA). We wished to detect a 50% reduction in the carbon monoxide-mediated protective effects by quercetin. Assuming an expected standard deviation of 15% based on our previously published data,3 an a priori power analysis (α=0.05 with two-sided hypothesis, β=0.1, power 90%) indicated that a sample size of seven animals per group would be sufficient to detect such reduction. The results are presented as means (SD) after normal distribution of data had been verified. One-way ANOVA for repeated measurements (haemodynamic, organ function, gas exchange parameters, mRNA analysis, and bandshift assay) and two-way ANOVA (protein analysis and histology) were used for within-group and between-group comparisons with post hoc Holmes–Sidak test, respectively. A P-value of <0.05 was considered statistically significant. Autoradiographies 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 survived the experiments and were included in the data analysis.

Homeostasis
Baseline homeostatic values (gas exchange parameters, concentrations of haemoglobin and carboxyhaemoglobin, and body temperature) were all within normal ranges and did not significantly differ between the groups (Supplementary Appendices 1 and 2, see British Journal of Anaesthesia online). At the onset of CPB, haemoglobin concentration decreased by ~20% in the CPB groups (P<0.05) without differences between the CPB groups (Supplementary Appendix 1). In the CPB groups, a respiratory alkalosis developed during CPB, which resolved after CPB. PaO2 was lower in the CO+CPB and Q+CO+CPB groups compared with the SHAM group (P<0.05; Supplementary Appendix 1). Pre-treatment with inhaled carbon monoxide did not further affect PaO2, post-CPB. After 1 h of carbon monoxide inhalation, carboxyhaemoglobin concentrations had increased from mean baseline values of 1.1 (0.5)% to 10.4 (2.2)% (P<0.05). By 2 h after CPB, carboxyhaemoglobin concentrations had decreased to 5.2 (2.7)%. In the SHAM and CPB groups, carboxyhaemoglobin concentrations remained unchanged in the normal range. Other homeostatic variables did not differ between the groups.

Systemic haemodynamics
The effects of carbon monoxide inhalation and quercetin administration on cardiovascular variables were
Table 1 Various plasma activities and concentrations of biochemical indicators of organ function in the six groups studied during the experimental protocol. SHAM, control group; CPB, cardiopulmonary bypass; CO, carbon monoxide; Q, quercetin; CK, creatine kinase; MB, muscle and brain subunit; pro-BNP, pro-natriuretic peptide and creatinine; MBg, myoglobin; LDH, lactate dehydrogenase; AP, alkaline phosphatase; AST, glutamate oxalacetate transaminase; ALT, glutamate pyruvate transaminase; GGT, gamma-glutamyl transferase. Values are means (±SD). *P<0.05 Q+CO+CPB vs Q+CPB; †P<0.05 Q+CO+CPB vs CPB; ‡P<0.05 Q+CPB vs CPB; §P<0.05 Q+SHAM vs SHAM; ¶P<0.05 Q+CO+CPB vs CPB; §§P<0.05 Q+CO+CPB vs Q+CPB.

<table>
<thead>
<tr>
<th>Activity</th>
<th>SHAM</th>
<th>Q+SHAM</th>
<th>CPB</th>
<th>Q+CPB</th>
<th>CO+CPB</th>
<th>Q+CO+CPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (IU litre⁻¹)</td>
<td>2023 (433)</td>
<td>3481 (931)*</td>
<td>3015 (484)</td>
<td>3646 (573)</td>
<td>1238 (747)*</td>
<td>2371 (713)*</td>
</tr>
<tr>
<td>CK-MB (IU litre⁻¹)</td>
<td>188 (45)</td>
<td>218 (63)</td>
<td>472 (212)</td>
<td>781 (312)</td>
<td>132 (87)*</td>
<td>203 (81)*</td>
</tr>
<tr>
<td>Pro-BNP (pg ml⁻¹)</td>
<td>6 (1)</td>
<td>7 (2)</td>
<td>7 (1)</td>
<td>7 (1)</td>
<td>7 (1)</td>
<td>6 (2)</td>
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<tr>
<td>Mgb (mg ml⁻¹)</td>
<td>23 (3)</td>
<td>24 (5)</td>
<td>28 (7)</td>
<td>32 (8)</td>
<td>19 (7)</td>
<td>22 (7)</td>
</tr>
<tr>
<td>LDH (IU litre⁻¹)</td>
<td>439 (229)</td>
<td>571 (197)</td>
<td>1058 (175)</td>
<td>1238 (418)</td>
<td>275 (175)*</td>
<td>599 (381)*</td>
</tr>
<tr>
<td>Bilirubin (mg dl⁻¹)</td>
<td>0.05 (0.01)</td>
<td>0.09 (0.03)</td>
<td>0.07 (0.03)</td>
<td>0.08 (0.05)</td>
<td>0.05 (0.01)</td>
<td>0.07 (0.02)</td>
</tr>
<tr>
<td>AP (IU litre⁻¹)</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>2 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>AST (IU litre⁻¹)</td>
<td>40 (6)</td>
<td>62 (12)*</td>
<td>123 (13)</td>
<td>167 (25)*</td>
<td>56 (34)*</td>
<td>181 (41)*</td>
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<tr>
<td>ALT (IU litre⁻¹)</td>
<td>45 (2)</td>
<td>68 (11)*</td>
<td>50 (4)</td>
<td>87 (18)*</td>
<td>33 (12)</td>
<td>53 (17)*</td>
</tr>
<tr>
<td>GGT (IU litre⁻¹)</td>
<td>32 (12)</td>
<td>44 (24)</td>
<td>263 (137)</td>
<td>315 (167)</td>
<td>52 (55)*</td>
<td>356 (194)*</td>
</tr>
<tr>
<td>Creatinine (mg dl⁻¹)</td>
<td>1 (0.1)</td>
<td>0.9 (0.2)</td>
<td>1.1 (0.2)</td>
<td>0.8 (0.2)</td>
<td>0.9 (0.2)</td>
<td>1.1 (0.3)</td>
</tr>
<tr>
<td>Urea (mg dl⁻¹)</td>
<td>20 (1)</td>
<td>20 (1)</td>
<td>21 (6)</td>
<td>25 (4)</td>
<td>18 (5)</td>
<td>19 (6)</td>
</tr>
<tr>
<td>Uric acid (mg dl⁻¹)</td>
<td>0.3 (0.1)</td>
<td>0.2 (0.1)</td>
<td>0.2 (0.1)</td>
<td>0.3 (0.2)</td>
<td>0.3 (0.2)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>Osmolality (mosmol kg⁻¹)</td>
<td>317 (5)</td>
<td>318 (5)</td>
<td>316 (8)</td>
<td>317 (6)</td>
<td>316 (6)</td>
<td>317 (5)</td>
</tr>
<tr>
<td>Albumin (g dl⁻¹)</td>
<td>3 (0)</td>
<td>3 (1)</td>
<td>3 (0)</td>
<td>3 (1)</td>
<td>2 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Amylase (IU litre⁻¹)</td>
<td>148 (156)</td>
<td>276 (124)</td>
<td>361 (185)</td>
<td>293 (171)</td>
<td>200 (145)</td>
<td>251 (193)</td>
</tr>
<tr>
<td>Lipase (IU litre⁻¹)</td>
<td>7 (1)</td>
<td>8 (2)</td>
<td>8 (1)</td>
<td>7 (1)</td>
<td>6 (1)</td>
<td>7 (1)</td>
</tr>
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</table>

Indicators of organ function

Plasma concentrations of pro-B-natriuretic peptide, myoglobin, bilirubin, creatinine, urea, uric acid, osmolality, albumin, amylase, and lipase were not significantly affected by any of the interventions and remained comparable between the groups throughout (Table 1). In contrast, the use of CPB and administration of quercetin was associated with increases in plasma concentrations of creatine kinase, lactate dehydrogenase, glutamate oxalacetate transaminase (quercetin), glutamate pyruvate transaminase (quercetin), and gamma-glutamyl transferase (all $P<0.05$). These increases were prevented by pre-treatment with inhaled carbon monoxide ($P<0.05$, CPB vs CO+CPB) (Table 1). This beneficial effect of pre-treatment with inhaled carbon monoxide was partially reversed by prior administration of quercetin ($P<0.05$, Q+CO+CPB vs CO+CPB; Table 1).

Pulmonary Hsps

In the SHAM control and the non-pre-treated CPB groups, mRNA expressions (Figs 1A and 2A) and protein concentrations (Figs 1C and 2C) of Hsp-70 and Hsp-90α remained unchanged throughout, and no DNA binding of heat shock factor (HSF)-1 was observed at any time (Fig. 3A, lanes 2, 4, 6, 8, 10 and 12; Fig. 3B and C, lane 2).

In contrast, pre-treatment with inhaled carbon monoxide (CO+CPB) was followed by immediate and sustained induction of mRNA transcription of Hsp-70 (Fig. 1A) and Hsp-90α (Fig. 2A), and of DNA binding of HSF-1 (Fig. 3A, lanes 1, 3, 5, 7, 9 and 11; Fig. 3B and C, lane 3). This was associated with increased protein concentrations of Hsp-70 at 60 and 120 min post-CPB (Fig. 1C), and of Hsp-90α at 10 and 120 min of CPB (Fig. 2C) (all at least $P<0.05$).

Pre-treatment with quercetin alone (Q+SHAM, Q+CPB) did neither affect protein concentrations of Hsp-70 and Hsp-90α (Figs 1C and 2C), nor DNA binding of HSF-1 (Fig. 3B and C, lanes 4 and 5). However, administration of quercetin before inhalation of carbon monoxide (Q+CO+CPB) abolished the carbon monoxide-induced increases in Hsp-70 protein concentration post-CPB (Fig. 1C) and in Hsp-90 protein concentration during CPB (Fig. 2C), and activation of HSF-1 (Fig. 3B–E).

At 120 min post-CPB, no binding of antibodies of Hsp-70 (Fig. 4A) and Hsp-90α (Fig. 4B) was detectable in the histological samples of the SHAM and CPB groups. In contrast, pre-treatment with inhaled carbon monoxide (CO+CPB) was associated with increased binding of Hsp-70 and Hsp-90α antibodies (brown colour, Fig. 4A and B). However, administration of quercetin before inhalation of carbon monoxide (Q+CO+CPB) prevented the expression of Hsp-70 and Hsp-90α almost completely (Fig. 4A and B). Interestingly, the anti-Hsp-90α antibody was detectable only in the epithelial cells of the bronchi (Q+CO+CPB, Fig. 4B).

Pulmonary IL-6 expression

In SHAM control animals, pulmonary IL-6 mRNA (Fig. 5A and B) and protein concentration remained unchanged throughout the experiment (Fig. 5C). In non-pre-treated animals (CPB), IL-6 mRNA (Fig. 5A and B) and protein concentration (Fig. 5C) increased during and after CPB (all at least $P<0.05$). In contrast, pre-treatment with inhaled carbon monoxide (CO+CPB) prevented these increases (Fig. 5A–C).
Pre-treatment with quercetin alone (Q+SHAM, Q+CPB) did not significantly modify the findings of IL-6 protein concentrations observed in the absence of quercetin pre-treatment (Fig. 5c). However, administration of quercetin before inhalation of carbon monoxide (Q+CO+CPB) prevented the carbon monoxide-associated suppression of increases in IL-6 protein concentration (Fig. 5c).

At 120 min post-CPB, there was macrophage-associated IL-6 staining. Pre-treatment with inhaled carbon monoxide (CO+CPB) suppressed macrophage-associated IL-6 staining (Fig. 6a and b). This was no longer the case after pre-treatment with inhaled carbon monoxide.

**Lung histopathology**

At 120 min post-CPB, no histopathological changes were seen in pulmonary samples of the SHAM control group (Fig. 7). In contrast, CPB was associated with histological...
findings of atelectasis, oedema, and macrophage infiltration. No such signs of lung injury were observed after pre-treatment with inhaled carbon monoxide (CO+CPB).

Pre-treatment with quercetin in the absence (Q+SHAM) or presence of CPB (Q+CPB) did not significantly modify lung histology when compared with that observed in the absence of quercetin pre-treatment (Fig. 7). However, administration of quercetin before inhalation of carbon monoxide (Q+CO+CPB) abolished the carbon monoxide-associated suppression of histological lung injury.

**Lung macrophages**

At 120 min post-CPB, pulmonary macrophage count was normal in the SHAM control group (Fig. 8A). CPB was associated with a larger number of macrophages (Fig. 8A

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**Fig 2** Pulmonary expression of Hsp-90. (A) mRNA expression of Hsp-90 without (CPB, grey bars) and with pre-treatment of inhaled CO (CO+CPB, black bars). (B) Representative northern blot of Hsp-90 mRNA and 18S rRNA before CPB (pre-CPB, lanes 1 and 2), at 10 (lanes 3 and 4) and 120 min (lanes 5 and 6) of CPB, and at 15 (lanes 7 and 8), 60 (lanes 9 and 10), and 120 min (lanes 11 and 12) post CPB—in the absence (−) and presence (+) of pre-treatment with inhaled CO. *P<0.05 and **P<0.001, CO+CPB vs CPB. (C) Pulmonary concentrations of Hsp-90. Respective concentrations in SHAM controls (SHAM and Q+SHAM), CPB with quercetin pre-treatment (Q+CPB) (left panel), and CPB alone (CPB), pre-treatment with inhaled CO (CO+CPB), and administration of quercetin before pre-treatment with inhaled CO (Q+CO+CPB) (right panel). Values are means (sd). #P<0.001 and *P<0.05, CO+CPB vs CPB and Q+CO+CPB.
and B). In contrast, pre-treatment with inhaled carbon monoxide (CO+CPB) inhibited macrophage infiltration (Fig. 8A and B). Administration of quercetin before inhalation of carbon monoxide (Q+CO+CPB) abolished the carbon monoxide-induced inhibition of macrophage infiltration (Fig. 8A and B).
The main findings of this randomized experimental in vivo study can be summarized as follows. (1) Inhalation of carbon monoxide before CPB: (a) induced pulmonary Hsp-70 and Hsp-90 gene and protein expression, (b) induced HSF-1 DNA binding activity, (c) prevented CPB-associated increases in pulmonary IL-6 mRNA and protein concentration, (d) prevented CPB-associated histological findings of lung injury, and (e) inhibited CPB-associated alveolar macrophage infiltration. (2) I.V. administration of quercetin before pre-treatment with inhaled CO antagonized all carbon monoxide-induced changes; it: (a) abolished expression of pulmonary Hsps, (b) abolished activation of HSF-1, (c) prevented suppression of increases in IL-6 protein concentration, (d) abolished suppression of histopathological lung injury, and (e) abolished inhibition of macrophage infiltration.
infiltration. These findings support our hypothesis that inhaled carbon monoxide before CPB mediates its pulmonary anti-inflammatory and cytoprotective effects via increased expression of Hsps.

We previously demonstrated that pre-treatment with inhaled carbon monoxide provides anti-inflammatory and cytoprotective effects during CPB. However, we did not investigate the molecular mechanism underlying this protective effect. Previous studies had shown that inhaled carbon monoxide suppressed the pro-inflammatory response and afforded cytoprotection. In 

\[\text{\textit{in vivo}}\] pulmonary models, carbon monoxide attenuated ischaemia/reperfusion injury. Carbon monoxide-mediated effects were diminished in HSF knockout mice, suggesting a possible role of the heat shock response in carbon monoxide-associated cytoprotection. In \textit{in vitro} administration of carbon monoxide prevented the lipopolysaccharide-induced production of pro-inflammatory cytokines in cultured macrophages. In our model, administration of quercetin reversed the cytoprotective effects of carbon monoxide, suggesting carbon monoxide-induced activation of Hsp-70 and Hsp-90 expression as the main underlying mechanism.
Several studies have examined the protective capacity of Hsp-70 and Hsp-90. In rats, ischaemic hepatic preconditioning induced Hsp-70, resulting in resistance to subsequent hepatic ischaemia/reperfusion injury.25 Whereas down-regulation of Hsp-70 facilitated tumour necrosis factor-induced apoptosis, up-regulation inhibited such apoptosis.26 – 28 Additionally, the expression of Hsp-70 conferred protection in models of cardiac ischaemia/reperfusion injury and acute respiratory distress syndrome.29 – 31 Accumulating evidence suggests that Hsps may mediate anti-inflammation by inhibiting pro-inflammatory gene expression.19 32 Our findings of antagonism of carbon monoxide-mediated inhibition of IL-6 expression by quercetin would suggest that carbon monoxide ameliorated tissue injury by early down-regulation of inflammatory regulators and by secondary down-regulation of stress protein gene expression.

As carbon monoxide abolished cytokine and chemokine release in the lung early on,5 24 we explored the role of carbon monoxide in modulation of HSF-1. In eukaryotic cells, HSF-1 has been identified as the primary stress-inducible molecule mediating heat shock gene expression.33 The data presented here provide evidence that carbon monoxide is able to induce a process which involves DNA binding of HSF-1 and subsequent transactivation of heat shock genes. In resting cells, HSF-1 is kept in an inactive form mainly by Hsp-90.34 During environmental stress, misfolded proteins occupy these repressing chaperones, resulting in the dissociation of the cytoplasm chaperone/HSF-1 complex.34 Alternatively, carbon monoxide might act via the modulation of transcriptional regulators such as PPARγ, Egr-1, or both. In lung macrophages and in a ventilator-induced lung injury, PPARγ acts as an anti-inflammatory mediator.35

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**Fig 8** (A) Representative histological lung sections at 120 min post-CPB. Lung sections (100-fold magnification) in SHAM control, and after CPB alone (CPB), pre-treatment with inhaled CO (CO+CPB), pre-treatment of SHAM controls with quercetin (Q+SHAM), pre-treatment of the CPB group with quercetin (Q+CPB), and administration of quercetin before pre-treatment with inhaled CO (Q+CO+CPB) after specific macrophage staining. (B) Macrophage count. Macrophages were counted in 10 randomly chosen fields of vision of each lung section sample. Values are means (sd). *P<0.05, CPB vs SHAM control and pre-treatment with inhaled CO (CO+CPB); #P<0.001, administration of quercetin before pre-treatment with inhaled CO (Q+CO+CPB) vs SHAM and CO+CPB.
Circulating humoral and cellular factors account primarily for the inflammatory processes and the subsequent pulmonary injury associated with exposure to foreign material during CPB. Additional factors such as ischaemia and reperfusion, fluid load, allogenic transfusions, and bacterial and endotoxin translocation as a result of gut hypoperfusion contribute to this process. The importance of the alveolar space as a source of inflammatory mediators is highlighted by the finding of increased concentrations of several inflammatory mediators in bronchoalveolar lavage fluid. Furthermore, gene expression of IL-6, IL-8, and tumour necrosis factor-α were up to eight times higher in alveolar macrophages than in plasma monocytes after CPB. The key role of Hsps in the times higher in alveolar macrophages than in plasma monocytes after CPB. The key role of Hsps in the aetiology of CPB-induced lung injury is emphasized by our finding that pre-treatment with quercetin before inhalation of carbon monoxide resulted in increased pulmonary macrophage infiltration and release of IL-6 in the alveolar space, thus counteracting the effects of pre-treatment with inhaled carbon monoxide alone.

We conclude that up-regulation of Hsps is an important mediator of the lung protective effect of inhaled carbon monoxide. The up-regulation of Hsps prevents the up-regulation of the pro-inflammatory cytokine IL-6, which, in turn, reduces pulmonary cytokine production and infiltration of macrophages. Conceivably, pre-treatment with inhaled carbon monoxide at low concentrations may constitute a therapeutic option for the reduction of CPB-mediated lung injury.

**Supplementary data**

Appendices 1 and 2 are available as Supplementary data at *British Journal of Anaesthesia* online.

**Acknowledgements**

The authors wish to thank Katharina Foerster (V.D., Department of Cardiovascular Surgery, University Medical Center, Freiburg, Germany) for her helpful assistance in animal care, and Heide Marninga (Technician, Department of Anaesthesia and Critical Care Medicine, University Medical Center, Freiburg, Germany) for lung immunohistopathology.

**Funding**

This work was supported by the Deutsche Forschungsgemeinschaft (DFG SCHL 604/2-1) and departmental funding.

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