Heme Oxygenase-1 Regulates Cardiac Mitochondrial Biogenesis via Nrf2-Mediated Transcriptional Control of Nuclear Respiratory Factor-1

Claude A. Piantadosi, Martha Sue Carraway, Abdelwahid Babiker, Hagir B. Suliman

Abstract—Heme oxygenase (HO)-1 is a protective antioxidant enzyme that prevents cardiomyocyte apoptosis, for instance, during progressive cardiomyopathy. Here we identify a fundamental aspect of the HO-1 protection mechanism by demonstrating that HO-1 activity in mouse heart stimulates the bigenomic mitochondrial biogenesis program via induction of NF-E2–related factor (Nrf)2 gene expression and nuclear translocation. Nrf2 upregulates the mRNA, protein, and activity for HO-1 as well as mRNA and protein for nuclear respiratory factor (NRF)-1. Mechanistically, in cardiomyocytes, endogenous carbon monoxide (CO) generated by HO-1 overexpression stimulates superoxide dismutase-2 upregulation and mitochondrial H$_2$O$_2$ production, which activates Akt/PKB. Akt deactivates glycogen synthase kinase-3β, which permits Nrf2 nuclear translocation and occupancy of 4 antioxidant response elements (AREs) in the NRF-1 promoter. The ensuing accumulation of nuclear NRF-1 protein leads to gene activation for mitochondrial biogenesis, which opposes apoptosis and necrosis caused by the cardio-toxic anthracycline chemotherapeutic agent, doxorubicin. In cardiac cells, Akt silencing exacerbates doxorubicin-induced apoptosis, and in vivo CO rescues wild-type but not Akt$^{-/-}$ mice from doxorubicin cardiomyopathy. These findings consign HO-1/CO signaling through Nrf2 and Akt to the myocardial transcriptional program for mitochondrial biogenesis, provide a rationale for targeted mitochondrial CO therapy, and connect cardiac mitochondrial volume expansion with the inducible network of xenobiotic and antioxidant cellular defenses. (Circ Res. 2008;103:1232-1240.)

Key Words: mitochondria ■ heme oxygenase ■ carbon monoxide ■ NF-E2–related factor 2 ■ nuclear respiratory factor-1

Progressive cardiomyopathy attends diverse stresses ranging from aberrant calcium signaling to inflammation to direct cardiomyocyte toxicity. The hallmarks of cardiac decompensation, such as apoptosis and myocyte depletion, often imply mitochondrial pathogenesis, particularly after inciting agents such as doxorubicin, which inhibits the expression of nuclear and mitochondrial encoded genes involved in mitochondrial biogenesis. This deficit, however, can be averted with low-dose carbon monoxide (CO), a product of endogenous heme oxygenase-1 (HO-1, Hmox1), which catalytically degrades potentially toxic heme to biliverdin.

Among the arsenal of antioxidant enzymes, HO-1 is strategically induced by its heme substrate, but also indirectly by endotoxin, hypoxia, and heavy metals against which it protects. HO-1/CO signaling operates in part and in similarity to the nitric oxide (NO) synthases, through heme–protein binding, eg, soluble guanylate cyclase (GC), and like NO, CO activates mitochondrial biogenesis. In the heart, however, CO acts independently of endothelial NO synthase on gene transactivation for nuclear respiratory factor (NRF)-1 and -2, as well as the PGC-1α coactivator and mitochondrial transcription factor A (Tfam), necessary for mitochondrial biogenesis.

CO binding to the reduced $a_{3}$ heme of cytochrome $c$ oxidase also enhances mitochondrial hydrogen peroxide ($H_{2}O_{2}$) production, which despite its potential toxicity, serves signal transduction and contributes to retrograde activation of mitochondrial biogenesis. CO activates the prosurvival phosphatidylinositol-3 (PI3)-kinase/Akt pathway, and Akt phosphorylates NRF-1, an integral transcription factor for mitochondrial biogenesis, before it enters the nucleus, but how CO activates the transcriptional programming is not known.

HO-1 activation by PI3-kinase/Akt as well as transcriptional regulation of Hmox-1 by NF-E2-related factor (Nrf)2 puts HO-1 in a position to exert oxidation-reduction (redox) control over cell processes. A basic leucine zipper transcription factor, Nrf2 is activated by electrophiles and reactive oxygen species, and enhances cell-protective gene expression by interacting with ARE motifs usually located 5′ to the transcription start site (TSS). Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein (Keap)1, which represses its transcriptional role, but
oxidation of cysteine-rich Keap1 frees Nrf2 to enter the nucleus to participate in ARE transactivation. Many electrophiles also enhance Nrf2 gene transcription, and the Nrf2/Keap1 complex is regulated by specific protein kinases such as glycogen synthase kinase (GSK)β, which inhibits Nrf2 nuclear translocation.

Here we analyzed the NRF-1 promoter for ARE motifs and examined the hypothesis that endogenous CO stimulates mitochondrial H$_2$O$_2$ production and induces NRF-1 responsive genes via Nrf2 occupancy of NRF-1 promoter sites to increase NRF-1 expression and activity. By implication, HO-1 would serve mitochondrial biogenesis through transcriptional integration with the major antioxidant enzyme defenses. Our findings delineate a new role for HO-1/CO in the coordination of mitochondrial biogenesis as well as a previously unsuspected Nrf2-based redox component for the regulation of mitochondrial mass in the heart.

Materials and Methods

Materials

Antibodies to citrate synthase, GSK3β, Nrf2, Bach1, Bid, Bcl-x, and PolRMT were obtained from Santa Cruz and to caspase 3, Akt, Ser473-phospho-Akt, phospho-GSK3β, and phospho-Bad from Cell Signaling. Phospho Ser/Thr/Tyr antibody was from AnaSpec and from the mid-dynamic range, and data expressed relative to tubulin.

Animals

Studies of mice were approved by the Institutional Animal Care and Use Committee: male 10 to 12 week-old C57BL/6 or Akt1+/− (Jackson) or transgenic mice that express green fluorescent protein (GFP) in mitochondria (mitoGFP-tg, from Hiroshi Shitara and Hiromichi Yonekawa of Tokyo Metropolitan Institute of Medical Science). Akt1+/−/mitoGFP-tg hybrids were generated by cross-breeding. Cardiomyopathy was induced by 1 injection of doxorubicin (Dox, Sigma-Aldrich; 15 mg/kg IP). Cardiomyopathy was induced by 1 injection of doxorubicin (Dox, Sigma-Aldrich; 15 mg/kg IP). Exponential C change in treated versus untreated control samples (ΔΔCt), relative to IgG. Exponential ΔΔCt, values were converted to linear values (2^-ΔΔCt) for graphics.

Statistical Analysis

Grouped data are expressed as the means±SEM for n=4 to 6 replicates. Statistical significance was tested with the unpaired Student’s t test or two-way analysis of variance using commercial software. Differences were considered significant at P<0.05.

Results

Nuclear Nrf2

One hour of CO breathing reinforced HO-1 gene expression and enzyme activity in the mouse heart in vivo (Figure 1A). Because Hmox-1 promoter contains multiple ARE motifs, we examined cardiac Nrf2 expression and activation by CO and found increased nuclear Nrf2 localization at 12 hours by confocal microscopy (Figure 1B), confirmed by Western analysis of nuclear protein (Figure 1C). This CO dose produced a 3-fold increase in cardiac Nrf2 mRNA levels by 6 hours (Figure 1D) and protected against Dox cardiotoxicity as shown by stability of ventricular ejection fraction using serial ultrasound measurements (Figure 1E).

The Nrf2 response to CO was examined in neonatal rat cardiomyoblasts and beating mouse cardiomyocytes (HL-1...
cells) with the same results. At low concentrations of DCM/CO (50 μmol/L), nuclear Nrf2 content increased by more than fivefold (data for HL-1 cells shown in Figure 2A, top). Also in HL-1 cells, DCM/CO disrupted the Nrf2 association with Keap1, an effect not observed in cells transfected with mitochondrial-targeted catalase (mCAT) and indicating an oxidant mechanism (Figure 2A, bottom).

DCM/CO increased HO-1 mRNA levels 3-fold and mitochondrial superoxide dismutase (SOD)2 mRNA fourfold, whereas Nrf2 gene silencing abrogated the ability of DCM/CO to increase mRNA levels for both enzymes (Figure 2B). After DCM/CO, HO-1 enzyme activity increased tenfold; once again this was abrogated by Nrf2 silencing (Figure 2C).

Nrf2 nuclear translocation involves not only Keap1 oxidation but Nrf2 phosphorylation. Because CO induces mitochondrial H₂O₂ generation and Akt activation, we compared the CO effects on Akt with Nrf2 nuclear translocation (Figure 2D, top). Treatment of HL-1 cells with DCM/CO or overexpression of active HO-1 provoked strong increases in Akt phosphorylation and Nrf2 nuclear translocation. When mitochondrial H₂O₂ release is attenuated by cotransfection with mCAT, Akt activation (68%) and Nrf2 nuclear translocation (60%) are attenuated (Figure 2D). To determine whether Akt regulates Nrf2 translocation directly or excludes the Bach1 repressor,32 HL-1 cells were treated with LY29 to inhibit PI3-kinase (Figure 2D, bottom). Here DCM/CO causes half as much nuclear Bach1 accumulation, whereas Akt inhibition has no effect on nuclear Bach1 but Nrf2 translocation is reduced by 60%. Bach1 siRNA increased nuclear Nrf2 content by 38% with no change in Akt activity (Figure 2D), implying that CO modulates Nrf2 nuclear translocation via Akt independently of Bach1.
HO-1 Increases Nrf2 Occupancy of NRF-1 Promoter ARE Motifs

CO induces NRF-1 mRNA and protein\(^{10}\), thus to investigate a similar effect of HO-1 on NRF-1 expression, HL-1 cells were transfected with HO-1. By confocal microscopy, active HO-1 compared with empty vector increased NRF-1 expression and nuclear protein accumulation (Figure 3A). Simultaneous cotransfection with HO-1 and active Akt containing an amino-terminal myristoylation signal (my-Akt) yielded a further 3-fold increase in nuclear NRF-1 protein (Figure 3A and 3B). These increases in nuclear NRF-1 are accompanied by 8- to 10-fold increases in the downstream expression of Tfam (Figure 3B), as well as in mtDNA copy number (Figure 3C).

Because HO-1 expression is regulated by Nrf2, we checked for Nrf2-binding sites in the NRF-1 promoter, core ARE motifs conforming to RTGA\(_Y\)nnnGC or its reverse, 10 kb upstream of the TSS.\(^{19}\) Seventeen consensus AREs were identified in the mouse and 13 on the plus-strand close to the NRF-1 TSS were investigated by ChIP analysis for Nrf2 interactions (Figure 3D) by analyzing immunoprecipitated DNA for enrichment by qPCR using primers flanking the ARE motifs. We found baseline Nrf2 occupancy to be minimal in control hearts, but after CO, found significant DNA enrichment at positions −1400, −3289, −3386 and −9829 bp (Figure 3D). The remaining ARE motifs did not respond to CO. These data demonstrate that after CO, Nrf2 regulates NRF-1 promoter activity at multiple sites.

Nrf2/HO-1 Induction of NRF-1 Regulates Mitochondrial Biogenesis and Opposes Cardiomyocyte Apoptosis

Cell protection assessed in rat H9c2 cardiomyoblasts with or without HO-1 overexpression before and after Dox is shown in Figure 4A. To connect nuclear Nrf2 to NRF-1 expression and the HO-1 protective function, we compared Nrf2 and NRF-1 expression before and after Dox as well as without or with HO-1 transfection. Nrf2-dependent NRF-1 responses transfection doubles mitochondrial Tfam and PolRMT protein and prevents Dox-mediated loss of these proteins (Figure 4A). The HO-1 protective effect is congruent with mitochondrial biogenesis at 24 hours as reflected in greater mitochondrial MTT reduction, increased citrate synthase (CS) expression (Figure 4B), and enhanced mRNA levels for mitochondrial-encoded OXPHOS proteins ND1 and COX subunit I (Figure 4C).

Dox toxicity is characterized by loss of mitochondrial number and hence a fall in Mitotracker signal intensity within 24 hours (Figure 4D). Mitochondrial structure is modified...
from its normal fine reticulum to vesicles and aggregates (Figure 4D, top images). In contrast, mitochondrial density in HO-1 overexpressing cardiomyocytes is greater than control, and the same Dox dose induces only sporadic mitochondrial structural changes (Figure 4D, bottom images). Dox causes cardiomyocyte apoptosis, confirmed by caspase-3 cleavage, whereas HO-1 overexpression prevents and HO-1 silencing exacerbates caspase-3 cleavage (Figure 4E, top gel). HO-1 overexpression promotes mitochondrial antiapoptotic protein expression, especially mitochondrial Bcl-X₇ (2.2-fold), compared with control cells (Figure 4E, bottom). HO-1 overexpression also decreases DOX-induced mitochondrial phosphorylated Bad (Ser-128) by 6-fold and Bid by 3-fold, whereas siHO-1 increases total mitochondrial pBad and Bid (Figure 4E). Thus HO-1 facilitates Bcl-X₇ and opposes Bid and Bad expression and/or mitochondrial translocation, which contributes to an antiapoptotic mitochondrial phenotype.

HO-1 Regulation of Cardiomyocyte Survival Through Akt

Cardiac protection by HO-1/CO in conjunction with Akt stimulates NRF-1 nuclear translocation to activate mitochondrial biogenesis. A role for Akt in HO-1/CO mitochondrial protection against Dox was tested in Akt1-deficient mice crossed with reporter mice expressing mitochondrial-targeted GFP. Cardiac pathology in the GFP reporter mice along with hematoxylin/eosin and TUNEL stains is shown in Figure 5. Akt⁻⁄⁻/GFP mice exposed to Dox show massive mitochondrial damage and cell death compared with wild-type (Wt) mice (Figure 5A) and interval CO treatment, which fully rescues mitochondrial damage and prevents necrosis in the Wt, is not effective in Akt⁻⁄⁻ mice (Figure 5A). In addition, CO ameliorates Dox-induced apoptosis in Wt but not in Akt⁻⁄⁻ mice (Figure 5B). Dox also introduces a spike in myocardial fibrosis, which CO prevents in Wt but not Akt⁻⁄⁻.
mice (not shown). Quantification of the mitochondrial reporter signal is shown in supplemental Figure I.

To further understand how Akt protects mitochondria, we examined GSK3β, which is negatively regulated by Akt phosphorylation of Ser-9 in the pseudosubstrate domain. A computer-assisted analysis of Nrf2 and Bach1 protein revealed no Akt but several canonical GSK3β motifs. Immunoprecipitation of Bach1 and Nrf2 and immunoblotting with anti–phospho-serine/threonine indicated that CO decreases phosphorylation of both Nrf2 and the suppressor, Bach1 (Figure 6A). In comparison, CO increases Akt and GSK3β protein phosphorylation in Wt but not in Akt1−/− mice (Figure 6B). The parsimonious reason is that Akt−/− mice are unable to phosphorylate and inactivate GSK3β during HO-1 activation. Moreover, nuclear translocation of NRF-1 induced by CO in Wt mice is abrogated in Akt−/− mice (Figure 6C). Collectively, these findings indicate that Akt critically offsets the GSK3β effect on nuclear exclusion of Nrf2 as well as facilitates NRF-1 nucleoprotein accumulation.

Discussion

The demonstration that HO-1 participates in cardiac mitochondrial biogenesis through the Nrf2 transcription factor, which confers resistance against electrophiles and xenobiotics, discloses several novel aspects of cell survival regulation with broad implications. Although we had previously implicated HO-1 in mitochondrial biogenesis through Akt1 activation, it was not known that the pathway involves Nrf2 expression, downstream GSK3β blockade, and Nrf2 nuclear translocation leading to Nrf2-dependent activation of NRF-1 transcription. The findings thus establish HO-1/CO, by sequentially activating these 2 transcription factors, as a remarkable component of a prosurvival program of mitochondrial biogenesis linked to the cellular antioxidant defenses.

The main antioxidant function of HO-1 has been thought to derive from the catalytic conversion of pro-oxidant heme to biliverdin, as well as from the induction of iron sequestration proteins. A role for endogenous CO has been more difficult to elucidate because CO inhibits respiration, generates reactive oxygen species, and causes apoptosis, yet recapitulates certain protective effects of HO-1 and as a pro-oxidant, exacerbates caspase 3 cleavage. Dox also decreases mitochondrial Bcl-XL and increases mitochondrial phosphorylated Bad (Ser-128) protein, which is counteracted by HO-1 overexpression. HO-1 silencing reverses the effects of HO-1 expression on mitochondrial pBad, Bid, and Bcl-XL.

Figure 4. Nrf2/HO-1 induction of NRF-1 activates mitochondrial biogenesis and prevents doxorubicin-induced cardiomyocyte apoptosis. Nuclear Nrf2 and NRF-1 expression were evaluated in H9c2 cardiomyoblasts without and with HO-1 overexpression or silencing compared with readouts of 2 nuclear-encoded downstream genes, Tfam and PolRMT, that regulate mitochondrial mRNA levels and with which Dox interferes (A, top). HO-1 overexpression doubles mitochondrial Tfam and PolRMT protein and attenuates Dox interference (A, middle). A, Bottom, demonstrates effective HO-1 silencing at 24 and 48 hours. Mitochondrial functional protection by HO-1 is reflected by enhanced MTT reduction and citrate synthase (CS) expression (B) and mRNA for mitochondrial-encoded subunits COX I and ND1 (C). Dox-induced changes in MitoTracker green show organelle modification from a fine reticulum to vesicles and aggregates within 24 hours (D, top). In contrast, mitochondrial density in cells overexpressing HO-1 is greater than control, and changes in mitochondrial structure occur sporadically after Dox (D, bottom). E, Dox-induced caspase 3 cleavage; HO-1 overexpression limits and HO-1 silencing exacerbates caspase 3 cleavage. Dox also decreases mitochondrial Bcl-XL and increases mitochondrial phosphorylated Bad (Ser-128) protein, which is counteracted by HO-1 overexpression. HO-1 silencing reverses the effects of HO-1 expression on mitochondrial pBad, Bid, and Bcl-XL.
activation by CO. Furthermore, cells transfected with mitochondrial-targeted catalase indicate that Nrf2/Akt activation is contingent on mitochondrial H$_2$O$_2$ production. Classic oxidant activation of Akt entails reciprocal phosphatase activation by CO. In contrast to Wt mice, neither Akt nor GSK3β is phosphorylated in Akt1−/− mice after CO (B). Also, nuclear translocation of NRF-1 induced by CO in Wt mice is absent in Akt1−/− mice (C).

The pro-oxidant chemistry of CO and regulation of HO-1 by Nrf2, a critical transcription factor for protection against electrophiles, inflammation, and chemical toxicity, offered the possibility that Nrf2 might connect mitochondrial biogenesis with the antioxidant and xenobiotic defenses through a CO-based redox mechanism. Nrf2 protects against toxicants and carcinogens in part by activating phase II detoxifying genes, and it mediates CO induction of the rate-limiting enzyme for glutathione biosynthesis, but the findings here are the first evidence of its role in mitochondrial biogenesis.

To explore nuclear regulation of mitochondrial genes by HO-1, we used CO to boost cardiac HO-1 activity, which also increased Nrf2 mRNA and Nrf2 nuclear protein levels. This HO-1 response, together with SOD2 induction was confirmed by gene silencing to depend in cardiomyocytes on Nrf2
Figure 7. Working diagram of the HO-1/CO/Nrf2 pathway involved in the regulation of mitochondrial biogenesis. Nrf2 constitutively docks with Keap1, which sequesters it in the cytoplasm, allowing it to undergo ubiquitination and degradation (upper left). HO-1/CO increases mitochondrial H2O2 (lower left), which stabilizes Keap1 and results in Nrf2 gene expression and nuclear protein translocation. Nuclear Nrf2 undergoes binding to AREs in the Hmox1, SOD2, and NRF-1 promoters (far right). HO-1 and SOD2 amplify the mitochondrial H2O2 signal, whereas NRF-1 entry into the nucleus drives transcription for Tfam, Pol, RMT, and other genes of mitochondrial biogenesis. 

oxidative mitochondrial damage, impaired NO signaling, mitochondrial biogenesis, respiratory gene suppression, and widespread intrinsic apoptosis. Mechanistically, Akt1 mice are especially susceptible to cardiac necrosis and apoptosis, but unlike WT mice, cannot be rescued by CO, which no longer activates NRF-1 and Tfam for mitochondrial biogenesis. This finding means that HO-1, in conjunction with other regulators of myocardial mitochondrial capacity, is fundamentally tied to the support of energy metabolism and the prevention of cell death.

Figure 7 summarizes how HO-1/CO acts on Nrf2-dependent gene expression to drive mitochondrial biogenesis. Endogenous CO enhances mitochondrial H2O2 production, which activates Akt, alleviates cytoplasmic Nrf2 inhibition, and promotes Nrf2 gene expression. Akt phosphorylates and deactivates GSK3β, releasing Nrf2 from inhibition. Nuclear Nrf2 occupies AREs in the HO-1, SOD2, and NRF-1 gene promoters, and facilitates the transcription and translation of all 3 proteins, whereas Akt phosphorylation of NRF-1 facilitates its translocation and activation of downstream genes required for mitochondrial biogenesis. SOD2 scavenges mitochondrial superoxide and in its regulatory capacity stabilizes Keap1 and results in nuclear protein translocation. Nuclear Nrf2 undergoes binding to AREs in the HO-1, SOD2, and NRF-1 gene promoters (far right). HO-1 and SOD2 amplify the mitochondrial H2O2 signal, whereas NRF-1 entry into the nucleus drives transcription for Tfam, Pol, RMT, and other genes of mitochondrial biogenesis.

In conclusion, HO-1/CO promotes Nrf2 gene expression via Nrf2, which couples mitochondrial biogenesis to the expression of phase II detoxifying and antioxidant enzyme defenses through the cis-acting ARE. In this role, HO-1/CO critically protects against doxorubicin-mediated mitochondrial damage and cardiomyocyte death, and by implication, pulse CO should have a place in targeted mitochondrial therapy. The most important new idea is that mitochondrial biogenesis as a prosurvival factor is integrally connected by redox control to the cellular defenses against xenobiotic toxicity and oxidative stress.

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Disclosures

None.

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## Online Table I: Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tr>
<td>Nrf2</td>
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<td>CCAGAGTGTTATCCAGCA</td>
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<tr>
<td>SOD2</td>
<td>CTGTCTTCAGCCACACCAGA</td>
<td>CTGCTTTCCAAAGTGTCCCTG</td>
</tr>
</tbody>
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Online Figure I

![Graph showing mean mitochondrial fluorescence intensity comparison between Wt and Akt^{+/-} groups across control, DOX, and DOX + CO conditions.]

- **Control:** Comparison between Wt and Akt^{+/-} groups.
- **DOX:** Significant difference indicated by *.
- **DOX + CO:** Significant difference indicated by *.

Bars represent mean fluorescence intensity with error bars indicating variability.