Activation of Mitochondrial Biogenesis by Heme Oxygenase-1–mediated NF-E2–related Factor-2 Induction Rescues Mice from Lethal *Staphylococcus aureus* Sepsis

Nancy Chou MacGarvey^{1*}, Hagir B. Suliman², Raquel R. Bartz^{2,3}, Ping Fu², Crystal M. Withers⁴, Karen E. Welty-Wolf^{1,5}, and Claude A. Piantadosi^{1,2,4,5}

¹Department of Medicine, ²Department of Anesthesiology, and ⁴Department of Pathology, Duke University Medical Center, Durham, North Carolina; and ³Department of Anesthesiology and ⁵Department of Medicine, Durham Veterans' Affairs Medical Center, Durham, North Carolina

Rationale: Mitochondrial damage is an important component of multiple organ failure syndrome, a highly lethal complication of severe sepsis that lacks specific therapy. Mitochondrial quality control is regulated in part by the heme oxygenase-1 (HO-1; *Hmox1*) system through the redox-regulated NF-E2–related factor-2 (Nrf2) transcription factor, but its role in mitochondrial biogenesis in *Staphylococcus aureus* sepsis is unknown.

Objectives: To test the hypothesis that Nrf2-dependent up-regulation of the HO-1/carbon monoxide (CO) system would preserve mitochondrial biogenesis and rescue mice from lethal *S. aureus* sepsis.

Methods: A controlled murine *S. aureus* peritonitis model with and without inhaled CO was examined for HO-1 and Nrf2 regulation of mitochondrial biogenesis and the resolution of hepatic mitochondrial damage.

Measurements and Main Results: Sepsis survival was significantly enhanced using inhaled CO (250 ppm once-daily for 1 h), and linked mechanistically to *Hmox1* induction and mitochondrial HO activity through Nrf2 transcriptional and Akt kinase activity. HO-1/CO stimulated Nrf2-dependent gene expression and nuclear accumulation of nuclear respiratory factor-1, -2 α (Gabpa), and peroxisome proliferator-activated receptor gamma coactivator-1 α ; increased mitochondrial transcription factor-A and citrate synthase protein levels; and augmented mtDNA copy number. CO enhanced antiinflammatory IL-10 and reduced proinflammatory tumor necrosis factor- α production. By contrast, *Nrf2*^{-/-} and *Akt1*^{-/-} mice lacked CO induction of *Hmox1* and mitochondrial biogenesis, and CO rescued neither strain from *S. aureus* sepsis.

Conclusions: We identify an inducible Nrf2/HO-1 regulatory cycle for mitochondrial biogenesis that is prosurvival and counter-inflammatory in sepsis, and describe targeted induction of mitochondrial biogenesis as a potential multiple organ failure therapy.

Keywords: Akt/PKB; carbon monoxide; cytokines; oxidative stress; mitochondrial DNA

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Multiple organ failure syndrome is a highly lethal complication of severe sepsis lacking directed therapy, and involving poorly understood pathogenic mechanisms including mitochondrial damage. This mitochondrial damage is opposed by the induction of heme oxygenase-1 and by mitochondrial biogenesis.

What This Study Adds to the Field

In *Staphylococcus aureus* sepsis in mice, we demonstrate activation of mitochondrial biogenesis through a redox-regulated network involving the heme oxygenase-1/carbon monoxide system and the NF-E2–related factor-2 transcription factor, and modulated by Akt1, that promotes survival and is amenable to pharmacologic intervention.

Hospitalization rates for sepsis continue to rise, especially in the elderly (1), and gram-positive bacteria, particularly *Staphylococcus aureus* species, are responsible for most of the infections (2). Severe sepsis and septic shock cause death from multiple organ failure (MOF) syndrome, most notably when three or more organs fail (3, 4). The pathogenesis of MOF involves damage to mitochondria from immune effectors, such as tumor necrosis factor (TNF)- α (5), and by reactive oxygen species (ROS) and reactive nitrogen species that escape the antioxidant defenses (6). MOF has no specific therapy, but survival is associated with the induction of mitochondrial antioxidant defenses and mitochondrial biogenesis (7, 8).

The cell's oxidation-reduction (redox) state regulates the antioxidant defenses and broader adaptive genetic responses (9, 10) including those that regenerate mitochondria (11). The induction of mitochondrial biogenesis (12, 13) and the clearance of irreparably damaged organelles by mitophagy (14) are responsible for the maintenance of mitochondrial structure and function. The mitochondrial biogenesis program is also coupled to the antioxidant defense network (15).

The antioxidant enzyme heme oxygenase-1 (HO-1; *Hmox-1*) catalyzes the rate-limiting step in heme degradation (16). HO-1 induction protects against prooxidant heme release by many agents including LPS, cytokines, and ROS, whereas its products (iron, biliverdin, and carbon monoxide) have important physiologic effects (17, 18). Endogenous CO binds heme proteins, such as hemoglobin, cytochrome-c oxidase, and guanylate cyclase, affecting their functions (19, 20). CO binding to cytochrome oxidase increases mitochondrial ROS leakage, which

(Received in original form June 29, 2011; accepted in final form January 17, 2012)

*Present address: Department of Medicine, Drexel College of Medicine, Philadelphia, PA 19102.

Supported by NIH RO1 AI064789, HL076528, and GM084116.

Author Contributions: N.C.M., H.B.S., and C.A.P., study conception and design; acquisition, interpretation, and analysis of data; drafting the article; and revising the article. R.R.B. and P.F., acquisition, analysis, and interpretation of data and revising the article. C.M.W., acquisition, analysis, interpretation of data and revising the article. K.E.W.-W., analysis and interpretation of data and revising the article. All authors approve of the final article.

Correspondence and requests for reprints should be addressed to Claude A. Piantadosi, M.D., Box 3315, Duke University Medical Center, 200 Trent Drive, Durham, NC 27710. E-mail: piant001@mc.duke.edu

Am J Respir Crit Care Med Vol 185, Iss. 8, pp 851–861, Apr 15, 2012

Copyright © 2012 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201106-1152OC on February 3, 2012

Internet address: www.atsjournals.org

Figure 1. *Staphylococcus aureus* peritonitis and hepatic heme oxygenase-1 (HO-1; *Hmox1*) gene, HO-1 protein, and HO enzyme activity in wild-type (WT) mice. (A) Survival of WT sepsis-control or sepsis-carbon monoxide (CO) mice (arrows) after implantation of fibrin clots containing 5×10^7 cfu of *S. aureus*. Data are pooled from experiments of 12 mice per group done in duplicate. Inhaled CO is protective in *S. aureus* sepsis in WT mice ($P = 0.014$ by Pearson chi square); however, CO protection is lost when HO is inhibited with SnPP. (B) Histologic sections of the mouse liver stained with hematoxylin and eosin demonstrate increased inflammatory foci after *S. aureus* peritonitis and protection after intervention with CO. (C) Western analysis for HO-1 protein in sepsis-control and sepsis-CO mice showing increases in the hepatic protein level in sepsis-control and sepsis-CO mice (densitometry bars are mean $\pm$ SD for n = 4 per group and time; * P < 0.05 vs. time 0; * P < 0.05, vs. sepsis-control). (D) Real-time reverse transcriptase polymerase chain reaction for hepatic HO-1 mRNA in WT sepsis-control and sepsis-CO mice. HO-1 mRNA is significantly increased by CO. Bars are means $\pm$ SD of duplicates in three experiments (* P < 0.05 vs. time 0; * P < 0.05 vs. sepsis-control group at the same time point). (E) Hepatic HO enzyme activity in WT sepsis-control and sepsis-CO mice. HO activity peaks at 24 hours in sepsis-control and at 6 hours in sepsis-CO mice. Bars are mean $\pm$ SD of duplicates in three experiments (* P < 0.05 vs. time 0; * P < 0.05 vs. sepsis-control group at the same time point). (F) Distribution of liver mitochondrial HO activity (per microgram mitochondrial protein) as a function of mitochondrial pH in control mice and after 24 hours in sepsis-control, sepsis-CO, and CO-only mice. HO enzyme activity was enriched in sepsis and highest in mitochondria at the extremes of pH, implying activation of heme degradation in these subpopulations.

promotes adaptive gene expression, including genes of mitochondrial biogenesis, through adjustments in redox state (21).

Hmox1 is thought to protect against sepsis-induced tissue damage, and it is induced by multiple transcriptional elements that respond to inflammation, especially the basic leucine zipper transcription factor, NF-E2 related factor-2 (Nrf2) (22). Nrf2 is normally sequestered in the cytosol by the cysteine-rich Kelch-like ECH-associated protein 1 (23), and Kelch-like ECH-associated protein 1 oxidation allows Nrf2 nuclear translocation (24) and binding to antioxidant response element (ARE) motifs located 5' to the *Hmox1* transcription start site (25). Nrf2 also occupies activating ARE motifs in the nuclear respiratory factor-1 (NRF-1)

promoter (15), and under the influence of CO, Nrf2 and NRF-1 along with NRF-2 (Gabpa) and the peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 coactivators stimulate mitochondrial biogenesis (13, 26). Nrf2 also influences the innate immune response and survival in cecal ligation and puncture (27) and modulates leukocyte function in sepsis (28). However, the connection of these events to mitochondrial protection is unknown.

In addition, HO-1 is post-translationally regulated by Akt/PKB (29), and CO activates the kinase (30, 31). Akt phosphorylates NRF-1 and promotes its nuclear localization (32) and inactivates GSK3 β , which improves Nrf2 nuclear translocation

Figure 2. Hepatic tumor necrosis factor (TNF)- α , nitric oxide synthase (NOS)-2, IL-10, and NF-E2-related factor-2 (Nrf2) antioxidant responses by real-time reverse transcriptase polymerase chain reaction during *Staphylococcus aureus* sepsis. (A) TNF- α mRNA up-regulation in sepsis-control mice is attenuated in sepsis-carbon monoxide (CO) mice (* P < 0.05 vs. time 0; # P < 0.05 vs. time 0 and sepsis-CO). (B) NOS2 mRNA is induced in sepsis-control mice by 6 hours, but is limited by inhaled CO (A and B: * P < 0.05 vs. time 0; # P < 0.05 vs. time 0 and with CO). (C) Nrf2 mRNA by real-time reverse transcriptase polymerase chain reaction is increased in sepsis-control mice by 6 hours, and expression of the gene is enhanced by CO inhalation. (D) Increased superoxide dismutase 2 mRNA levels in sepsis-control mice are further increased in sepsis-CO mice. (E) Increased IL-10 mRNA levels in sepsis-control mice are further increased in sepsis-CO mice (C, D, and E: * P < 0.05 vs. time 0; # P < 0.05 vs. time 0 and sepsis-control mice).

(33). These events form a basis for the role of HO-1 in the adaptive induction of mitochondrial biogenesis (15).

Endogenous CO contributes to the protective effects of HO-1 illustrated nicely by modulation of the antiinflammatory response (34) (i.e., through IL-10 [35], an antiinflammatory cytokine involved in LPS tolerance [36]). Mortality in rodents after LPS and after cecal ligation and puncture is reduced by CO or by CO-releasing molecules (37–39), and one study found an association between mitochondrial biogenesis and protection by CO (38). The link between Nrf2 and HO-1 suggests that CO could benefit sepsis resolution through Nrf2-mediated induction of mitochondrial biogenesis in concert with HO-1 and other prosurvival responses. We tested this hypothesis through intervention with CO in wild-type (WT) and Nrf2 and Akt1 null mice with *S. aureus* peritonitis. A portion of this work has been published in abstract form (40).

METHODS

Materials

Antibodies were obtained from Santa Cruz, except for HO-1 (Assay Designs, Farmingdale, NY), PGC-1 α (Cayman, Ann Arbor, MI), and phospho-Akt (S473 and T308; Cell Signaling, Danvers, MA). Polyclonal NRF-1, NRF-2 (Gabpa), PGC-1-related coactivator (PRC), and transcription factor-A (Tfam) were developed and characterized in our laboratory (11).

Mice

Studies were preapproved by our Institutional Animal Care and Use Committee. C57Bl6/J (WT) mice were obtained from Jackson

Laboratory (Bar Harbor, ME). Nrf2^{-/-} mice (Riken, Saitama, Japan) and Akt1^{-/-} mice (Jackson) were bred institutionally and both sexes used at 9–15 weeks of age. *S. aureus* (clinical isolate Rosenbach Seattle 1945; ATCC [Manassas, VA] #25923; 1×10^6 to 10^8 cfu) was prepared, counted, and implanted abdominally in fibrin clots (7). Based on preliminary studies, we empirically administered CO at 250 ppm in air for 1 hour in breathing chambers on Days 1–3. This transiently increased carboxyhemoglobin level to approximately 20% without significant hypothermia or tissue hypoxia (21, 41). Some mice received HO inhibitor tin protoporphyrin (SnPP, 20 μ mol/kg; Frontier Scientific) at surgery and again 24 hours later. Mice were killed at the indicated times and livers harvested for RNA, mtDNA, or mitochondrial or nuclear protein. Mitochondrial and nuclear pellets were stored in radioimmunoprecipitation assay buffer at -80°C .

Real-Time Polymerase Chain Reaction

Quantitative polymerase chain reaction was performed on an ABI PRISM 7000 with TaqMan expression assays (Applied Biosystems, Carlsbad, CA). 18S rRNA served as an endogenous control. Quantification of mRNA was determined by comparative threshold cycle C_T and RQ method.

Protein Methods

Protein was measured with bicinchoninic acid using bovine serum albumin standards. Fresh liver was hand-homogenized in standard isolation buffer and mitochondria isolated on 0.25% sucrose. Mitochondria were loaded onto a Rotofor (BioRad, Hercules, CA) containing an ampholyte (pH range, 3–10) and separated over 90 minutes by isoelectric focusing. For Western blotting, 10–20 μ g protein were resolved by gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20%), transferred

Figure 3. Transcriptional up-regulation of mitochondrial biogenesis and protection of mtDNA copy number in *Staphylococcus aureus* sepsis. (A) Hepatic nuclear respiratory factor (NRF)-1 expression by real-time reverse transcriptase polymerase chain reaction increases in sepsis-control mice, reaching a maximum at 24 hours, with further increases in sepsis-carbon monoxide (CO) mice. (B) Hepatic up-regulation of Gabpa (NRF-2α) mRNA in sepsis-control mice parallels NRF-1 and further increases in sepsis-CO mice. (C) The peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α coactivator is up-regulated by 6 hours of sepsis and further increases in sepsis-CO mice. (D) The PGC-1 family member PGC-1-related coactivator increases in sepsis-control mice and increases further in sepsis-CO mice. (E) Hepatic transcription factor-A (Tfam) mRNA level increases in sepsis-control mice and increases further in sepsis-CO mice. (A–E: #*P* < 0.05 vs. time 0; **P* < 0.05 vs. time 0 and sepsis-control for *n* = 4 per time per group). (F) Log hepatic mitochondrial DNA copy number in sepsis-control and sepsis-CO mice. Inhaled CO reverses the decline in mtDNA copy number in sepsis (*n* = 4 per time per group; #*P* < 0.05 less than time 0 control; **P* < 0.05 higher than the respective sepsis-control group).

to Immobilon P, and blocked with 4% nonfat milk. Membranes were incubated at 4°C overnight with polyclonal anti-HO-1, p-Akt, Akt, porin, NRF-1, and Tfam (all 1:1,000); His3 (1:500); Nrf2 (1:800); PGC-1α (1:600); and PRC (1:200). The membranes were washed with Tris-buffered saline with Tween and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA); rewash; and developed with enhanced chemiluminescence (Amersham, Piscataway, NJ). Digitized images were quantified in the mid-dynamic range with BioRad ImageQuant. HO activity was measured in tissue and mitochondria using the CO reduction method (42).

Cells

Human HepG2 cells (ATCC) were cultured at 37°C in 5% CO₂ in RMPI 1640 with 10% fetal calf serum, glutamine, and antibiotics. Near-confluent cells were exposed to peptidoglycan (PGN; 25 ng/ml) or dichloromethane (DCM/CO; 50 μM) to generate CO. Cells were transfected with scrambled or *Nrf2* targeted siRNA using FuGene HD (Roche, Indianapolis, IN) to efficiencies and gene suppression of greater than 70% (43). Some cells were treated with DCM/CO and GSK-3β inhibitor TDZD-8 (25 μM; Calbiochem, St. Louis, MO) for 6 hours and some pretreated for 30 minutes with PI-3K/Akt inhibitor, Ly294002 (25 μM; Promega, Madison, WI).

Data Analysis

Data for real-time reverse transcriptase polymerase chain reaction and protein densitometry are expressed as means ± SD for four to six samples. Significance was tested by Student unpaired *t* test or by two-way analysis of variance. Survival at 7 days was tested by Pearson chi square. Significance was set at *P* less than 0.05.

RESULTS

In C57BL/6J mice (WT), sepsis induced by implanting fibrin clots containing live *S. aureus* into the peritoneum followed by fluid resuscitation produces dose-dependent organ damage and lethality (7). Using 5×10^7 cfu *S. aureus*, we tested inhaled CO at 250 ppm for 1 hour at 2 hours after surgery, repeated daily for 3 days. In WT mice, 7-day mortality in the sepsis-control group was 48%, whereas mortality in the sepsis-CO treatment group was 23% (Figure 1A) (*P* < 0.05). CO was comparably protective at 1×10^8 cfu of *S. aureus* (not shown). We also performed sepsis studies on WT mice after HO inhibition and found them significantly more susceptible to mortality than sepsis-control mice (*P* < 0.05). They could not be rescued by CO (Figure 1A). We evaluated the liver histologically and found many inflammatory foci and areas of cell vacuolization and cell death in sepsis-control mice that were averted by the CO intervention (Figure 1B).

To assess HO-1, we examined hepatic HO-1 protein, mRNA, and HO enzyme activity in C57BL/6J mice at 0, 6, 24, and 48 hours after implantation of 5×10^7 cfu *S. aureus* with or without CO. HO-1 protein and *Hmox1* gene were up-regulated in sepsis-control mice at 6 and 24 hours, but sepsis-CO mice showed significantly greater *Hmox1* up-regulation than sepsis-control mice (Figures 1C and 1D). CO also increased hepatic HO enzyme activity (Figure 1E), resulting in a higher CO production capacity and initiating a positive feedback cycle.

In healthy control mice (no sepsis), CO increased blood carboxyhemoglobin and tissue CO content commensurately with

Figure 4. Up-regulation of mitochondrial biogenesis proteins during *Staphylococcus aureus* sepsis in wild-type mice by Western blot analysis. (A) Hepatic nuclear respiratory factor (NRF)-1 and peroxisome proliferator-activated receptor gamma coactivator-1 α nuclear proteins increase in sepsis-control mice and increase further with carbon-monoxide (CO) (His3 is a nuclear reference protein). (B) Western analysis of hepatic Gabpa (NRF-2 α) showing enhanced nuclear protein levels in sepsis-control and sepsis-CO mice. Nuclear NF-E2-related factor-2 (Nrf2) protein in sepsis-control and sepsis-CO mice is shown from the same nuclear samples compared with His3. For panels A and B, the densitometry confirms significant up-regulation of all four proteins by 24 hours in sepsis-control mice and further increases in sepsis-CO mice (for the densitometry, $n = 4$; $\#P < 0.05$ vs. time 0; $*P < 0.05$ for the CO effect). (C) To verify the induction of mitochondrial biogenesis, we checked three mitochondrial marker proteins (nuclear-encoded citrate synthase [CS], mtDNA-encoded Complex I subunit NADH dehydrogenase 1, and the imported mitochondrial transcription factor transcription factor-A [Tfam]) (for the densitometry $n = 4$; $\#P < 0.05$ above time 0 control; $\&P < 0.05$ below time 0; $*P < 0.05$ for the CO effect). (D) Akt phosphorylation in sepsis-control mice is shown to increase at S473 and T308, but CO only enhances S473 phosphorylation (for densitometry, $n = 4$; $\#P < 0.05$ vs. time 0; $*P < 0.05$ for the CO effect).

return to baseline within 2 hours (not shown); thus, we measured HO enzyme activity at 2 hours after CO breathing. In sepsis-control mice, HO activity gradually increased over 24 hours, whereas in sepsis-CO mice, HO activity increased within 6 hours and remained elevated.

We isolated liver mitochondria and separated them into populations by isoelectric point, and found enrichment of HO activity in certain subpopulations. Figure 1F shows the distribution of HO activity in liver mitochondria of control mice and at 24 hours in sepsis-control, sepsis-CO, and CO-alone mice. Mitochondrial HO activity in the liver was enhanced at high and low pH_i in sepsis and after CO breathing, implying activation of heme catabolism by these stresses in specific groups of organelles.

Staphylococcus aureus sepsis rapidly induced proinflammatory TNF- α (Figure 2A) and nitric oxide synthase-2 (NOS2)

production (Figure 2B). TNF- α increases mitochondrial ROS production and mitochondrial oxidative stress (44), whereas the effect of NO varies. Excessive NO inhibits respiration and damages cells, whereas physiologic NO stimulates mitochondrial biogenesis (45). Here TNF- α and NOS2 induction were decreased in sepsis-CO mice. Sepsis-control mice showed an oxidative stress response indicated by Nrf2 (Figure 2C) and superoxide dismutase 2 (Figure 2D) gene induction that was enhanced by CO. Sepsis-control mice also showed robust increases in antiinflammatory IL-10 mRNA by 6 hours; this too was enhanced by CO (Figure 2E).

During *S. aureus* sepsis in mice, aerobic function depends on mitochondrial replacement, which in turn depends on specific transcription factors, such as NRF-1 and NRF-2 (Gabpa), and coactivators, in particular PGC-1 α , that up-regulate nuclear-

Figure 5. Nuclear NF-E2-related factor-2 (Nrf2) and nuclear respiratory factor (NRF)-1 accumulation after *Staphylococcus aureus* peptidoglycan (PGN) or dichloromethane (DCM)/carbon monoxide (CO) administration in HepG2 cells. (A) HepG2 cells were examined by confocal microscopy for the localization and expression of NRF-1 (red) and Nrf2 (green). Before PGN, both transcription factors are detectable only in the cytoplasm. Nuclear staining with Dapi (blue) is shown in the bottom panels of the columns. (B) After PGN exposure, both transcription factors accumulate in cell nuclei. (C) After exposure to PGN plus DCM/CO, intense homogeneous nuclear staining is noted for Nrf2 and NRF-1. (D) Nrf2 siRNA (*siNrf2*) blocks the effects of PGN plus DCM/CO on the nuclear accumulation of Nrf2 and NRF-1. Scrambled siRNA had no effect (no images shown).

encoded mitochondrial genes, such as *Tfam*, whose protein products are imported into mitochondria for mtDNA transcription and replication for mitochondrial biogenesis (46). In WT mice, hepatic NRF-1, Gabpa, PGC-1 α , PRC, and *Tfam* mRNA levels increased in sepsis-control mice, but these effectors were further up-regulated in sepsis-CO mice (Figures 3A–3E). Sepsis-control

mice confirmed a postinoculation fall in hepatic mtDNA copy number, which was abrogated by CO (Figure 3F).

Nuclear NRF-1 and PGC-1 α protein levels increased in sepsis-control, but this response occurred earlier in sepsis-CO mice (Figure 4A). Nuclear Gabpa and Nrf2 protein levels increased in sepsis-control mice with further up-regulation of

Figure 6. NF-E2-related factor-2 (*Nrf2*) deletion and *Staphylococcus aureus* sepsis. (A) *Nrf2*^{-/-} mice were implanted with fibrin clots containing *S. aureus* (5×10^6 cfu). The mice in the sepsis-control group all died by 54 hours and carbon monoxide (CO) offered no advantage (100% mortality by ~ 36 h; $P = \text{NS}$; $n = 12$ mice per group). (B) Hematoxylin and eosin-stained liver sections in wild-type (WT) (left) and *Nrf2*^{-/-} mice (right) at 0 and 24 hours after *S. aureus* inoculation (5×10^6 cfu). Extensive cell vacuolization, autolysis, and cell death are present in *Nrf2*^{-/-} mouse liver. Horizontal arrows indicate inflammatory foci after sepsis, which are larger and more numerous in *Nrf2*^{-/-} mice. Vertical arrows indicate foci of necrosis or apoptosis. (C) Heme oxygenase (HO)-1 mRNA levels in WT and *Nrf2*^{-/-} mice. The WT response to CO is a positive control. In *Nrf2*^{-/-} mice, *S. aureus* sepsis but not CO induces HO-1 mRNA expression ($*P < 0.05$ vs. sepsis-CO). (D) He-

hepatic HO-1 protein in *Nrf2*^{-/-} mice by Western blot. *S. aureus* increases HO-1 protein, whereas CO does not. (E) Loss of hepatic mtDNA copy number in *Nrf2*^{-/-} and *Akt1*^{-/-} mice compared with WT mice at 24 hours of sepsis ($*P < 0.05$ compared with WT at time 0; $**P < 0.05$ compared with WT at 0 and 24 h). Note the higher *S. aureus* inoculation dose in WT mice. SA = *S. aureus*.

Figure 7. Transcriptional regulators of mitochondrial biogenesis in NF-E2-related factor-2 (*Nrf2*)^{-/-} mice during *Staphylococcus aureus* sepsis. (A) Liver nuclear respiratory factor (NRF)-1 mRNA levels by real-time reverse transcriptase polymerase chain reaction increase less than twofold in *Nrf2*^{-/-} sepsis-control mice and there is no significant response to CO. CO response in wild-type (WT) mice is provided as a positive control. (B) *Gabpa* mRNA levels in *Nrf2*^{-/-} sepsis-control mice increase 2.5-fold, but there is no effect of CO. (C) Transcription factor-A (Tfam) mRNA expression does not increase in *Nrf2*^{-/-} sepsis-control or sepsis-CO mice. Values are means $\pm$ SD of duplicates of three experiments (**P* < 0.05 vs. baseline control). SA = *S. aureus*.

these two proteins in sepsis-CO mice (Figure 4B). Mitochondrial ROS strongly influence the cellular oxidant-antioxidant balance, also involved in redox signaling (47). Overwhelming ROS production damages mitochondria, but oxidative stress stimulates Nrf2 translocation and binding to AREs in protective phase II antioxidant genes, including *Hmox1* (48). HO-1/CO increases Nrf2 occupancy of the *NRF1* promoter and increases NRF-1 production (15).

To confirm the induction of mitochondrial biogenesis, we measured mitochondrial levels of the nuclear-encoded tricarboxylic acid cycle enzyme citrate synthase, the mitochondrial-encoded NADH dehydrogenase 1 (ND1) subunit of Complex I, and mitochondrial transcription factor Tfam (Figure 4C). There were losses in mitochondrial citrate synthase and ND1 induced by sepsis that were reversed earlier with CO than by

the native responses. Tfam protein levels increased along with the activation of mitochondrial biogenesis (Figure 4C). CO promotes mitochondrial biogenesis through Akt and phosphorylation of NRF-1. In sepsis-control mice, hepatic Akt displayed greater phosphorylation at T308 and S473, but CO increased Akt phosphorylation only at S473, implying a relatively specific effect on Akt activation (Figure 4D).

Because of pronounced nuclear accumulation of Nrf2 in sepsis-control and sepsis-CO mice, we checked for overlapping mechanisms in HepG cells using *S. aureus* PGN and DCM/CO. PGN (25 ng/ml) increased the nuclear translocation of Nrf2 and that of NRF-1 (Figures 5A and 5B), and both effects were enhanced by DCM/CO (Figure 5C). Nrf2 was upstream of NRF-1 as demonstrated in Nrf2-silenced cells in which neither PGN nor DCM/CO, alone or in combination, stimulated the nuclear accumulation of NRF-1 (Figure 5D) (scrambled siRNA controls were negative, data not shown).

Sepsis mortality in *Nrf2*^{-/-} mice was checked at a lower *S. aureus* inoculation dose of 5×10^6 cfu than in WT mice because *Nrf2*^{-/-} mice were more susceptible to *S. aureus* mortality in preliminary studies. At the lower inoculation dose, *Nrf2*^{-/-} sepsis-control mice died by 54 hours (Figure 6A). Moreover, all *Nrf2*^{-/-} sepsis-CO mice died before the third CO treatment (implying toxicity). At 24 hours, the extent of hepatic congestion, cell damage, and inflammation in *Nrf2*^{-/-} mice was more severe than in WT mice (Figure 6B). These findings support a survival role for Nrf2, a requirement for Nrf2 in CO protection, and the importance of early mitochondrial biogenesis in this *S. aureus* model.

For *Hmox1* studies, separate *Nrf2*^{-/-} mice were implanted with 5×10^6 *S. aureus*; half received two CO treatments and the livers harvested at approximately 28 hours. In *Nrf2*^{-/-} mice, HO-1 mRNA increased approximately 2.5-fold in the sepsis-control group, but did not respond further in the sepsis-CO group (Figure 6C). *Nrf2*^{-/-} sepsis-control mice also displayed normal hepatic HO-1 protein induction (Figure 6D); thus, Nrf2 was not the sole induction mechanism in *S. aureus* sepsis, but Nrf2 was required for CO to up-regulate HO-1. We assessed mtDNA copy number in the livers of the *Nrf2*^{-/-} mice and in *Akt1*^{-/-} mice and found a greater loss of mtDNA at 24 hours in *Nrf2*^{-/-} compared with WT mice (Figure 6E).

Nrf2^{-/-} mice in *S. aureus* sepsis did not induce mitochondrial biogenesis transcription factor genes normally; only *Gabpa* mRNA doubled, to half the WT levels (Figures 7A–7C). In *Nrf2*^{-/-} mice, CO had no effect on NRF-1, *Gabpa*, or Tfam mRNA in sepsis. NRF-1 and PGC-1 α protein increased in *Nrf2*^{-/-} sepsis-control mice, but did not respond to CO (Figure 8A) and Tfam levels did not improve in *Nrf2*^{-/-} sepsis-control mice or in *Nrf2*^{-/-} sepsis-CO mice (Figure 8B). Akt phosphorylation increased in *Nrf2*^{-/-} sepsis-control mice (Figure 8C), but CO had no effect, implying an interaction with Nrf2.

We exposed *Akt1*^{-/-} mice to *S. aureus* at 5×10^6 cfu with or without CO intervention. In *Akt1*^{-/-} sepsis-control mice, two-thirds of the group died by Day 7 (Figure 9A), and CO inhalation did not improve survival, indicating an Akt requirement for CO rescue (Figure 9A). *Hmox1* induction was lost in *Akt1*^{-/-} sepsis-control mice and CO actually interfered with HO-1 mRNA up-regulation (Figure 9B). *Akt1*^{-/-} sepsis-control mice showed slight increases in hepatic HO-1 protein, but HO-1 did not respond to CO (Figure 9C). NRF-1 and Tfam protein increased slightly in *Akt1*^{-/-} sepsis-control mice at 24 hours, but CO had no effect (Figure 9D). This implies impaired mitochondrial biogenesis connected to the absence of Akt phosphorylation of NRF-1 and the loss of Nrf2 regulation of *NRF1*.

Nrf2 nuclear translocation is inhibited by Gsk3 β , which is prevented by Akt (33), and we detected transient Gsk3 β

Figure 8. Regulation of mitochondrial biogenesis regulatory proteins in NF-E2-related factor-2 (*Nrf2*)^{-/-} mice during *Staphylococcus aureus* sepsis. (A) Western blots of total hepatic nuclear respiratory factor (NRF)-1 and PGC-1α protein showing minimal increases in *Nrf2*^{-/-} sepsis-control mice, but none in carbon monoxide (CO) alone or in sepsis-CO mice (for densitometry, n = 4 per group). (B) Transcription factor-A (Tfam) is unaffected in *Nrf2*^{-/-} sepsis-control mice, CO-only, or sepsis-CO mice (densitometry n = 4 per group). (C) In *Nrf2*^{-/-} mice, Akt (S473) is phosphorylated in *Nrf2*^{-/-} sepsis-control mice, but there are no effects of CO (densitometry n = 4 per group). SA = *S. aureus*.

phosphorylation in *Akt1*^{-/-} sepsis-control mice at 6 but not 24 hours. CO did not increase phosphoGsk3β levels, implying an Akt requirement for the inhibition of Gsk3β after CO (Figure 9E). This was checked in HepG2 cells, and the effects of DCM/CO on HO-1 were consistent with the mouse, and Akt inhibition blocked CO induction of HO-1, whereas Gsk3β inhibition enhanced it, indicating a critical role for Akt activation by CO in HO-1 induction (Figure 9F).

DISCUSSION

The resolution of mitochondrial damage after severe sepsis is fundamental to the recovery from sepsis-induced multiple organ dysfunction syndrome, a major cause of intensive care unit mortality. The mitochondrial quality control process in sepsis must be understood in the context of organ recovery to devise suitable mitochondrial therapy. The defense of mitochondria in sepsis is initiated by activation of innate immunity, which induces mitochondrial biogenesis through the Toll-like receptor system (43, 49, 50).

A role for the HO-1/CO system and the antiinflammatory effect of CO are well recognized, and despite potential CO toxicity, low doses of the gas can be administered without tissue hypoxia or cell damage (18). This presumably mimics the protective mechanisms of endogenous CO, and the affinity of CO for reduced heme proteins has implicated certain heme targets, including cytochrome oxidase, and physiologic CO stimulates mitochondrial biogenesis and leads to improved respiratory

capacity (21). HO-1 is protective in sepsis, but the impact of the HO-1/CO system on mitochondrial integrity has remained ill-defined (38). To investigate this aspect, we tested whether pulse CO administration in mice would enhance HO-1 activity, thereby facilitating the induction of mitochondrial biogenesis and improving survival in *S. aureus* sepsis. A 1 hour per day CO regimen at 250 ppm for 3 days reduced 7-day sepsis mortality by half, and in a sentinel organ, the liver, induced *Hmox1* through Nrf2 activation. The CO intervention expedited HO activity in mitochondria and the induction of mitochondrial biogenesis, promptly restoring mtDNA copy number. Moreover, Nrf2 stimulated antiinflammatory IL-10 expression, which dampens proinflammatory TNF-α and NOS2 production.

This mechanism was established by taking advantage of four facts. (1) *Hmox1* is induced by Nrf2 (22). (2) HO-1/CO can activate mitochondrial biogenesis through Nrf2-inducible genes (15). (3) HO-1 is activated by and activates Akt (30, 51) and Akt phosphorylates NRF-1 during mitochondrial biogenesis (32). (4) HO-1 is linked to counter-inflammation, most discernibly through IL-10 (35), and coupling of counter-inflammation to mitochondrial biogenesis by Nrf2 (52).

HO-1 and its products lessen inflammatory tissue damage partly because increased heme catabolism decreases oxidative stress (17, 18, 53). In our *S. aureus* peritonitis model, this system reaches quasistability by 48 hours, but additional CO accelerated *Hmox1* induction, leading to higher total and mitochondrial HO activity and a higher capacity for heme clearance. Lacking an obvious HO-1 importation mechanism, active enzyme

spiratory factor (NRF)-1 and transcription factor-A (Tfam) did not increase in *Akt*^{-/-} sepsis-control or sepsis-CO mice. (E) Hepatic GSK3β phosphorylation increased in *Akt*^{-/-} sepsis-control mice only at 6 hours, and CO had no effect. (F) HepG2 cells incubated with dichloromethane (DCM)/CO demonstrate the effect of CO on HO-1 protein expression. HO-1 induction by DCM/CO was blocked by inhibition of Akt and enhanced by inhibition of Gsk3β (densitometry n = 3; *P < 0.05 compared with lane 1).

associates with a subset of mitochondria to mitigate heme-induced oxidative stress.

In addition to *Hmox1*, Nrf2 activates other antioxidant and phase II detoxifying genes (54–56). Some Nrf2 is also associated

with mitochondria (56), and Nrf2 activation is augmented by a surge in mitochondrial ROS generation after CO binding to cytochrome oxidase (15). Thus, HO-1 acting through CO generates a feed-forward cycle, apparently unique among Nrf2

Figure 10. The heme oxygenase (HO)-1/carbon monoxide (CO) system and activation of mitochondrial biogenesis in *Staphylococcus aureus* sepsis. The diagram illustrates the redox cycle that activates NF-E2-related factor-2 (Nrf2) and the HO-1/CO system in response to *S. aureus* and to CO. Both the oxidative stress of innate immune hyperactivation and the CO intervention induce the mitochondrial biogenesis transcriptional program through Nrf2. The two known sites of Akt involvement are also indicated. Keap1 = Kelch-like ECH-associated protein 1; NF- κ B = nuclear factor- κ B; NRF1 = nuclear respiratory factor-1; PGC = peroxisome proliferator-activated receptor gamma coactivator; Tfam = transcription factor-A; ROS = reactive oxygen species; TLR = Toll-like receptor; TNF = tumor necrosis factor.

network proteins, that meets the requirement for this transcription factor in mitochondrial biogenesis induction.

In fluid-resuscitated sepsis models, ROS produce sufficient damage to mitochondrial ultrastructure and function (57, 58) to be mitigated by antioxidant therapy (59, 60). In WT mice in sepsis, *Nrf2* is a potent survival gene evidenced by a high sensitivity of *Nrf2*^{-/-} mice to *S. aureus* lethality. *Nrf2*^{-/-} sepsis-control mice show modest increases in hepatic HO-1 levels, but significantly less *Hmox1* induction than WT mice. Thus, *Hmox1* is induced by Nrf2-dependent and Nrf2-independent pathways in sepsis, but in *Nrf2*^{-/-} mice, CO does not up-regulate HO-1, indicating an essential role for Nrf2 in CO protection. Accordingly, despite smaller *S. aureus* inoculations, CO fails to rescue *Nrf2*^{-/-} mice. Moreover, when HO is inhibited, CO does not rescue WT mice, indicating a requirement for the Nrf2/HO-1 cycle. This also implies that the use of CO here does not depend on CO-dependent inhibition of *S. aureus* growth (39).

Nrf2 also up-regulates *NRF1*, and NRF-1 activates *Tfam* and other genes that control mtDNA transcription and replication. Oxidative mtDNA damage in sepsis is a result of innate immune activation, leading to a distinctive fall in hepatic mtDNA copy number (61), which is abrogated by CO intervention. Because Nrf2 regulates NRF-1 expression, we anticipated the weak induction of NRF-1 in *Nrf2*^{-/-} sepsis-control mice, which along with disruption of the HO-1/CO cycle and suboptimal *Gabpa* and *Tfam* induction indicates impaired redox regulation of the mitochondrial biogenesis program. Moreover, failure of *Nrf2*^{-/-} mice to activate mitochondrial biogenesis impedes counter-inflammation, which perpetuates mitochondrial damage caused by inflammatory cytokines, such as TNF- α (62).

Oxidative stress also activates Akt through the inactivation of specific phosphatases, such as phosphatase and tensin homolog, that counter-regulate PI-3K (62). CO enhances phosphatase inactivation through mitochondrial ROS production, leading to increased phosphorylation of Akt and NRF-1, which promotes mitochondrial biogenesis (32). Akt is phosphorylated by PI-3K at S473 and T308, and in WT and *Nrf2*^{-/-} sepsis-control mice, Akt phosphorylation occurs at both sites, whereas CO enhances S473 phosphorylation. In *Akt1*^{-/-} sepsis-control mice, Nrf2 activation is impaired by an unopposed inhibitory effect of GSK3 β ; *Akt1*^{-/-} mice therefore lack *NRF1* induction and cannot be rescued by CO.

Mechanistically, Nrf2 activation and *Hmox1* expression improve survival in mice in *S. aureus* sepsis, presumably through a set of redox-activated Nrf2-dependent genes that support mitochondrial biogenesis. The pathways activated through this system are diagrammed in Figure 10. *S. aureus* and CO also both activate Akt, which acts upstream and directly through HO-1 on mitochondrial biogenesis, although we have an incomplete picture of its role. *Nrf2*^{-/-} and *Akt1*^{-/-} mice show increased sepsis mortality, and CO rescues WT but not mice lacking either of the two genes.

We used CO to induce HO-1 through Nrf2, but other activators may also work, potentially with less toxicity, and CO may be less effective in lung injury in patients on high FI_{O2} because of low CO/O₂ ratios. Thus, a novel sepsis survival mechanism involving mitochondrial heme sensing activates genes through Nrf2 that protect mtDNA and induce mitochondrial biogenesis. More specifically, HO-1, in the degradation of prooxidant heme, is the linchpin of an inducible mitochondrial quality control network that is transcriptionally integrated with the antioxidant and antiinflammatory defenses and amenable to pharmacologic exploitation.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Craig Marshall, Marta Salinas, Lynn Tatro, John Patterson, and Kathy Stempel for excellent technical assistance.

References

- Dombrovskiy VY, Martin AA, Sunderram J, Paz HL. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. *Crit Care Med* 2007;35:1244-1250.
- Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003;348:1546-1554.
- Brealey D, Brand M, Hargreaves I, Heales S, Land J, Smolenski R, Davies NA, Cooper CE, Singer M. Association between mitochondrial dysfunction and severity and outcome of septic shock. *Lancet* 2002;360:219-223.
- Vincent JL, Bruzzi de Carvalho F. Severity of illness. *Semin Respir Crit Care Med* 2010;31:31-38.
- Bradley JR. TNF-mediated inflammatory disease. *J Pathol* 2008;214:149-160.
- Svistunenko DA, Davies N, Brealey D, Singer M, Cooper CE. Mitochondrial dysfunction in patients with severe sepsis: an EPR interrogation of individual respiratory chain components. *Biochim Biophys Acta* 2006;1757:262-272.
- Haden DW, Suliman HB, Carraway MS, Welty-Wolf KE, Ali AS, Shitara H, Yonekawa H, Piantadosi CA. Mitochondrial biogenesis restores oxidative metabolism during *Staphylococcus aureus* sepsis. *Am J Respir Crit Care Med* 2007;176:768-777.
- Carre JE, Orban JC, Re L, Felsmann K, Iffert W, Bauer M, Suliman HB, Piantadosi CA, Mayhew TM, Breen P, et al. Survival in critical illness is associated with early activation of mitochondrial biogenesis. *Am J Respir Crit Care Med* 2010;182:745-751.
- Avery SV. Molecular targets of oxidative stress. *Biochem J* 2011;434:201-210.
- Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 2010;48:749-762.
- Piantadosi CA, Suliman HB. Mitochondrial transcription factor a induction by redox activation of nuclear respiratory factor 1. *J Biol Chem* 2006;281:324-333.
- Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 2004;18:357-368.
- Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* 2008;88:611-638.
- Wang K, Klionsky DJ. Mitochondria removal by autophagy. *Autophagy* 2011;7:297-300.
- Piantadosi CA, Carraway MS, Babiker A, Suliman HB. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ Res* 2008;103:1232-1240.
- Maines MD. The heme oxygenase system: update 2005. *Antioxid Redox Signal* 2005;7:1761-1766.
- Wagner FA, Volk HD, Willis D, Abraham NG, Soares MP, Adema GJ, Figdor CG. Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol Rev* 2003;55:551-571.
- Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;86:583-650.
- Piantadosi CA. Biological chemistry of carbon monoxide. *Antioxid Redox Signal* 2002;4:259-270.
- Desmard M, Boczkowski J, Poderoso J, Motterlini R. Mitochondrial and cellular heme-dependent proteins as targets for the bioactive function of the heme oxygenase/carbon monoxide system. *Antioxid Redox Signal* 2007;9:2139-2155.
- Suliman HB, Carraway MS, Tatro LG, Piantadosi CA. A new activating role for co in cardiac mitochondrial biogenesis. *J Cell Sci* 2007;120:299-308.
- Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a cap'n'collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 1999;274:26071-26078.
- Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999;13:76-86.
- Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* 2002;99:11908-11913.
- Alam J, Igarashi K, Immenschuh S, Shibahara S, Tyrrell RM. Regulation of heme oxygenase-1 gene transcription: Recent advances and

- highlights from the international conference (Uppsala, 2003) on heme oxygenase. *Antioxid Redox Signal* 2004;6:924–933.
26. Scarpulla RC. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* 2011; 1813:1269–1278.
27. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, Biswal S. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006;116:984–995.
28. Kong X, Thimmulappa R, Craciun F, Harvey C, Singh A, Kombairaju P, Reddy SP, Remick D, Biswal S. Enhancing Nrf2 pathway by disruption of Keap1 in myeloid leukocytes protects against sepsis. *Am J Respir Crit Care Med* 2011;184:928–938.
29. Salinas M, Wang J, Rosa de Sagarra M, Martin D, Rojo AI, Martin-Perez J, Ortiz de Montellano PR, Cuadrado A. Protein kinase Akt/PKB phosphorylates heme oxygenase-1 in vitro and in vivo. *FEBS Lett* 2004;578:90–94.
30. Zhang X, Shan P, Alam J, Fu XY, Lee PJ. Carbon monoxide differentially modulates Stat1 and Stat3 and inhibits apoptosis via a phosphatidylinositol 3-kinase/Akt and p38 kinase-dependent Stat3 pathway during anoxia-reoxygenation injury. *J Biol Chem* 2005;280:8714–8721.
31. Li MH, Jang JH, Na HK, Cha YN, Surh YJ. Carbon monoxide produced by heme oxygenase-1 in response to nitrosative stress induces expression of glutamate-cysteine ligase in pc12 cells via activation of phosphatidylinositol 3-kinase and Nrf2 signaling. *J Biol Chem* 2007; 282:28577–28586.
32. Suliman HB, Carraway MS, Welty-Wolf KE, Whorton AR, Piantadosi CA. Lipopolysaccharide stimulates mitochondrial biogenesis via activation of nuclear respiratory factor-1. *J Biol Chem* 2003;278:41510–41518.
33. Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. Glycogen synthase kinase-3 β inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor nrf2. *J Biol Chem* 2006;281:14841–14851.
34. Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* 2010;80:1895–1903.
35. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 2002;8:240–246.
36. Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, Fiers W, Goldman M, Velu T. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J Exp Med* 1993;177:547–550.
37. Sarady JK, Zuckerbraun BS, Bilban M, Wagner O, Usheva A, Liu F, Ifedigbo E, Zamora R, Choi AM, Otterbein LE. Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. *FASEB J* 2004;18:854–856.
38. Lancel S, Hassoun SM, Favory R, Decoster B, Motterlini R, Neviere R. Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis. *J Pharmacol Exp Ther* 2009;329:641–648.
39. Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, et al. A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia. *FASEB J* 2009;23:1023–1031.
40. MacGarvey NC, Suliman HB, Piantadosi CA. The HO/CO system promotes hepatic mitochondrial biogenesis during *Staphylococcus aureus* sepsis. *Am J Respir Crit Care Med* 2010;181:A1141.
41. Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, Piantadosi CA. The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. *J Clin Invest* 2007;117:3730–3741.
42. Vreman HJ, Wong RJ, Kadotani T, Stevenson DK. Determination of carbon monoxide (CO) in rodent tissue: effect of heme administration and environmental CO exposure. *Anal Biochem* 2005;341:280–289.
43. Suliman HB, Sweeney TE, Withers CM, Piantadosi CA. Co-regulation of nuclear respiratory factor-1 by NF[κ]B and CREB links LPS-induced inflammation to mitochondrial biogenesis. *J Cell Sci* 2010; 123:2565–2575.
44. Wong GH, Elwell JH, Oberley LW, Goeddel DV. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. *Cell* 1989;58:923–931.
45. Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science* 2003;299:896–899.
46. Scarpulla RC. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. *Gene* 2002;286: 81–89.
47. Kemp M, Go YM, Jones DP. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med* 2008;44:921–937.
48. Alam J, Cook JL. How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Respir Cell Mol Biol* 2007;36:166–174.
49. Suliman HB, Welty-Wolf KE, Carraway MS, Schwartz DA, Hollingsworth JW, Piantadosi CA. Toll-like receptor 4 mediates mitochondrial DNA damage and biogenic responses after heat-inactivated *e. coli*. *FASEB J* 2005;19:1531–1533.
50. Sweeney TE, Suliman HB, Hollingsworth JW, Piantadosi CA. Differential regulation of the PGC family of genes in a mouse model of *Staphylococcus aureus* sepsis. *PLoS ONE* 2010;5:e11606.
51. Pischke SE, Zhou Z, Song R, Ning W, Alam J, Ryter SW, Choi AM. Phosphatidylinositol 3-kinase/Akt pathway mediates heme oxygenase-1 regulation by lipopolysaccharide. *Cell Mol Biol (Noisy-le-grand)* 2005; 51:461–470.
52. Piantadosi CA, Withers CM, Bartz RR, MacGarvey NC, Fu P, Sweeney TE, Welty-Wolf KE, Suliman HB. Heme oxygenase-1 couples activation of mitochondrial biogenesis to anti-inflammatory cytokine expression. *J Biol Chem* 2011;286:16374–16385.
53. Bilban M, Haschemi A, Wegiel B, Chin BY, Wagner O, Otterbein LE. Heme oxygenase and carbon monoxide initiate homeostatic signaling. *J Mol Med* 2008;86:267–279.
54. Leiser SF, Miller RA. Nrf2 signaling, a mechanism for cellular stress resistance in long-lived mice. *Mol Cell Biol* 2010;30:871–884.
55. Klaassen CD, Reisman SA. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicol Appl Pharmacol* 2010;244: 57–65.
56. Lo SC, Hannink M. Pgms5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria. *Exp Cell Res* 2008;314:1789–1803.
57. Welty-Wolf KE, Simonson SG, Huang YC, Fracica PJ, Patterson JW, Piantadosi CA. Ultrastructural changes in skeletal muscle mitochondria in gram-negative sepsis. *Shock* 1996;5:378–384.
58. Suliman HB, Welty-Wolf KE, Carraway M, Tatro L, Piantadosi CA. Lipopolysaccharide induces oxidative cardiac mitochondrial damage and biogenesis. *Cardiovasc Res* 2004;64:279–288.
59. Lowes DA, Thottakam BM, Webster NR, Murphy MP, Galley HF. The mitochondria-targeted antioxidant mitoq protects against organ damage in a lipopolysaccharide-peptidoglycan model of sepsis. *Free Radic Biol Med* 2008;45:1559–1565.
60. Zapelini PH, Rezin GT, Cardoso MR, Ritter C, Klamt F, Moreira JC, Streck EL, Dal-Pizzol F. Antioxidant treatment reverses mitochondrial dysfunction in a sepsis animal model. *Mitochondrion* 2008;8:211–218.
61. Suliman HB, Carraway MS, Piantadosi CA. Postlipopolysaccharide oxidative damage of mitochondrial DNA. *Am J Respir Crit Care Med* 2003;167:570–579.
62. Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J* 2003;22:5501–5510.