CARBON MONOXIDE RESCUES MICE FROM LETHAL SEPSIS BY
SUPPORTING MITOCHONDRIAL ENERGETIC METABOLISM AND
ACTIVATING MITOCHONDRIAL BIOGENESIS

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Running title page

Running title: Carbon monoxide improves mitochondrial energetics in sepsis

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List of nonstandard abbreviations:
3-(N-morpholino)propanesulfonic acid (MOPS), carbon monoxide (CO), carbon monoxide - releasing molecules (CO-RMs), cecal ligation and puncture (CLP), enhanced chemiluminescence (ECL), glutathione (GSH), GSH disulfide (GSSG), interleukin (IL), inactive CORM-3 (iCORM-3), mitochondrial DNA (mtDNA), mitochondrial membrane potential (ΔΨm), peroxisome proliferator-activated receptor γ co-activator (PGC-1α), respiratory control ratio (RCR), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), tricarboxylchoro(glycinato)ruthenium (II) (CORM-3), tetraphenylphosphonium (TPP+), tumor necrosis factor (TNF).

Section: cardiovascular
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 anti-apoptotic effects. 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 (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 (CLP). Tumor necrosis factor (TNF)-α, interleukin (IL)-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 H₂O₂, carbonyl protein and glutathione levels. Mitochondrial biogenesis was assessed by peroxisome proliferator-activated receptor γ co-activator 1 (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. Interestingly, 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.
INTRODUCTION

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 recently emerged 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 (Hoetzel, et al., 2007; Mayr, et al., 2005; 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 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, as well as heme-containing molecules and mitochondrial oxidases (Kim, et al., 2006; Boczkowski 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; Suliman, et al., 2007b).

Sepsis is a complex syndrome characterized by inflammation, oxidative damage, hypercoagulation, tissue hypoperfusion, immune suppression as well as mitochondrial dysfunction (Carre 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; Motterlini, 2007), are emerging as a potential therapy for sepsis via their anti-oxidant, anti-inflammatory, and anti-apoptotic 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 pro-adhesive 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). Interestingly, 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. As 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 (CORM-3) (Clark, et al., 2003) can improve sepsis-induced cardiac mitochondrial dysfunction and survival in peritonitis-challenged mice.
METHODS

Animals used

Six to eight week-old (25 to 30 g) outbred (ICR) male mice (Harlan France) 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 Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Sepsis model

Cecal ligation and puncture (CLP) was used to induce intra-abdominal peritonitis and sepsis. Under anesthesia (intra peritoneal ketamine 2.5 mg/kg and xylazine 0.25 mg/kg), 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 the cecum was neither ligated nor punctured. Animals were maintained on 12-h light/dark cycles with free access to water.

Animal treatments

Tricarbonylchloroglycinato) ruthenium (II) CORM-3, synthesized as previously described (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 subcutaneous injection of either (a) 10 mg/kg tricarbonylchloroglycinato) ruthenium (II)
(CORM-3) in normal saline or (b) 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 N₂ 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, Abingdon OX, UK). Reading was realized in a microplate reader Digiscan (Spectracount Packard Packard; Meriden CT USA).

**Mitochondrial respiration**

Mouse hearts were placed in isolation buffer A containing (in mmol/L): sucrose 300, 2-morpholinoethanesulphonic acid N-[tris(hydroxymethyl)methyl]-2-aminoethane sulphonic acid (TES) 5, ethylene glycol tetraacetic acid (EGTA) 0.2, 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 8,800g for 5 min. Mitochondrial pellet was resuspended in buffer A and centrifuged one more time at 8,800g, 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, Innsbruck, Austria) was evaluated, i.e., state 4 respiration rate (oxygen uptake with glutamate 5 mM malate 2 mM in the absence of exogenous ADP; pmol oxygen/s/mg), state 3 respiration rate (oxygen uptake during ADP 1 mM phosphorylation; pmol oxygen/s/mg) and respiratory control ratio (RCR): ratio of state 3 and state 4 oxygen uptake rates.

**Mitochondrial oxidative stress**

Hydrogen peroxide (H$_2$O$_2$) generated from isolated mitochondria was measured using the Amplex red assay technique according to manufacturer's instructions (Molecular Probes, Eugene, OR). In brief, aliquots of freshly prepared mitochondrial suspensions were incubated with malate/pyruvate in assay buffer. Amplex red was added to initiate reactions: this indicator reacts with H$_2$O$_2$ to generate a fluorescent signal with a 540 nm excitation and 590 nm emission wavelengths. H$_2$O$_2$ standards, supplied with the Amplex red kit, were used to calibrate assays. Assay results are reported in pmol of H$_2$O$_2$ per mg protein.

Protein carbonyl evaluation in isolated mitochondria was based on spectrophotometric detection of protein hydrazones, the reaction product of 2,4-dinitrophenylhydrazine with protein carbonyls, using a commercially available kit (Cayman, VWR, Fontenay-sous-Bois, France).

Mitochondrial glutathione (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 (Cayman, VWR, Fontenay-sous-Bois, France).

**Mitochondrial transmembrane potential**

Isolated mitochondria (1 mg/mL proteins) were suspended in buffer C (in mM): sucrose 250; 3-(N-morpholino)propanesulfonic acid MOPS 10; glutamate-Tris 5; malate-Tris
2; Pi-Tris 1; EGTA-Tris 0.02; pH 7.4 at 25°C in a multiport measurement chamber equipped with tetraphenylphosphonium (TPP⁺)-selective microelectrodes and reference electrodes (WPI, Aston, UK). First, mitochondria were gently stirred for 1.5 min in buffer C containing 1.5µM TPP⁺ (Sigma, Saint Quentin Fallavier, France). Mitochondrial transmembrane potential was estimated by calculating the transmembrane distribution of TPP⁺. Transmembrane potential \( \Delta \Psi_m \) was calculated as

\[
59 \log(v/V) - 59 \log(10^{\Delta E/59} - 1),
\]

where \( v \) is matrix volume (1.1µl/mg mitochondrial protein), \( V \) is volume chamber (1 mL), and \( \Delta E \) is voltage difference before and after calcium induced permeability transition expressed in mV.

**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 which detects NAD and NADH was used according to manufacturer’s instructions (Biovision, Mountain View, California).

**Western blotting**

Proteins isolated from mitochondria (50µg) were run on a 10% polyacrylamide gel (SDS-PAGE). Proteins in gel were electrophoretically transferred to nitrocellulose membranes. After blocking, membranes were treated with rabbit polyclonal anti-PGC-1α and anti-glyceraldehyde 3-phosphate dehydrogenase GAPDH antibodies (Cell Signaling Technology, MA, USA). Membranes were then incubated with horseradish peroxidase-conjugated sheep anti-rabbit and mouse immunoglobulin G secondary antibody (Biorad, Marnes-la-Coquette, France), washed, and bound antibodies were detected by the use of ECL Plus kit (Amersham Biosciences Europe GmbH, Freiburg, Germany).

**Mitochondrial DNA copy number determination**

Total DNA (genomic + mitochondrial) was extracted from heart tissue using QIAamp DNA Mini Kit (Qiagen, 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°C, 58°C and 72°C as denaturation, annealing and 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. Relative amount of mtDNA was expressed as mtCOII/18S ratio.

**Statistics**

Results were analyzed with the SPSS 11.0.1 (SPSS France, Paris-la-Défense, France). 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 isolated 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 (10-50µM). Overall, the respiratory control ratio (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 F1F0-ATPase was assayed by adding oligomycin (10 µM). In the presence of oligomycin, the mitochondrial membrane potential returned to control values (ΔΨm −225±1.5 vs. −215±1.5 mvols; P< 0.05, 5-6 experiments in each group).

Secondly, the effects of CORM-3 were evaluated in vivo using a model of CLP-induced sepsis (Figure 1). Septic mice began to die between 12 and 24 hours 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 100mg/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 (Figure 1), whereas a much higher dose regimen (100 mg/kg) had deleterious effects on the survival of CLP mice (Figure 1). Inactive CORM-3 (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 (Figure 2A) and IL-10 (Figure 2B) levels which subsequently decreased at 48 h post-treatment. Treatment with CORM-3 during sepsis had no significant effects on TNF-α while modestly increased IL-10 levels at 48 h post-treatment. In addition, CORM-3 prevented the increase in nitrite/nitrate production, an indicator of NO synthesis (Figure 2C). CLP-induced sepsis was accompanied by deterioration of mitochondrial respiration, as indicated by a marked increase in the redox state (NADH/NAD ratio) (Figure 3A) and membrane potential dissipation (Figure 3B). In this context, administration of CORM-3 largely prevented CLP-induced deterioration of mitochondrial state 3 respiration rates at 24 and 48 h post-treatment (Figure 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$_2$O$_2$ production (Figure 4A), carbonyl proteins (Figure 4B) and GSH/GSSG ratio (Figure 4C). In contrast, CORM-3 treatment in CLP-mediated sepsis consistently promoted an increase in both mitochondrial H$_2$O$_2$ production and carbonyl protein levels. Protein expression of PGC-1α, a specific marker of mitochondrial biogenesis, decreased following CLP-induced sepsis (Figure 5). In contrast, CLP mice treated with CORM-3 resulted in a significant stimulation of PGC-1α protein expression (Figure 5) and mtDNA copy number (Figure 6), 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. Interestingly, 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, as 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; Carre and Singer, 2008). Thus, strategies aimed of preventing the impairment of mitochondrial energy production may be beneficial. In this context, carbon monoxide (CO) has emerged as a potential therapeutic stratagem as 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 (Hoetzel, et al., 2007; Kim, et al., 2006; 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, et al., 2005; Motterlini, 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 pro-inflammatory cytokines 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 (Mazzola, et al., 2005; Otterbein, et al., 2000). In our model of sepsis, CORM-3 had no effects on TNF-α and IL-10 levels at 24 h, while 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 effects can involve either increased or reduced nitric oxide production (Cepinskas, et al., 2008; Sarady, et al., 2004). 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 due to microbial infections such as experimental peritonitis, inhibition of the inflammatory response could disrupt 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.,
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. It is
thus 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 favours 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 as 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 following 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; Suliman, et al., 2007b). Mitochondrial biogenesis requires nuclear and mitochondrial genomic activation driven by nuclear respiratory factors, mitochondrial transcription factors, and master peroxisome proliferator-activated receptor γ co-activator 1 (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 activities 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


Footnotes

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- EA 2689 Ministère de l’Enseignement Supérieur et de la Recherche (2006-2009)
LEGENDS FOR FIGURES

**Figure 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 100mg/kg) treated mice. Sample size is 20 mice in each group. Survival studies have been conducted twice. Comparison between groups was assessed by Kaplan-Meier, log rank.

**Figure 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±SEM and were analyzed with one way ANOVA and Bonferroni's multiple comparison posthoc adjustment. * indicates P<0.05 vs sham+iCORM-3; ** indicates P<0.05 vs CLP+iCORM-3.

**Figure 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±SEM and were analyzed with one way ANOVA and Bonferroni's multiple comparison posthoc adjustment. * indicates P<0.05 vs sham+iCORM-3; ** indicates P<0.05 vs CLP+iCORM-3.

**Figure 4:** Effects of CORM-3 on sepsis-induced oxidative stress response. Mitochondrial H₂O₂ 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±SEM and were analyzed with one way ANOVA and Bonferroni's multiple comparison posthoc adjustment. * indicates P<0.05 vs sham+iCORM-3.
**Figure 5**: Effects of CORM-3 on sepsis-induced mitochondrial biogenesis deficit (A) Representative micrographs of PGC-1α Western-blot at 48h post treatment. (B) Densitometric analysis of PGC-1α to GAPDH protein expression ratio in sham+iCORM-3, sham+CORM-3, CLP+iCORM-3 and CLP+CORM-3. Sample size is 5-6 mice in each group. Results are presented as mean±SEM and were analyzed with one way ANOVA and Bonferroni’s multiple comparison posthoc adjustment. * indicates P<0.05 vs sham+iCORM-3. ** indicates P<0.05 vs CLP+iCORM-3.

**Figure 6**: CORM-3 increases mtDNA copy number in septic heart at 48h post 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±SEM and were analyzed with one way ANOVA and Bonferroni’s multiple comparison posthoc adjustment. *indicates P<0.05 compared with sham+iCORM-3.
TABLE 1

Respiration parameters and membrane potential of isolated heart mitochondria

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

Respiration rates (pmol oxygen/s/mg) with glutamate (5 mM) and malate (2 mM) in absence of exogenous ADP (state 4) and with 1 mM ADP (state 3). RCR: respiratory control ratio of state 3 and state 4 respiration rates. $\Delta \Psi_m$: mitochondrial membrane potential (mvolts) measured with glutamate/malate in absence of exogenous ADP. Sample size is 5-6 mitochondrial preparation in each group. Results are presented as mean±SEM and analyzed with one way ANOVA and Bonferroni's multiple comparison posthoc adjustment. * indicates P<0.05 vs. controls.
Figure 1

Survival of mice treated with active CORM-3 and inactive iCORM-3

- sham + iCORM-3 (10 mg/kg)
- sham + CORM-3 (10 mg/kg)
- CLP + CORM-3 (10 mg/kg)
- CLP + iCORM-3 (10 mg/kg)
- CLP + CORM-3 (100 mg/kg)

Percent survival

Time (hours)
Figure 3

A

NADH/NAD ratio

B

Mitochondrial membrane potential ($\Delta\psi_m$)

C

Respiratory control ratio (RCR)
Figure 4

A. 

H$_2$O$_2$ concentration

B. 

Carbonyl protein concentration

C. 

GSH/GSSG ratio
Figure 5

A

PGC-1α

- 91kDa

B

PGC1-α/GAPDH protein expression ratio

24 hr  48 hr

*  **
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>sham+iCORM-3</th>
<th>sham+CORM-3</th>
<th>CLP+iCORM-3</th>
<th>CLP+CORM-3</th>
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B

mtCOII / 18S

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