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Carbon Monoxide Rescues Mice from Lethal Sepsis by Supporting Mitochondrial Energetic Metabolism and Activating Mitochondrial Biogenesis

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ABSTRACT

Use of metal carbonyl-based compounds capable of releasing carbon monoxide (CO) in biological systems have emerged as a potential adjunctive therapy for sepsis via their antioxidant, anti-inflammatory, and antiapoptotic effects. The role of CO in regulation of mitochondrial dysfunction and biogenesis associated with sepsis has not been investigated. In the present study, we employed a ruthenium-based water-soluble CO carrier, tricarbonylchoro(glycinato)ruthenium (II) (CORM-3), one of the novel CO-releasing molecules (CO-RMs), to test whether CO can improve cardiac mitochondrial dysfunction and survival in peritonitis-induced sepsis. Peritonitis was performed in mice by cecal ligation and perforation. Tumor necrosis factor-α, interleukin-10, and nitrite/nitrate plasma levels were tested to evaluate the systemic inflammatory response. Functional mitochondrial studies included determination of membrane potential, respiration, and redox status. Oxidative stress was evaluated by measurements of mitochondrial hydrogen peroxide, carbonyl protein and GSH levels. Mitochondrial biogenesis was assessed by peroxisome proliferator-activated receptor γ coactivator (PGC)-1α protein expression and mitochondrial DNA (mtDNA) copy number. The systemic inflammatory response elicited by peritonitis was accompanied by mitochondrial energetic metabolism deterioration and reduced PGC-1α protein expression. CORM-3 treatment in septic mice restored the deleterious effects of sepsis on mitochondrial membrane potential, respiratory control ratio, and energetics. It is interesting that administration of CORM-3 during sepsis elicited a mild oxidative stress response that stimulated mitochondrial biogenesis with PGC-1α protein expression and mtDNA copy number increases. Our results reveal that delivery of controlled amounts of CO dramatically reduced mortality in septic mice, indicating that CO-RMs could be used therapeutically to prevent organ dysfunction and death in sepsis.

Carbon monoxide (CO) represents a major air pollutant with a well established reputation of toxic effects at high inspired doses (Ernst and Zibrak, 1998). As a different concept, administration of small amounts of CO has emerged recently as a potential therapy for human diseases (Desmard et al., 2007; Ryter and Choi, 2007). The possibility that CO could be used clinically arose from observations of dramatic tissue protection after the application of low concentrations of this gas in animal models of inflammation, sepsis, oxidative stress, and ischemia-reperfusion injury (Mayr et al., 2005; Hoetzel et al., 2007; Ryter and Choi, 2007). CO has long been known to inhibit cytochrome *c* oxidase by competing with oxygen binding, which would collapse mitochondrial membrane potential, decrease ATP synthesis, and ultimately lead to cell death (Iheagwara et al., 2007). Although the majority of evidence supporting this concept originates from studies using very high concentrations of CO, there is also evidence that low levels of CO, which still inhibit cytochrome

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ABBREVIATIONS: CO, carbon monoxide; ROS, reactive oxygen species; CO-RM, carbon monoxide-releasing molecule; CORM-3, tricarbonylchoro(glycinato)ruthenium (II); CLP, cecal ligation and puncture; iCORM-3, inactive CORM-3; TNF, tumor necrosis factor; IL, interleukin; TES, 2-morpholinoethanesulphonic acid *N*-[tris(hydroxymethyl)methyl]-2-aminoethane sulfonic acid; RCR, respiratory control ratio; H_2O_2, hydrogen peroxide; GSSG, GSH disulfide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TPP^+, tetraphenylphosphonium; PGC, peroxisome proliferator-activated receptor γ coactivator; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; mtCOII, mouse mitochondrial cytochrome *c* oxidase subunit II; ANOVA, analysis of variance.

c oxidase, preserve ATP generation and cell function (Kim et al., 2006; Zuckerbraun et al., 2007). In this context, CO acts via cytochrome *c* oxidase inhibition, leading to the generation of low levels of reactive oxygen species (ROS) that in turn mediate subsequent adaptive mechanism(s) to counteract cellular dysfunction (Zuckerbraun et al., 2007). Mechanisms of protection conferred by CO are only partially elucidated and are thought to be mediated by activation of mitogen-activated protein kinase pathways, peroxisome proliferator-activated receptors, and heme-containing molecules and mitochondrial oxidases (Boczkowski et al., 2006; Kim et al., 2006). Moreover, growing evidence suggest that modest increases in cellular CO concentrations may activate mitochondrial biogenesis by a set of molecular responses that includes mitochondrial hydrogen peroxide production, direct activation of guanylate cyclase and phosphatidylinositide 3-kinase/akt, and induction of heme-oxygenase-1 (Suliman et al., 2007a,b).

Sepsis is a complex syndrome characterized by inflammation, oxidative damage, hypercoagulation, tissue hypoperfusion, immune suppression, and mitochondrial dysfunction (Carré and Singer, 2008). Existing therapeutic approaches have failed to reduce mortality of this syndrome. Metal carbonyl-based compounds [CO-releasing molecules (CO-RMs)], capable of delivering small amounts of CO to biological systems in a controlled manner (Motterlini et al., 2005, 2007), are emerging as a potential therapy for sepsis via their antioxidant, anti-inflammatory, and antiapoptotic effects (Hoetzel et al., 2007). For example, CO-RMs reduce cytokine release in LPS-stimulated macrophages (Sawle et al., 2005) and decrease inflammatory response and oxidative stress in LPS-stimulated endothelial cells (Sun et al., 2008). In vivo, CO-RMs attenuate systemic inflammation and proadhesive vascular endothelial cell properties in septic and thermally injured mice by reducing nuclear factor κB activation, protein expression of ICAM-1, and tissue granulocyte infiltration (Sun et al., 2007; Cepinskas et al., 2008). It is interesting that CO-RMs doses that are protective in vivo are well below the threshold needed to raise blood CO hemoglobin levels, implying that at least some of the CO released by CO-RMs escapes the reaction with hemoglobin in the blood and is delivered to tissues.

The role of CO in the modulation of mitochondrial dysfunction and regulation of mitochondrial biogenesis associated with prolonged sepsis has not been investigated yet. Because the cardiovascular system is severely affected by sepsis and its dysfunction related to poor outcome (Martin et al., 2003), we focused our investigation on how sepsis alters cardiac mitochondrial activities. We tested whether a ruthenium-based water-soluble CO carrier, tricarbonylchoro(glycinato)ruthenium (II) (CORM-3) (Clark et al., 2003), can improve sepsis-induced cardiac mitochondrial dysfunction and survival in peritonitis-challenged mice.

Materials and Methods

Animals Used. Six- to eight week-old (25–30 g) outbred (ICR) male mice (Harlan, Indianapolis, IN) were housed for 6 days before manipulation. All experiments were conducted in accordance with the National and European Institutes of Health guidelines for the use of laboratory animals and were approved by the Lille University. The investigation conforms to the PHS Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/olaw.htm).

Sepsis Model. Cecal ligation and puncture (CLP) was used to induce intra-abdominal peritonitis and sepsis. Under anesthesia (2.5 mg/kg i.p. ketamine and 0.25 mg/kg i.p. xylazine), the cecum was ligated with 4-0 silk suture immediately distal to the ileocecal valve, punctured once with a 21-gauge needle, and gently squeezed to extrude some stool. The cecum was then replaced into the abdomen, which was closed in two layers, followed by a 1.0-ml subcutaneous injection of 0.9% saline. Sham-operated animals were treated identically, except that the cecum was neither ligated nor punctured. Animals were maintained on 12-h light/dark cycles with free access to water.

Animal Treatments. CORM-3, synthesized as described previously (Clark et al., 2003), was kindly provided by Hemocorm Ltd, Harrow, UK. It was prepared as a 10 mM stock by dissolving the compound in pure distilled water and was kept at −20°C and defrosted before each experiment. After surgery procedures, sham and CLP mice were randomized to receive immediately after surgery and 12 h later 1.0 ml s.c. injection of either: 1) 10 mg/kg CORM-3 in normal saline or 2) 10 mg/kg inactive CORM-3 (iCORM-3). To prepare iCORM-3, the required amount of compound was dissolved in saline and left at room temperature for 24 h to liberate its entire CO content, and the residual CO present was finally removed by bubbling N_2 for 10 min (Clark et al., 2003). Four groups of mice were then studied: sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3.

Survival Studies. Survival studies after CLP were repeated twice. An investigator blinded to the identity of the mice performed a 72-h follow-up in two separate experiments using 20 mice per group.

Systemic Inflammation Evaluation. Plasma levels of nitrite/nitrate, an index of NO production, were measured by the Griess reaction. Plasma levels of TNF-α and interleukin (IL)-10 levels were determined by using commercially immunoassay kits specific for cytokines (Quantikine Mouse TNF-α and interleukin IL-10, R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK). Reading was realized in a microplate reader Digiscan (Spectracount Packard; Perkin Elmer Life and Analytical Sciences, Boston, MA).

Mitochondrial Respiration. Mouse hearts were placed in isolation buffer A containing 300 mM sucrose, 5 mM TES, and 0.2 mM EGTA, pH 7.2 (4°C). Cardiac tissue was then finely minced and homogenized by the use of a Kontes tissue grinder. After centrifugation (800g), supernatant was centrifuged at 8800*g* for 5 min. Mitochondrial pellet was resuspended in buffer A and centrifuged one more time at 8800*g* for 5 min. Protein concentration was determined according to the Bradford method. Purity and integrity of isolated mitochondria were assessed by measuring specific activities of nicotinamide adenine dinucleotide phosphate NADPH-cytochrome *c* reductase, as an endoplasmic reticulum marker enzyme, and cytochrome *c* oxidase, as an inner mitochondrial membrane marker enzyme.

For in vivo experiments, enriched mitochondrial fraction (200 μg/ml) obtained from sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3 were suspended in respiration medium. Mitochondrial respiration (Oroboros Instruments, Innsbruck, Austria) was evaluated, i.e., state 4 respiration rate (oxygen uptake with 5 mM glutamate and 2 mM malate in the absence of exogenous ADP; picomoles of oxygen per second per milligram), state 3 respiration rate (oxygen uptake during 1 mM ADP phosphorylation; picomoles of oxygen per second per milligram) and respiratory control ratio (RCR): ratio of states 3 and 4 oxygen uptake rates.

Mitochondrial Oxidative Stress. Hydrogen peroxide (H_2O_2) generated from isolated mitochondria was measured using the Amplex red assay technique according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In brief, aliquots of freshly prepared mitochondrial suspensions were incubated with malate/pyruvate in assay buffer. Amplex red was added to initiate reactions;

TABLE 1

Respiration parameters and membrane potential of isolated heart mitochondria

Respiration rates (picomoles of oxygen per second per milligram) with glutamate (5 mM) and malate (2 mM) in the absence of exogenous ADP (state 4) and with 1 mM ADP (state 3). Sample size is five to six mitochondrial preparations in each group. Results are presented as mean ± S.E.M. and analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment.

CORM-3	Control	0.5 μM	1 μM	10 μM	50 μM
State 4	164 ± 12	188 ± 8	208 ± 6*	260 ± 40*	316 ± 11*
State 3	670 ± 75	715 ± 10	1050 ± 140*	655 ± 88	320 ± 25*
RCR	4.0 ± 0.7	4.1 ± 0.3	5.2 ± 03*	2.5 ± 0.2*	1.0 ± 0.1*
$\Delta\Psi_m$	−215 ± 1.0	−215 ± 1.5*	−225 ± 1.5*	−205 ± 1.2*	−195 ± 3.5*

RCR, respiratory control ratio of states 3 and 4 respiration rates; Δ Ψm, mitochondrial membrane potential (millivolts) measured with glutamate/malate in absence of exogenous ADP.

* $P < 0.05$ vs. controls.

this indicator reacts with H_2O_2 to generate a fluorescent signal with a 540-nm excitation and 590-nm emission wavelengths. H_2O_2 standards, supplied with the Amplex red kit, were used to calibrate assays. Assay results are reported in picomoles of H_2O_2 per milligram of protein.

Protein carbonyl evaluation in isolated mitochondria was based on spectrophotometric detection of protein hydrazones, the reaction product of 2,4-dinitrophenylhidrazine with protein carbonyls, using a commercially available kit (VWR, West Chester, PA). Mitochondrial GSH and GSH disulfide (GSSG) were determined by an optimized enzymatic recycling method, using glutathione reductase for the quantification of GSH according to manufacturer's instructions (VWR).

Mitochondrial Transmembrane Potential. Isolated mitochondria (1 mg/ml proteins) were suspended in buffer C: 250 mM sucrose, 10 mM MOPS, 5 mM glutamate-Tris 5, 2 mM malate-Tris 2, 1 mM π-Tris, and 0.02 mM EGTA-Tris, pH 7.4, at 25°C in a multiport measurement chamber equipped with tetraphenylphosphonium (TPP^+)-selective microelectrodes and reference electrodes (WPI, Sarasota, FL). First, mitochondria were gently stirred for 1.5 min in buffer C containing 1.5 μM TPP^+ (Sigma-Aldrich, St. Louis, MO). Mitochondrial transmembrane potential was estimated by calculating the transmembrane distribution of TPP^+. Transmembrane potential ΔΨm was calculated as $59\log(v/V) - 59\log(10^{\Delta E/59} - 1)$, where v is matrix volume (1.1 μl/mg mitochondrial protein), V is volume chamber (1 ml), and ΔE is voltage difference before and after calcium-induced permeability transition, expressed in millivolts.

Mitochondrial Nicotinamide Nucleotide Concentrations. Assay of nicotinamide nucleotides NAD and NADH was used to evaluate energy production capacity and redox state of mitochondria. A specific enzyme cycling reaction that detects NAD and NADH was used according to the manufacturer's instructions (BioVision, Mountain View, CA).

Western Blotting. Proteins isolated from mitochondria (50 μg) were run on a 10% polyacrylamide gel. Proteins in gel were electrophoretically transferred to nitrocellulose membranes. After blocking, membranes were treated with rabbit polyclonal anti-peroxisome proliferator-activated receptor γ coactivator (PGC)-1α and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies (Cell Signaling Technology Inc., Danvers, MA). Membranes were then incubated with horseradish peroxidase-conjugated sheep anti-rabbit and mouse immunoglobulin G secondary antibody (Bio-Rad S.A, Marnes-la-Coquette, France), washed, and bound antibodies were detected by the use of the ECL Plus kit (GE Healthcare, Chalfont St. Giles, UK).

Mitochondrial DNA Copy Number Determination. Total DNA (genomic + mitochondrial) was extracted from heart tissue using QIAamp DNA Mini Kit (QIAGEN S.A., Courtaboeuf, France). The relative mitochondrial DNA (mtDNA) copy number was measured by PCR and corrected by simultaneous measurement of the nuclear DNA. The forward and reverse primers for mtDNA, which are complementary to the sequence of the mouse mitochondrial cytochrome *c* oxidase subunit II (mtCOII) gene, were 5′-AACCATAGGGCACCAATGATAC-3′ and 5′-GGATGGCATCAGTTTTAAGTCC-3′. 5′-CGGCGACGACCCATTCGAAC-3′ and 5′-GAATCGAACCCTGATTCCCCGTC-3′, sequences complementary to the 18S gene, were the primers used for the detection of nuclear DNA. After 33 cycles (92, 58, and 72°C as denaturation, annealing, and

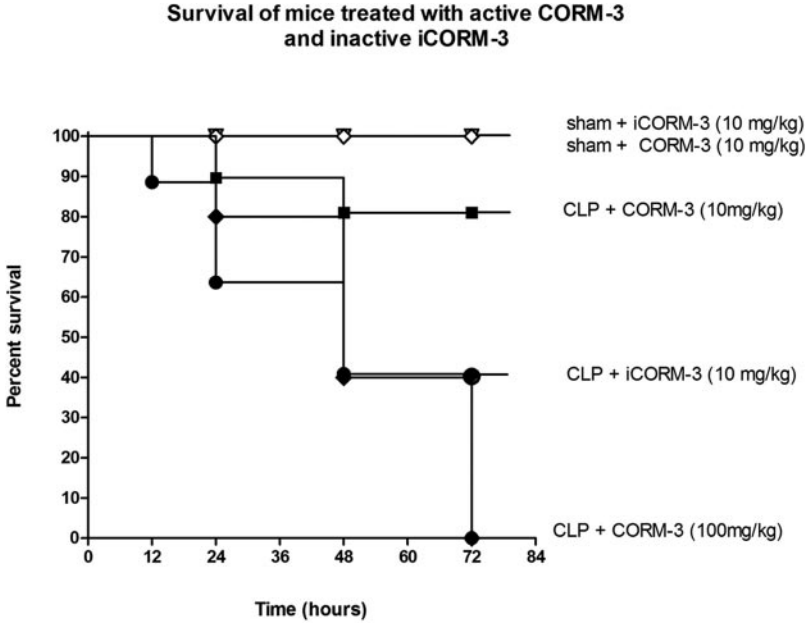
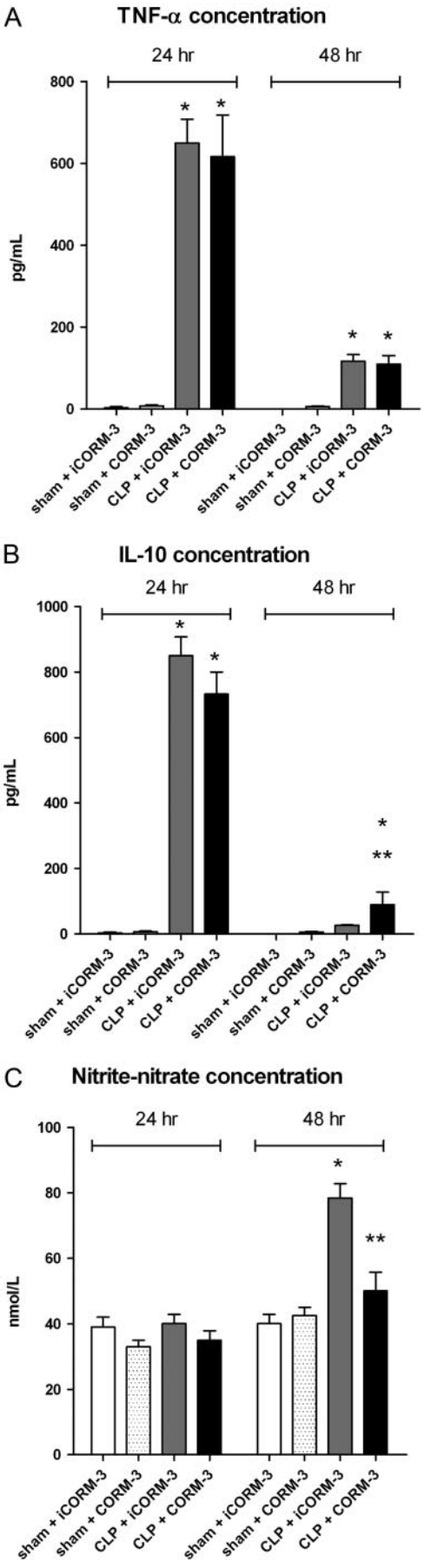


Fig. 1. Effect of CORM-3 in CLP-induced sepsis. The survival rate was followed in sham + iCORM-3-, sham + CORM-3-, CLP + iCORM-3-, and CLP + CORM-3 (10 and 100 mg/kg)-treated mice. The sample size is 20 mice in each group. Survival studies have been conducted twice. Comparison between groups was assessed by Kaplan-Meier, log rank.



extension temperatures, respectively), products were resolved in agarose gel-containing ethidium bromide and scanned with a gel documentation system. Relative intensities of mtCOII and 18S products were analyzed with ImageJ. A relative amount of mtDNA was expressed as the mtCOII/18S ratio.

Statistics. Results were analyzed with the SPSS 11.0.1 (SPSS Inc., Chicago, IL). Data represent means ± S.E.M and were analyzed by ANOVA procedures. We identified specific differences between groups using a sequentially rejective Bonferroni procedure. After application of the Bonferroni correction, $P < 0.05$ was taken as a level of statistical significance. Survival was evaluated using the Fisher's exact test.

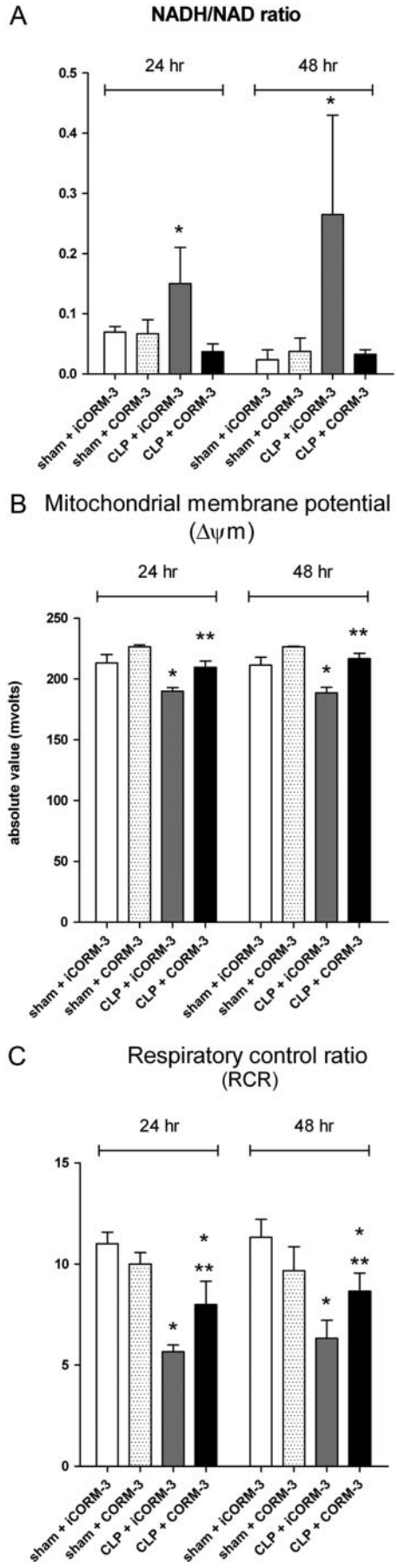
Results

First, the effects of CORM-3 and iCORM-3 were evaluated on mitochondrial fractions from control hearts (Table 1). Inactive CORM-3 had no effects on the measured mitochondrial parameters. CORM-3 increased state 4 (uncoupled) respiration in a dose-dependent manner (0.5–50 μM). CORM-3 (1 μM) increased state 3 respiration and mitochondrial membrane potential compared with controls. Above the concentration of 1 μM, CORM-3 reduced state 3 respiration in a dose-dependent manner (10–50 μM). Overall, the RCR was increased with 1 μM CORM-3 and decreased above 1 μM. Compared with controls, CORM-3 at 1 μM increased mitochondrial membrane potential, whereas it was dissipated at higher concentrations of CORM-3. The dependence of hyperpolarization induced by CORM-3 (1 μM) on the F_1F_0-ATPase was assayed by adding oligomycin (10 μM). In the presence of oligomycin, the mitochondrial membrane potential returned to control values ($\Delta\Psi_m$, −225 ± 1.5 versus −215 ± 1.5 mV; $P < 0.05$, five to six experiments in each group).

Secondly, the effects of CORM-3 were evaluated in vivo using a model of CLP-induced sepsis (Fig. 1). Septic mice began to die between 12 and 24 h post-CLP surgery. The highest mortality rate (60%) was observed 48 h post-CLP and remained stable at 84 h. To determine whether CORM-3 alters sepsis-induced mortality, mice were treated with increasing doses of CORM-3 (10 and 100 mg/kg), and the survival rate was followed for 84 h. A consistent level of protection against CLP-induced mortality was observed in mice treated with 10 mg/kg CORM-3 (Fig. 1), whereas a much higher dose regimen (100 mg/kg) had deleterious effects on the survival of CLP mice (Fig. 1). iCORM-3, which does not liberate CO, had no effect on the survival rate of sham and CLP-treated mice.

Pro- and anti-inflammatory responses induced by CLP-mediated sepsis were assessed by measuring the plasma levels of TNF-α and IL-10. After 24 h, sepsis induced a significant increase in TNF-α (Fig. 2A) and IL-10 (Fig. 2B) levels, which subsequently decreased at 48 h after treatment. Treatment with CORM-3 during sepsis had no significant effects on TNF-α, whereas it modestly increased IL-10 levels at 48 h after treatment. In addition, CORM-3 prevented the increase in nitrite/nitrate production, an indicator of NO

Fig. 2. Effects of CORM-3 on sepsis-induced systemic inflammatory response. TNF-α, IL-10, and nitrite-nitrate plasma levels in sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3. Sample size is 10 mice in each group. Results are presented as mean ± S.E.M. and were analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment. *, $P < 0.05$ versus sham + iCORM-3; **, $P < 0.05$ versus CLP + iCORM-3.



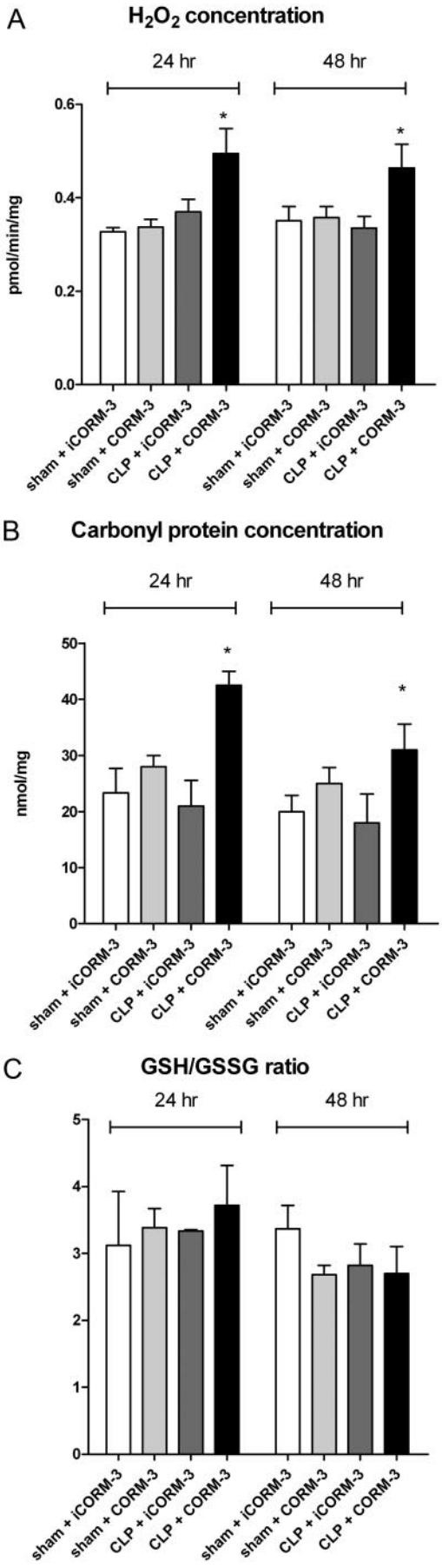
synthesis (Fig. 2C). CLP-induced sepsis was accompanied by deterioration of mitochondrial respiration, as indicated by a marked increase in the redox state (NADH/NAD ratio) (Fig. 3A) and membrane potential dissipation (Fig. 3B). In this context, administration of CORM-3 largely prevented CLP-induced deterioration of mitochondrial state 3 respiration rates at 24 and 48 h after treatment (Fig. 3C). CORM-3-treated CLP mice displayed normal mitochondrial membrane potential and redox state at 48 h. We observed a minor effect of CLP-induced sepsis on mitochondrial oxidative stress assessed by H_2O_2 production (Fig. 4A), carbonyl proteins (Fig. 4B), and GSH/GSSG ratio (Fig. 4C). In contrast, CORM-3 treatment in CLP-mediated sepsis consistently promoted an increase in both mitochondrial H_2O_2 production and carbonyl protein levels. Protein expression of PGC-1α, a specific marker of mitochondrial biogenesis, decreased after CLP-induced sepsis (Fig. 5). In contrast, CLP mice treated with CORM-3 resulted in a significant stimulation of PGC-1α protein expression (Fig. 5) and mtDNA copy number (Fig. 6), of which levels increased above sham levels.

Discussion

In the present study, we report for the first time that the water-soluble compound that liberates CO (CORM-3) restored cardiac mitochondrial membrane potential, respiratory control ratio, and cellular energetics in sepsis induced by experimental peritonitis. It is interesting that administration of CORM-3 during sepsis elicited a mild oxidative stress response that stimulated mitochondrial biogenesis. Overall, our data reveal the ability of CORM-3 to dramatically reduce mortality in septic mice.

Sepsis has attracted extensive investigation because this syndrome remains a leading cause of mortality in hospitalized patients (Martin et al., 2003). Sepsis is described as a combination of clinical manifestations of systemic inflammation specifically related to an infectious insult (Annane et al., 2005). Existing therapeutic approaches that target the systemic inflammatory response syndrome have failed to significantly reduce mortality. In the course of sepsis, fatalities are often preceded by multiple organ dysfunction, and emerging data implicate mitochondrial damage and dysfunction as prognosis factors (Abraham and Singer, 2007; Carré and Singer, 2008). Thus, strategies aimed of preventing the impairment of mitochondrial energy production may be beneficial. In this context, CO has emerged as a potential therapeutic stratagem because low concentrations of CO confer cytoprotection via inhibition of cytochrome *c* oxidase, which in turn induces preservation of mitochondrial membrane potential and cellular ATP levels (Kim et al., 2006; Hoetzel et al., 2007; Ryter and Choi, 2007; Zuckerbraun et al., 2007). In this report, we show for the first time that a CO-releasing metal carbonyl-based compound (CORM-3), previously characterized for its versatile pharmacological effects (Motterlini

Fig. 3. Effects of CORM-3 on sepsis-induced mitochondrial dysfunction. Mitochondrial membrane potential, respiratory control ratio, and NADH/NAD ratio in sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3. Sample size is 10 mice in each group. Results are presented as mean ± S.E.M. and were analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment. *, $P < 0.05$ versus sham + iCORM-3; **, $P < 0.05$ versus CLP + iCORM-3.



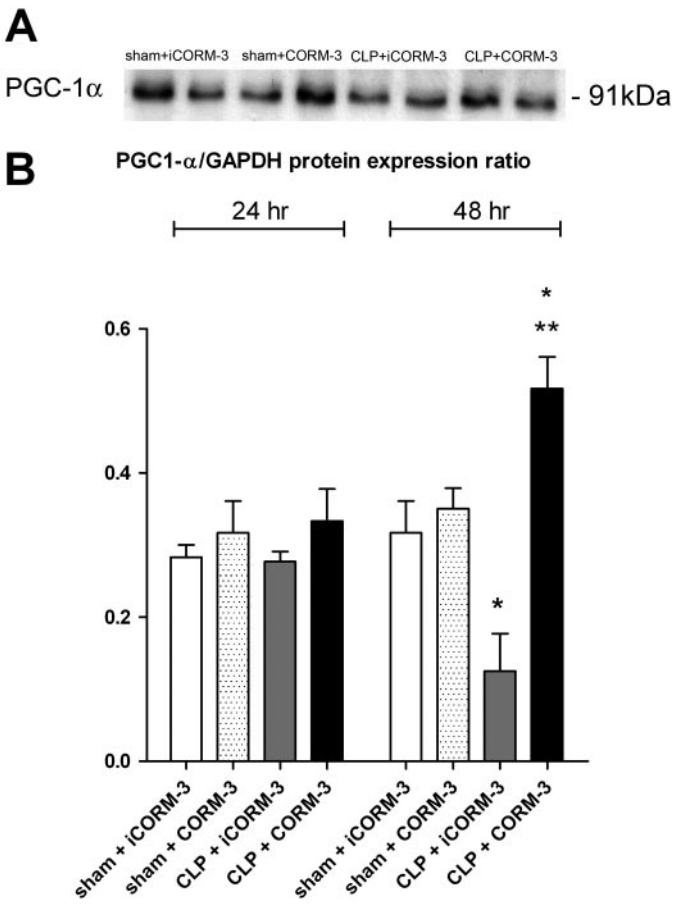


Fig. 5. Effects of CORM-3 on sepsis-induced mitochondrial biogenesis deficit. A, representative micrographs of PGC-1α Western blot at 48 h after treatment. B, densitometric analysis of PGC-1α to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression ratio in sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3. Sample size is five to six mice in each group. Results are presented as mean ± S.E.M. and were analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment. *, $P < 0.05$ versus sham + iCORM-3. **, $P < 0.05$ versus CLP + iCORM-3.

et al., 2005, 2007), improved cellular energetics and mortality in sepsis.

The effects of CORM-3 illustrated in our data are consistent with those demonstrating that CO affords protection against endotoxin challenge in vitro and in vivo by inhibiting of proinflammatory cytokine production such as TNF-α and IL-1β, in a mechanism involving the modulation of p38 mitogen-activated protein kinase (Otterbein et al., 2000; Sawle et al., 2005). In addition, CO gas promotes anti-inflammatory cytokine IL-10 production in animal models of endotoxemia (Otterbein et al., 2000; Mazzola et al., 2005). In our model of sepsis, CORM-3 had no effects on TNF-α and IL-10 levels at 24 h, whereas CORM-3 increased IL-10 levels at 48 h. Likewise, in vitro application of CO can inhibit nitric oxide synthase activity and subsequently reduce nitric oxide production (Sawle et al., 2005). In vivo, CO-mediated protective

Fig. 4. Effects of CORM-3 on sepsis-induced oxidative stress response. Mitochondrial H_2O_2 and carbonyl protein concentrations and GSH/GSSG ratio in sham + iCORM-3, sham + CORM-3, CLP + iCORM-3, and CLP + CORM-3. Sample size is 10 mice in each group. Results are presented as mean ± S.E.M. and were analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment. *, $P < 0.05$ versus sham + iCORM-3.

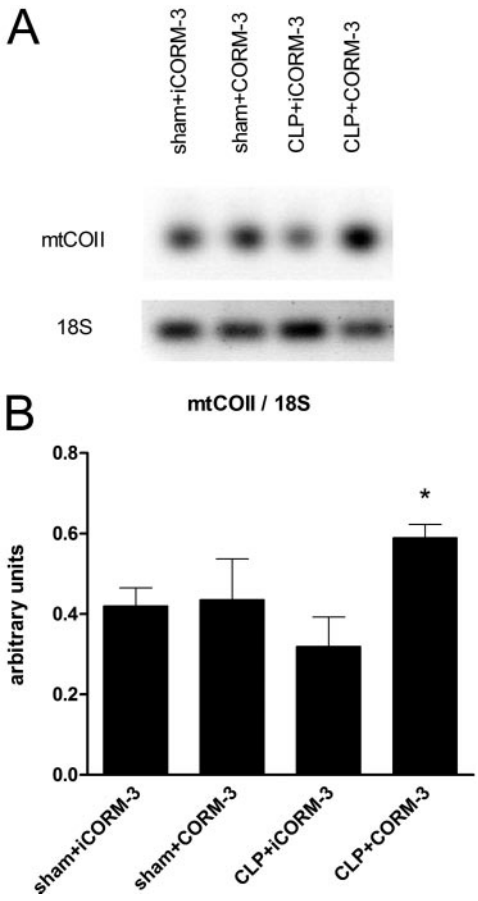


Fig. 6. CORM-3 increases mtDNA copy number in septic heart at 48 h after treatment. A, representative 1.2% agarose gel with ethidium bromide-containing PCR products. B, relative mtDNA (cytochrome *c* oxidase subunit II, mtCOII) copy number measured by PCR normalized to 18S rRNA. Results are presented as mean ± S.E.M. and were analyzed with one-way ANOVA and Bonferroni's multiple comparison post hoc adjustment. *, $P < 0.05$ compared with sham + iCORM-3.

effects can involve either increased or reduced nitric oxide production (Sarady et al., 2004; Cepinskas et al., 2008). Here, we found that CORM-3 reduced nitrite/nitrate plasma levels in CLP-induced sepsis. Overall, CORM-3 modestly inhibited the inflammatory signaling response to CLP at 48 h. However, when investigating diseases because of microbial infections, such as experimental peritonitis, inhibition of the inflammatory response could disrupt the ability of the immune system to eradicate invading pathogens. Although changes in blood and end-organ bacterial counts were not evaluated in our CLP model of polymicrobial sepsis, CO has been shown to enhance bacterial clearance in CLP by increasing phagocytosis and the endogenous antimicrobial response (Chung et al., 2008). Combination of properties, controlling infection without producing major immunosuppressive environment, could allow the eradication of bacteria and improved survival from CLP sepsis.

The biological effects of CO are complex because CO binds to diverse heme-containing proteins, including cytochrome P450, guanylate cyclase, and cytochrome *c* oxidase (Kim et al., 2006). Inhibition of cytochrome *c* oxidase by elevated concentrations of CO and the subsequent reduced mitochondrial electron transport activity may be deleterious to organ function. As a different and perhaps counterintuitive concept, we and others (Suliman et al., 2007b) suggest that modest increases in cellular CO concentrations might exert protective effects through an improvement of oxidative metabolism and/or mitochondrial biogenesis. The principal findings of our studies in isolated mitochondria are that relatively low concentrations of CORM-3 (~1 μM) elicited mitochondrial hyperpolarization (under conditions of state 4 respiration) and substantially improved the respiratory control ratio. Thus, it is likely that the mechanisms of protection conferred by CO involve, at least in part, an interaction with components that directly control mitochondrial oxidative metabolism. Akin to nitric oxide, hyperpolarization induced by CO may be attributed to cytochrome *c* oxidase inhibition (Zuckerbraun et al., 2007). Initial transient collapse of membrane potential that follows cytochrome *c* oxidase inhibition results in a more reduced state of the electron transport chain, proton leak closure, and the reversal of the ATP synthase, which effectively extrudes protons (Moncada and Erusalimsky, 2002). Combination of reduced electron transport chain state and hyperpolarization favors mitochondrial energy generation when ADP is again available, as shown by an increased state 3 respiration observed in our studies.

The principal finding of our in vivo studies is that administration of CORM-3 (10 mg/kg) improved mitochondrial energetic metabolism, consequently leading to a better outcome in septic mice. CORM-3 suppressed sepsis-related increase in NADH/NAD ratio, suggesting a clear improvement of mitochondrial energetics (Lavitrano et al., 2004). Treatment of septic mice with CORM-3 also improved mitochondrial respiration in association with a moderate increase in mitochondrial oxidative stress. These results are provocative because it has been suggested that increased ROS generation, even at low levels, can lead to damage to the electron transport chain (Ryter et al., 2007). However, increasing data suggest that a transient increase of ROS may play an important role in normal cell signaling (Murphy and Steenbergen, 2008). For example, CO-induced ROS generation has been associated with improved mitochondrial respiration after ischemia reperfusion injury (Sandouka et al., 2006), suggesting that heme-dependent cytochromes present in the mitochondria could potentially serve as a possible target for CO to confer cytoprotection. Alternatively, CO-induced generation of reactive oxygen species has been linked to increased respiratory complex mitochondrial protein content and mitochondrial biogenesis (Suliman et al., 2007a,b). Mitochondrial biogenesis requires nuclear and mitochondrial genomic activation driven by nuclear respiratory factors, mitochondrial transcription factors, and master PGC-1α (Scarpulla, 2008). Consistent with the notion that reactive oxygen species are an important stimulus for mitochondrial biogenesis, we observed that CORM-3 elicited a mitochondrial oxidative stress response in septic mice, which was accompanied by an increased protein PGC-1α expression and mitochondrial DNA copy number. Altogether, our in vivo results support the contention that CORM-3 ameliorates cellular energetics and stimulates mitochondrial biogenesis via a modest mitochondrial oxidative stress.

In summary, our results emphasize that CORM-3 has protective effects on cardiac mitochondrial energetics and improved survival in septic mice. In our in vivo model, CO-induced generation of reactive oxygen species could be responsible for the observed increased mitochondrial activi-

ties, which seem to be crucial to prevent multiple organ dysfunction. These findings suggest that CO-RMs could be used therapeutically to prevent organ injury and death in clinical sepsis.

References

Abraham E and Singer M (2007) Mechanisms of sepsis-induced organ dysfunction. *Crit Care Med* **35:**2408–2416.

Annane D, Bellissant E, and Cavaillon JM (2005) Septic shock. *Lancet* **365:**63–78.

Boczkowski J, Poderoso JJ, and Motterlini R (2006) CO-metal interaction: vital signaling from a lethal gas. *Trends Biochem Sci* **31:**614–621.

Carré JE and Singer M (2008) Cellular energetic metabolism in sepsis: the need for a systems approach. *Biochim Biophys Acta* **1777:**763–771.

Cepinskas G, Katada K, Bihari A, and Potter RF (2008) Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am J Physiol Gastrointest Liver Physiol* **294:**G184–G191.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, and Motterlini R (2003) Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* **93:**e2–8.

Chung SW, Liu X, Macias AA, Baron RM, and Perrella MA (2008) Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* **118:**239–247.

Desmard M, Boczkowski J, Poderoso J, and Motterlini R (2007) Mitochondrial and cellular heme-dependent proteins as targets for the bioactive function of the heme oxygenase/carbon monoxide system. *Antioxid Redox Signal* **9:**2139–2155.

Ernst A and Zibrak JD (1998) Carbon monoxide poisoning. *N Engl J Med* **339:**1603–1608.

Hoetzel A, Dolinay T, Schmidt R, Choi AM, and Ryter SW (2007) Carbon monoxide in sepsis. *Antioxid Redox Signal* **9:**2013–2026.

Iheagwara KN, Thom SR, Deutschman CS, and Levy RJ (2007) Myocardial cytochrome oxidase activity is decreased following carbon monoxide exposure. *Biochim Biophys Acta* **1772:**1112–1116.

Kim HP, Ryter SW, and Choi AM (2006) CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* **46:**411–449.

Lavitrano M, Smolenski RT, Musumeci A, Maccherini M, Slominska E, Di Florio E, Bracco A, Mancini A, Stassi G, Patti M, et al. (2004) Carbon monoxide improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass in pigs. *FASEB J* **18:**1093–1095.

Martin GS, Mannino DM, Eaton S, and Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* **348:**1546–1554.

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

Mazzola S, Forni M, Albertini M, Bacci ML, Zannoni A, Gentilini F, Lavitrano M, Bach FH, Otterbein LE, and Clement MG (2005) Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. *FASEB J* **19:**2045–2047.

Moncada S and Erusalimsky JD (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol* **3:**214–220.

Motterlini R (2007) Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. *Biochem Soc Trans* **35:**1142–1146.

Motterlini R, Mann BE, and Foresti R (2005) Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Investig Drugs* **14:**1305–1318.

Murphy E and Steenbergen C (2008) Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* **88:**581–609.

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

Ryter SW and Choi AM (2007) Cytoprotective and anti-inflammatory actions of carbon monoxide in organ injury and sepsis models. *Novartis Found Symp* **280:**165–175; discussion 175–181.

Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, and Choi AM (2007) Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* **9:**49–89.

Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R, and Motterlini R (2006) Treatment with CO-RMs during cold storage improves renal function at reperfusion. *Kidney Int* **69:**239–247.

Sarady JK, Zuckerbraun BS, Bilban M, Wagner O, Usheva A, Liu F, Ifedigbo E, Zamora R, Choi AM, and Otterbein LE (2004) Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. *FASEB J* **18:**854–856.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, and Motterlini R (2005) Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* **145:**800–810.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* **88:**611–638.

Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, and Piantadosi CA (2007a) The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. *J Clin Invest* **117:**3730–3741.

Suliman HB, Carraway MS, Tatro LG, and Piantadosi CA (2007b) A new activating role for CO in cardiac mitochondrial biogenesis. *J Cell Sci* **120:**299–308.

Sun BW, Jin Q, Sun Y, Sun ZW, Chen X, Chen ZY, and Cepinskas G (2007) Carbon liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice. *World J Gastroenterol* **13:**6183–6190.

Sun B, Zou X, Chen Y, Zhang P, and Shi G (2008) Preconditioning of carbon monoxide releasing molecule-derived CO attenuates LPS-induced activation of HUVEC. *Int J Biol Sci* **4:**270–278.

Zuckerbraun BS, Chin BY, Bilban M, d'Avila JC, Rao J, Billiar TR, and Otterbein LE (2007) Carbon monoxide signals via inhibition of cytochrome *c* oxidase and generation of mitochondrial reactive oxygen species. *FASEB J* **21:**1099–1106.

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