A new activating role for CO in cardiac mitochondrial biogenesis

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Summary

To investigate a possible new physiological role of carbon monoxide (CO), an endogenous gas involved in cell signaling and cytotoxicity, we tested the hypothesis that the mitochondrial generation of reactive oxygen species by CO activates mitochondrial biogenesis in the heart. In mice, transient elevations of cellular CO by five- to 20-fold increased the copy number of cardiac mitochondrial DNA, the content of respiratory complex I-V and interfibrillar mitochondrial density within 24 hours. Mitochondrial biogenesis is activated by gene and protein expression of the nuclear respiratory factor 1 (NRF1) and NRF2, of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha), and of mitochondrial transcription factor A (TFAM), which augmented the copy number of mitochondrial DNA (mtDNA). This is independent of nitric oxide synthase (NOS), as demonstrated by the identical responses in wild-type and endothelial NOS (eNOS)-deficient mice, and by the inhibition of inducible NOS (iNOS). In the heart and in isolated cardiomyocytes, CO activation involved both guanylate cyclase and the pro-survival kinase Akt/PKB. Akt activation was facilitated by mitochondrial binding of CO and by production of hydrogen peroxide (H2O2). Interference with Akt activity by blocking PI 3-kinase and by mitochondrial targeting of catalase to scavenge H2O2 prevented binding of NRF1 to the Tfam promoter, thereby connecting mitochondrial H2O2 to the pathway leading to mtDNA replication. The findings disclose mitochondrial CO and H2O2 as new activating factors in cardiac mitochondrial biogenesis.

Key words: Reactive oxygen species, Carbon monoxide, Heme oxygenase, Nitric oxide, Mitochondrial DNA

Introduction

Pleiotropic effects of carbon monoxide (CO), attributed to heme protein binding, produce cellular hypoxia and interfere with enzymatic function (Coburn and Forman, 1987; Piantadosi, 2002), but also allow for adaptation (Maines, 1988; Thom et al., 2000). Endogenous CO produced by heme catabolism has physiological roles in eukaryotic cells (Verma et al., 1993), and both endogenous and exogenous CO ameliorate experimental cardiac, lung and vascular injuries (Otterbein et al., 2003; Sato et al., 2001; Song et al., 2003), and protect against certain inflammatory states (Fujita et al., 2001). Thus, CO can exhibit anti-inflammatory (Wagener et al., 2003), anti-proliferative (Taille et al., 2003) and anti-apoptotic effects (Zhang et al., 2003) by largely undetermined physiological mechanisms.

The cellular physiology of CO is complex because the gas binds to multiple heme proteins, including cytochrome P450, guanylate cyclase and cytochrome c oxidase (Coburn and Forman, 1987). The latter inhibits mitochondrial electron transport, which can be deleterious to aerobic contractile function (Liao et al., 1996). However, we considered that the physiological activities of CO might be linked to oxidative metabolism (especially with respect to adaptation) by optimizing mitochondrial biogenesis. This requires nuclear and mitochondrial genomic orchestration by regulatory factors, including nitric oxide (NO), which activates guanylate cyclase and yields transcriptional response of, for example, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1A, hereafter referred to as PGC-1alpha) (Nisoli and Carruba, 2006; Nisoli et al., 2003).

CO specifically binds to reduced cytochrome a3 but has a weak affinity for guanylate cyclase – much less than NO – and, therefore, may be poised to play a complementary role in mitochondrial biogenesis. This idea derives from observations that cytochrome c oxidase naturally metabolizes CO to carbon dioxide (CO2) (Young and Caughey, 1986), binding of CO to cytochrome oxidase a3 heme increases the mitochondrial H2O2 leak rate (Zhang and Piantadosi, 1992), and the pro-survival phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Cantley, 2002) activates replication of mitochondrial DNA (mtDNA) by oxidant-dependent regulation of phosphorylation of nuclear respiratory factor 1 (NRF1) and expression of Tfam (Piantadosi and Suliman, 2006). Our hypothesis was that mitochondrial H2O2 production deriving from CO binding to cytochrome c oxidase is an activating factor in mitochondrial biogenesis.

Results

Preliminary studies of metabolism and mitochondrial biogenesis

We empirically identified by light microscopy brief CO exposures in mice that produced no mortality or evidence of histological damage. Exposures of 1 hour were chosen, which transiently depressed the resting oxygen consumption (VO2) and the CO2 production rates (VCO2), but then allowed resting-energy expenditure to recover to normal or above (Fig. 1A).
The molecular work focused on the heart because of its high metabolic rate and rapid response.

In mice, cardiac mitochondrial protein content increased for all five respiratory complexes by 2D gel electrophoresis within 24 hours of CO exposure (Fig. 1B). Mitochondrial state-4–state-3 respiration and coupling were preserved, although succinate-linked state-3 increased after CO (Fig. 1C). Low-temperature difference spectra (77°C) of cardiac mitochondria showing CO–cytochrome-a3 complex formation (CO-a3) and selective cytochrome b-c1 reduction (Cyt bc1). (E) TEM of longitudinal sections of hearts of control (left) and 24 hours after CO exposure (center) in Wt mice. Biogenesis is seen after CO in interfibrillar mitochondria (8000x magnification). Enlargement (right panel) shows budding (arrowhead) and dividing (arrows) mitochondria characteristic of mitochondrial biogenesis (22,000x magnification). The increase in mitochondrial density was approximately 30% 24 hours after treatment with CO by point counting.

In mouse heart tissue, the physiological CO concentration was on average 9 picomoles/mg and increased five- to 20-fold after 1 hour of incubation with CO (Fig. 2A, left panel). The intracellular CO content rose rapidly with the formation of carboxyhemoglobin (COHb), which increases the tissue capillary diffusion barrier and causes retention of endogenous CO (Cronje et al., 2004). At 6 hours, cellular CO concentration had declined to baseline (not shown) but expression of PGC-1α mRNA had increased (Fig. 2A, middle) in relation to the measured cardiac CO concentration (Fig. 2A, right).
A comparison of wild-type (Wt) mice and endothelial nitric oxide synthase (eNOS)-deficient (eNOS –/–) mice was performed to evaluate the role of NO in CO-induced mitochondrial biogenesis (Clementi and Nisoli, 2005; Nisoli and Carruba, 2006; Nisoli et al., 2003; Nisoli et al., 2004). In Wt and eNOS –/– strains, mRNA expression for the mitochondrial transcription factor A (TFAM) and DNA polymerase γ (Polγ) was increased after treatment with CO, and cardiac TFAM and Polγ protein had tripled at 24 hours (Fig. 2B, left and middle panels for western blots). Cardiac mtDNA content increased in both strains (Fig. 2B, right). The initial mtDNA content was lower in eNOS –/– than Wt hearts, but increased 2.5- to 3-fold in both strains after treatment with CO, demonstrating eNOS-independence. The possibility that inducible NOS (iNOS) caused the CO response in eNOS –/– mice was excluded by inhibiting iNOS with 1400 W (Cayman Chemical, Ann Arbor, MI), which did not alter the responses (data not shown).

The levels of mRNA for NRF1, NRF2 and PGCo1α, the transcription factors and the coactivator that regulate mitochondrial biogenesis, responded to the presence of CO in Wt and eNOS –/– mouse hearts followed by Tfam mRNA expression (Fig. 3A-D). In Wt mice, mRNA of PGCo1α peaked at 2 hours (Fig. 3A, fourfold; *P<0.05), of NRF1 and NRF2 at 2-6 hours (Fig. 3B,C, four- to sixfold; *P<0.05) and of Tfam at 24 hours (Fig. 3D, fivefold; *P<0.01). In eNOS –/– mice, levels of these transcripts were lower than in Wt mice but the temporal profiles were comparable.

To establish whether these responses were produced by CO or by hypoxia, Wt mice were exposed to hypoxia for 1 hour (a simulated altitude of 24,000 ft) to control for the hypoxic effect of COHb by lowering cardiac pressure of oxygen (PO2) levels. Hypoxia had no effect on the expression of cardiac PGCo1α or NRF1 (Fig. 3E,F). To confirm the requirement for binding of CO to heme protein in vivo, CO treatment was given in conjunction with hyperbaric oxygen to prevent binding of CO to reduced cytochrome a3 by raising mitochondrial PO2 levels (Piantadosi, 2002). Hyperbaric oxygen abrogated the effect of CO on mRNA levels of both proteins.

Activation of p38 MAP kinase and PI3-K/Akt
Mitogen-activated protein (MAP) kinases, most notably p38, are activated by reactive oxygen species (ROS) (Sugden and Clerk, 1998) and by CO (Zhang et al., 2003). We used the p38 inhibitor SB203580 in vivo to prevent CO-induced phosphorylation of cardiac p38 (Fig. 4A). SB203580 did not affect mRNA levels of...
PGC-1α, NRF1 or Tfam, or the increase in mtDNA in Wt or eNOS−/− mice after treatment with CO (Fig. 4A). The pro-survival, anti-apoptotic serine/threonine kinase Akt is cardioprotective, in part by phosphorylating and activating eNOS (Dimmeler et al., 1999). CO activates cardiac Akt in Wt and eNOS−/− mice (Fig. 4B, top, \( P < 0.05 \), densitometry not shown). Akt activation had the expected anti-apoptotic effect, e.g. phosphorylation of Bad (Fig. 4B, middle) (Matsui et al., 2001). In Wt mice, hypoxia (or treatment with hyperbaric oxygen and CO as negative control) did not yield phosphorylation of Akt; moreover, inhibition of p38 did not prevent Akt activation (Fig. 4B, bottom panel). Akt activation by CO, however, was attenuated more than 50% by inhibiting heme oxygenase (data not shown), supporting the hypothesis that cardiac Akt is also activated by endogenous CO.

Role of H2O2
Rat heart H9c2 cells were treated with the CO-generating molecule dichloromethane (DCM) to increase cellular CO levels via cytochrome P450 activity (Kubic and Anders, 1975). CO dose and CO time responses are shown in Fig. 5A (left panel) with cGMP production (middle panel). Mitochondrial viability and function, assessed by MTT assay, was initially unaffected (at 12-24 hours) but increased significantly 36 and 48 hours after treatment with DCM and CO generation, commensurate with mitochondrial biogenesis (Fig. 5A, right panel).

In H9c2 cells, like in the heart, an oxidative stress response to CO was demonstrated by the production of the proteins SOD2 (Fig. 5B), UCP2 (not shown) and HO-1 (not shown). SOD2 protein increased most dramatically (approximately tenfold at 6 hours). ROS generation by CO in H9c2 cells was proximate to mitochondria using the oxidant-sensitive probe Redox Sensor Red CC-1 dye (CC-1) and the mitochondrial-selective dye MitoTracker Green FM (MitoTracker) (Fig. 5C). Control cells showed little CC-1 oxidation and no CC-1 localization to mitochondria (Fig. 5C A-C). After DCM, CC-1 localized early to mitochondria (D-F) and dissipated by 24 hours (G-I). This ROS production depended on CO because the CC-1 fluorescence was eliminated by cytochrome P450 blockade (J-M).

In H9c2 cells, CO led to Akt and p38 phosphorylation, but we focused on Akt because the in vivo data did not implicate p38. Moreover, CO-activated Akt phosphorylation in H9c2 cells was unaffected by p38 inhibition, partially abrogated by guanylate cyclase inhibition, and blocked by PI3-K inhibition (Fig. 5D). Full Akt expression was also reduced by heme oxygenase inhibition (data not shown). Akt activation by CO was disrupted by the insertion of catalase into the mitochondria (Fig. 5D), thus demonstrating a role for mitochondrial H2O2 in Akt regulation.

Activation of mitochondrial biogenesis in H9c2 cells by CO was indicated by three- to fourfold increases in NRF1, NRF2α, PGC-1α and TFAM protein expression by western blot.
analysis (Fig. 5E). Guanylate cyclase blockade halved the CO response, but p38 blockade had no effect. By contrast, inhibition of PI3-K/Akt blocked expression of NRF1 and TFAM and attenuated that of PGC-1α and NRF2 (Fig. 5E). CO also increased the copy number of mtDNA, which was prevented by inhibition of guanylate cyclase or PI3-K but not p38 (Fig. 5F).

NRF1 and PGC-1α are binding partners in Tfam expression; therefore NRF1 binding to PGC-1α was checked by co-immunoprecipitation (Fig. 6). NRF1–PGC-1α binding was enhanced in H9c2 cells after treatment with CO, and was sensitive to PI3-K/Akt inhibition. Tfam promoter activation by NRF1 and NRF2 by CO was demonstrated by chromatin immunoprecipitation assay (Fig. 6B). NRF1–Tfam promoter binding after CO in H9c2 cells was abrogated by mitochondrial-targeted catalase (Fig. 6B).

MtDNA synthesis and mitochondrial biogenesis in H9c2 cells was evaluated using bromodeoxyuridine (BrdU) and anti-BrdU antibody (Ab) (Fig. 6C). In control cells, association of BrdU (red) with mitochondria (green) was minimal (Fig. 6CA-C). After CO treatment, in situ BrdU incorporation increased in conjunction with mtDNA replication, commensurate with an increase in mitochondrial mass (green fluorescence intensity, D-F). This finding was confirmed quantitatively by using MTT (not shown). BrdU incorporation into mtDNA was blocked by cytochrome P450 inhibition, confirming the requirement of CO (G-I).

Discussion
Overall, the work demonstrates that mitochondrial production of ROS by CO binding acts as a retrograde activating-factor for mitochondrial biogenesis in the heart. In mice, CO in the

Fig. 4. CO activation of cardiac p38 MAP kinase and PI3-K/Akt. (A, top) Western blot of phosphorylated p38 (P-p38) in Wt and eNOS−/− mice after treatment with CO (1250 ppm, 1 hour) with or without p38 inhibition (SB20). (Middle) Graphs indicate time courses for PGC-1α, NRF1 and Tfam mRNA expression by real-time RT-PCR in Wt and eNOS−/− mice after treatment with CO with or without the p38 inhibitor (SB20). Values are the mean ± s.e. normalized to GAPDH multiplied by total RNA mg/wet weight (*P < 0.05 Wt vs eNOS−/−). Inhibition of p38 did not attenuate the CO effect in either strain. (Bottom) Gel showing cardiac mtDNA copy number by competitive PCR in Wt and eNOS−/− mice 24 hours after treatment with CO. The top band corresponds to the 571 bp of target mtDNA and the bottom band to the 332 bp DNA fragment. p38 inhibition did not alter the copy number. (B, top) Western blot of unphosphorylated Akt and phosphorylated Akt (pAkt) demonstrating an increase in the ratio of pAkt:Akt in Wt and eNOS−/− mice after treatment with CO. (Middle) Western blot of cardiac unphosphorylated Bad and phosphorylated Bad (pBAD), showing the pBad:Bad ratio in Wt and eNOS−/− mice after treatment with CO demonstrating phosphorylation of the mitochondrial anti-apoptotic protein. (Bottom) Western blot of Akt and pAkt showing the pAkt:Akt ratio in Wt heart after treatment with CO with or without hyperbaric oxygen (HBO), hypoxia (HH) or the p38 inhibitor (SB203580). Akt phosphorylation was not caused by hypoxia, and was prevented by hyperbaric oxygen but not by p38 inhibition (n=3).
physiological range acts on cardiac mitochondrial biogenesis demonstrated by a combination of molecular, biochemical and morphological evidence. This new finding has importance for both exogenous CO and that generated as an endogenous gas.

Mitochondrial biogenesis is regulated by the coordinated expression of specialized nuclear transcription factors that activate genes for mitochondrial proteins (Hood, 2001; Hood et al., 2003). These transcriptional elements were stimulated by CO in the mouse heart by a hypoxia-independent effect. The transcriptional activation mechanisms are not delineated here, but the responses produced significant increases in the copy number of mtDNA, content of OXPHOS protein and mitochondrial density in vivo. The response to CO was confirmed in cardiomyocytes, which in association with cGMP expression and mitochondrial ROS production also showed apposite increases mitochondrial DNA synthesis and functional mitochondria.

The increases in cardiac mtDNA content in Wt and eNOS−/− mice were the same, unaffected by iNOS inhibition and, in view of the low nNOS content of the heart, support NO-independent effects of CO on mitochondrial biogenesis. The molecular data imply an overlap in the mechanism of action of
the two gases: NO relies on guanylate cyclase (Nisoli et al., 2003), whereas CO has dual guanylate-cyclase-dependent and guanylate-cyclase-independent effects – the latter requires mitochondrial production of H$_2$O$_2$ and activation of Akt.

CO requires classical O$_2$-dependent heme-protein binding demonstrated by the effect of hyperbaric oxygen, which keeps oxylabile transition metal centers in the oxidized state. This test is definitive because CO binds only reduced cytochrome oxidase $\alpha_3$ heme in intact mitochondria (Coburn and Forman, 1987; Piantadosi, 2002). CO elicits other features of mitochondrial redox signaling: inhibition of electron transport (Coburn and Forman, 1987), H$_2$O$_2$ generation (Zhang and Piantadosi, 1992) and reversibility by oxygenation (Young and Caughey, 1986).

The binding of CO to cytochrome $\alpha_3$ promotes reduction of the respiratory carriers in the cytochrome bc$_1$ region, raising PO$_2$ levels and increasing mitochondrial H$_2$O$_2$ production (Zhang and Piantadosi, 1992), shown here by low-temperature heart-muscle spectra and CC-1 cell labeling studies suggesting an accelerated mitochondrial H$_2$O$_2$ leak rate. CO also upregulates SOD2, which scavenges superoxide, augments mitochondrial H$_2$O$_2$ release, and may modulate redox signaling (Zhang et al., 2002). In cardiomyocytes, mitochondrial CC-1 uptake in the presence of CO was blocked by mitochondrial-targeted catalase. The loss of mitochondrial H$_2$O$_2$ disrupted Akt activation, NRF1 phosphorylation and its binding to the Tфam promoter, events that can be regulated by H$_2$O$_2$ (Piantadosi and Suliman, 2006). This role of mitochondrial H$_2$O$_2$ is also consistent with the ability of exogenous peroxides to increase cell mitochondrial mass (Lee et al., 2002; Piantadosi and Suliman, 2006).

Mitochondrial biogenesis entails mtDNA replication and transcription, which depend on nuclear-encoded transcription of Tфam (Moraes, 2001) regulated by NRF1 and NRF2 in association with PGC-1α (Virbasius and Scarpulla, 1994; Wu et al., 1999). NRF1 and NRF2 also partner with PGC-1α to activate other components of mitochondrial biogenesis (Kelly and Scarpulla, 2004; Schreiber et al., 2003) including genes encoding components of the respiratory subunit, e.g. cytochrome c and ATP synthase (Kelly and Scarpulla, 2004; Schreiber et al., 2003). PGC-1α is activated by cGMP (Nisoli et al., 2003) and transcriptional activity of GA-repeat-binding protein (mouse NRF2 homologue) forms a positive feedback loop that drives nuclear gene expression for mitochondrial proteins (Mootha et al., 2004).

CO activates two requisite processes in the current paradigm for mitochondrial biogenesis. First, like NO, CO activates guanulate cyclase which promotes mitochondrial biogenesis. Second, CO activates Akt through an established oxidant mechanism involving NRF1, which in concert with NRF2 activates Tфам expression. cGMP may also influence PI3-K-Akt signaling (Li et al., 2000), but H$_2$O$_2$ activates Akt by oxidizing cysteine in counter-regulatory phosphatases, such as PTEN, that oppose PI3-K (Leslie et al., 2003). In H9c2 cells, Akt activation by CO was eliminated by two structurally dissimilar PI3-K inhibitors. Likewise, Tфам expression was inhibited, confirming the importance of this pathway in Tфам regulation (Piantadosi and Suliman, 2006). Akt activation was abrogated by mitochondrial-targeted catalase, establishing mitochondrial H$_2$O$_2$ as a signal. By contrast, guanulate cyclase inhibition interfered little with Akt activation by CO, thereby
implicating H₂O₂ as the primary PI3-K/Akt activation mechanism in this case.

CO also activates p38 MAP kinase (Zhang et al., 2003) but we could not implicate it in early activation of mitochondrial biogenesis. We did not explore the disruption of PGC-1α binding to p160 myb – a negative regulator of its transcriptional activity and of respiration (Fan et al., 2004) – by p38 or the cross-talk postulated between p38 and PI3-K/Akt (Iwasa et al., 2003), because p38 signaling in the heart is complex. Some studies have found p38 protective (Baines et al., 1999), whereas others find it pro-apoptotic (Petrich and Wang, 2004; Ren et al., 2005).

An interpretation of picomolar CO as a respiratory inhibitor must be placed in the context of micromolar cytochrome oxidase in the heart. The CO to cytochrome a₃ stoichiometry was too low to compromise aerobic energy production to the point of damage, and indicated a high gain of mitochondrial biogenesis to mitochondrial oxidation-reduction state. For instance, the spectral data suggest that the CO/O₂ ratio adjusts electron transport in conjunction with the Q cycle to regulate superoxide production and the H₂O₂ leak rate via SOD2 (Trumpower, 1990; Zhang et al., 2002) (Fig. 7). CO is advantaged by binding only the reduced cytochrome oxidase a₃ heme, whereas other terminal inhibitors, such as CN and NO, bind both ferrous (Fe²⁺) and ferric (Fe³⁺) heme. Work on those gases will be of interest but, like CO, requires care to avoid ATP depletion and additional stimulation of biogenesis. Because the Michaelis-Menten constant (Kₘ) of cytochrome c oxidase for O₂ is low, the reduced cytochrome oxidase a₃ heme available to react with CO is also low, but increases under hypoxic and State 3 conditions (Chance et al., 1970).

Two observations regarding CO and hypoxia are relevant from a physiological perspective. First, the induction of hypoxia by COHb might directly activate Akt, eNOS and certain MAP kinases. This possibility was excluded by hypoxia-controls in mice and in H9c2 cells; picomolar CO stimulated mitochondrial biogenesis in aerobic conditions, mitigating hypoxia as the direct cause. Second, COHb delays endogenous CO clearance both by decreasing the PO₂ level and by increasing the gas back-pressure in tissue (Cronje et al., 2004). This is a simple explanation for the rapid cytoprotection by CO that may recapitulate HO-1 induction (Akamatsu et al., 2004; Fujita et al., 2001). CO and hypoxia also induce HO-1 and mitochondrial heme release (Cronje et al., 2004; Piantadosi et al., 2006), and heme oxygenase inhibition attenuates Akt activation, thus also implicating endogenous CO in mitochondrial biogenesis. The effect is not simple, however, and was set aside for the mitochondrial H₂O₂ mechanism which gives purview to endogenous CO.

With respect to adaptation and cell survival, our evidence is preliminary: CO influences the mitochondrial phenotype by upregulating TFAM, SOD2, the anti-apoptotic pBAD and the resting energy expenditure. We noticed relative activation of complex II after CO despite uniform increases in respiratory complex proteins, which could, in principle, reflect differential assembly, regulation or other adaptive strategies that require energy (Fujita et al., 2001; Wagener et al., 2003). These speculations require confirmation and future investigation.

In summary, modest increases in cellular CO concentration activate mitochondrial biogenesis by a set of molecular responses that includes mitochondrial H₂O₂ production, activation of guanylate cyclase and Akt, and induction of HO-1, independently of eNOS, iNOS and hypoxia. We impute from the physiological behavior of CO that exogenous and endogenous CO interact, and conjecture that endogenous CO participates in accordance with heme turnover and CO clearance rates. These findings have both adaptive and pathogenic implications for conditions that substantially raise the tissue CO content and produce oxidative stress, such as smoking, air pollution, and CO poisoning, and hemolytic or inflammatory states that accelerate heme turnover.

Materials and Methods

Mouse studies

Male 8-week- to 12-week-old mice (C57BL/6, Wt) and eNOS-null mice (eNOS–/– bred onto C57BL/6, Jackson) were housed in a barrier facility with ad libitum access to standard chow and water. The procedures were approved by the University IACUC. The resting steady-state O₂ consumption and CO₂ production rates (VO₂ and VCO₂, respectively) were measured at the same time of day at constant temperature in a metabolic chamber by timed collections of expired gas and by gas chromatography (Varian model 3800). VO₂ and VCO₂ were computed with standard formulae corrected to standard temperature and pressure dry.

Pre-mixed CO was directed into exposure chambers at 6 l/min (Cronje et al., 2004) and levels were monitored with a CO analyzer (Interscan, Chatsworth, CA). To control for changes in PO₂, some mice were exposed to conditions mimicking higher altitude (24,000 ft) or hyperbaric conditions (final pressure 5ATA±CO). Others received intraperitoneal (i.p.) injections of 50 µg of the heme oxygenase inhibitor Sn-protoporphyrin (SnPp), of vehicle (DMSO) with or without CO or of p38 inhibitor at 4 hours and 1 hour before treatment with CO (i.p. SB2035801, TOCRIS, Ellisville, MO); control mice received vehicle (0.2 ml 5% DMSO). After euthanasia, hearts were removed for immediate use, were flash-frozen or fixed by perfusion for light microscopy and transmission electron microscopy (TEM) (Suliman et al., 2004). For EM, hearts underwent retro-aortic perfusion first with cold PBS, then with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4). Multiple 1-2-mm cuboids of the left ventricle were cut and randomly

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Mitochondrial DNA copy number
The mtDNA copy number was quantified by competitive PCR using co-amplification of a target template with a known amount of competitor (Suliman et al., 2003a). Mouse cytochrome-b-specific primers 14335 5'-CATCAGTAACAC-ACATTGFG-3' and 14906 5'-GATTAGCTTTTCCCTTCGCAGCCAT-3' amplify 361 bp of the target mtDNA and 332 bp of the competitor DNA when cut with SphI. Triplicate sets of 2×104 copies of competitor and 50 ng of target mtDNA were co-amplified in a thermal cycler (Applied Biosystems, Foster City, CA) using product optimization on etidium bromide (EB)-stained gels in the exponential phase. PCRs were separated by electrophoresis in 2% agarose. EB-stained band intensities were measured densitometrically and mtDNA-specific products plotted against competitor density on a log-scale. Copy number was determined from the x intercept or the point at which the ratio of the 361-bp and 332-bp products was 1. A factor of 1.7 was used to correct for molecular weight.

Mitochondrial protein, function and spectra
Fresh cardiac interfibrillar mitochondria were isolated, their yield was determined and respiratory complexes were separated by Blue native polyacrylamide gel electrophoresis (BN-PAGE) (Suliman et al., 2004). Samples (2 mg) were mixed in 6-aminoacaproic acid (200 µM 0.75 M) Bis-Tris (50 mM, pH 7.0) and 37.5 µM dDMD (10%), centrifuged (100,000 g) and Serva Blue was added to the supernatant before 4-12% gradient electrophoresis. Complexes were identified by elution order and in-gel activity stains. Peptides were resolved by 2D Tricine-SDS-PAGE electrophoresis; BN-PAGE bands were excised, incubated in 1% SDS/1% mercaptoethanol for 2 hours and loaded onto 16% separating gels. After electrophoresis, gels were stained with Coo massie Blue followed by silver. Multiple sets of gels, each with tissue from one control and one CO-exposed mouse were carried simultaneously through separation, staining and densitometry. Low-temperature difference spectra of rat heart mitochondria, frozen in 50% glycerol, after reduction with sodium dithionite or 1% CO gas, using oxygenated temperature difference spectra of rat heart mitochondria, frozen in 50% glycerol, after reduction with sodium dithionite or 1% CO gas, using oxygenated temperature difference spectra of rat heart mitochondria, frozen in 50% glycerol, after reduction with sodium dithionite or 1% CO gas, using oxygenated

Plasmids expressing mitochondrial-targeted catalase
Mitochondrial-targeted catalase (mCAT)-expression vectors were constructed as a chimeric cDNA of mouse MnSOD mitochondrial leader sequence and mouse CAT. The MnSOD leader sequence was amplified using a sense primer flanked by a NheI restriction site (5'-GGCACTGTGGGAGGCCCA-3') and an antisense primer flanked by an Hpy188I restriction site (5'-GTCGCCGACATCCGGGGGGCCCA-3'). The mouse CAT-coding sequence (GenBank accession number X52108) was equipped with an Hpy188I site at the 5' end with oligonucleotide linkers, using the primers 5'-AGTGGGACATCCGGGGGGCCC-3' and 5'-CAAGGTATGTTTTTCCCCGGAGCCCAT-3'. PCR fragments of the leader sequence and CAT-coding region were cloned into pGEM-T-System 1 (Promega, Madison, WI). The pGEM-T vector containing the mitochondrial leader sequence was linearized with Hpy188I, the catalase region was removed, treated with T4 polymerases and ligated into the pGEM-T-MnSOD linearized vector. The MnSOD-CAT construct was subcloned in-frame into pCDNA3 expression vector. H9c2 cells were transfected with the DNA vector using FuGENE-HD (Roche) and expression verified by lack of H2O2 production using CC-1 dye. Cells transfected with the pcDNA3 vector lacking the insert were the controls.

Immunoprecipitation and CHIP assays
H9c2 cells were lysed and centrifuged at 16,000 g for 30 minutes. Supernatant proteins were cleared with mouse or rabbit IgG and immunoprecipitated with anti-PGC-1. Immunoprecipitates were resolved by SDS-10% PAGE and transferred to polyvinylidene membranes. NFR1 was detected by western blot analysis using a validated Ab. H9c2 cells (~4×10^5) cultured in 15 cm plates were treated with DCM (100 µM) for indicated times and then cross-linked with 1% formaldehyde for 7.5 minutes, harvested, and sonicated to ~500bp fragments, incubated for 30 min at 37°C and quenched with 0.125 M glycine. Cells were washed with PBS, harvested and processed for Chip It assay with anti-NRF1 or anti-NRF2. After ethanol precipitation, DNA was resuspended in 200 µl of 10% cells and 2.5 µl as PCR template. Input samples representing 1% of total DNA were hybridized to mouse genome microarrays (Roche Applied Science, Manassas, VA). The probes were hybridized in Slidehyb with standard protocol (Roche). The arrays were scanned with a Cyclone (Perkin-Elmer) and quantified using GenePix Pro 6.0 software (Molecular Devices). The data were normalized, and processed for statistical analysis using GeneSpring (Silicon Genetics). High-confidence LOCs with gene expression changes >2 fold and p<0.05 were retained for further analysis.

Bromodeoxyuridine incorporation
H9c2 cells were grown in chemoattractant and treated with DCM (100 µM) for 48 hours. Twelve hours before fixation, medium was changed to grow medium without pyruvate, uridine or antibiotics supplemented with 1 µM 5-bromo-2'-deoxy-uridine (BrDU) (Roche). After 12 hours, the medium was removed and the cultures were washed with pre-warmed HBSS (37°C). Cells were fixed at room temperature in 4% phosphate-buffered paraformaldehyde and 4% sucrose and washed in PBS. Fixed cells were incubated in methanol (−20°C) for 10 minutes and washed again with PBS. Cultures were incubated for 25 minutes at 37°C in 200 nM MitoTracker (Molecular Probes), washed in PBS and incubated 10 minutes at 37°C with mouse anti-BrDU Ab (Roche) in buffer, or buffer only. Cells were washed and incubated for 10 minutes at 37°C in 50 µg/ml anti-mouse FluoroLink, Cy3-labeled Ab (Molecular Probes), rinsed, coverslipped and observed by fluorescence/confocal microscopy (Zeiss 410 LSM, 620x).

Statistics
Mouse and cell data were expressed as the mean ± standard error (s.e.) for n=3-8.
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