Heart Failure

Cardioprotective and Antiapoptotic Effects of Heme Oxygenase-1 in the Failing Heart

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Background—Heme oxygenase-1 (HO-1) is an inducible stress-response protein that imparts antioxidant and antiapoptotic effects. However, its pathophysiological role in cardiac remodeling and chronic heart failure (HF) is unknown. We hypothesized that induction of HO-1 in HF alleviates pathological remodeling.

Methods and Results—Adult male nontransgenic and myocyte-restricted HO-1 transgenic mice underwent either sham operation or coronary ligation to induce HF. Four weeks after ligation, nontransgenic HF mice exhibited postinfarction left ventricular (LV) remodeling and dysfunction, hypertrophy, fibrosis, oxidative stress, apoptosis, and reduced capillary density, associated with a 2-fold increase in HO-1 expression in noninfarcted myocardium. Compared with nontransgenic mice, HO-1 transgenic HF mice exhibited significantly (P<0.05) improved postinfarction survival (94% versus 57%) and less LV dilatation (end-diastolic volume, 46±8 versus 85±32 μL), mechanical dysfunction (ejection fraction, 65±9% versus 49±16%), hypertrophy (LV/tibia length 4.4±0.4 versus 5.2±0.6 mm/mm), interstitial fibrosis (11.2±3.1% versus 18.5±3.5%), and oxidative stress (3-fold reduction in tissue malondialdehyde). Moreover, myocyte-specific HO-1 overexpression in HF promoted tissue neovascularization and ameliorated myocardial p53 expression (2-fold reduction) and apoptosis. In isolated mitochondria, mitochondrial permeability transition was inhibited by HO-1 in a carbon monoxide (CO)–dependent manner and was recapitulated by the CO donor tricarbonylchloro(glycinato)ruthenium(II) (CORM-3). HO-1–derived CO also prevented H2O2-induced cardiomyocyte apoptosis and cell death. Finally, in vivo treatment with CORM-3 alleviated postinfarction LV remodeling, p53 expression, and apoptosis.

Conclusions—HO-1 induction in the failing heart is an important cardioprotective adaptation that opposes pathological LV remodeling, and this effect is mediated, at least in part, by CO-dependent inhibition of mitochondrial permeability transition and apoptosis. Augmentation of HO-1 or its product, CO, may represent a novel therapeutic strategy for ameliorating HF. (Circulation. 2010;121:1912-1925.)

Key Words: apoptosis ◼ carbon monoxide ◼ heart failure ◼ heme oxygenase-1 ◼ cardiac remodeling, ventricular

Heme oxygenase-1 (HO-1) is a rapidly inducible cytoprotective protein that degrades heme to biliverdin, ferrous iron, and carbon monoxide (CO).1 HO-1 mitigates cellular injury by exerting antioxidant, antiapoptotic, and antiinflammatory effects;1–4; these benefits of HO-1 have been widely demonstrated in a variety of pathological states including hypoxic lung disease,5 vascular injury,6 and cardiac transplant rejection.7 Conversely, mice with germ-line HO-1 disruption exhibit progressive anemia, inflammation, and oxidative stress with age.8,9 HO-1–mediated cytoprotection likely reflects the effects of its catalytic products, especially CO, because CO recapitulates the cytoprotective profile of HO-1 and can rescue the injurious effects of HO-1 deficiency.1–4,7,10

Clinical Perspective on p 1925

Short-term HO-1–mediated cytoprotection extends to the heart. Heterozygous HO-1+/- mice exhibit exaggerated cardiac injury and dysfunction after ischemia/reperfusion, findings partially rescued by antioxidants.11 In contrast, mice with cardiac-restricted HO-1 overexpression are resistant to ischemia/reperfusion injury, with improved contractile recovery and reduced infarct size, inflammatory cell infiltration, oxi-
H9c2 Cardiomyocytes and HO-1 Transfection

H9c2 cells (rat embryonic cardiomyoblasts) were obtained from the American Type Culture Collection. Ad5/HO-1, the replication-deficient adenoviral vector containing the entire coding region of rat HO-1 complementary DNA, was generated in 293 cells by homologous recombination of pCMV/HO-1 and pJM17, a circularized adenovirus genome lacking its E1 region and a portion of the E3 region. Plaque-isolated viral clones were propagated in 293 cells and then purified over 2 CaCl gradients and titered by plaque assay as described previously.19 Ad5/LacZ containing the β-galactosidase gene driven by the CMV promoter was used as a control vector. H9c2 cells were seeded in 100-mm tissue culture dishes and transiently transfected with Ad5/HO-1 or Ad5/LacZ for 45 minutes at a multiplicity of infection of 10 for 72 hours before treatments.

Immunohistology

Masson trichrome staining for collagen was performed as described previously.15 Myocyte cross-sectional area was determined in sections stained with rhodamine-conjugated wheat germ agglutinin ( Molecular Probes, Carlsbad, Calif). Oxidative stress was indexed by immunostaining for malondialdehyde-adducted proteins as described previously.20 For HO-1 immunofluorescent staining, we used anti-mouse HO-1 antibody ( Stressgen ) and tetramethylrhodamine isothiocyanate-conjugated secondary antibody. Immunostaining with fluorescein isothiocyanate–conjugated isoleucin B4 ( Vector Laboratories, Burlingame, Calif ) was performed to determine tissue capillary density. Apoptosis in tissue and cells was determined with the use of the DeadEnd fluorometric terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling ( TUNEL ) system ( Promega ). Optical sections were obtained with a Zeiss LSM510 inverted confocal scanning laser microscope equipped with Enterprise/argon/HeNe lasers and excitation wavelengths appropriate for multichannel scanning. For immunohistological analyses, we typically analyzed 6 fields per heart with an area of 66 mm² per field (total area, 396 mm² per heart).

HO-1 Gene Expression

Total RNA was isolated from cardiac tissue as described previously.17,20 HO-1 mRNA was quantified and normalized to mouse β-actin mRNA with the use of real-time polymerase chain reaction with LUX gene-specific primers ( Invitrogen , Carlsbad, Calif).

Western Immunoblotting

Protein extraction, Western immunoblotting, and densitometry were performed as described previously.17,18,20 Primary antibodies used included anti-p53, anti-poly-ADP ribose polymerase ( PARP ), anti-Bax, anti-Bcl-2, anti-β-actin, and anti-α-tubulin from Santa Cruz Biotechnology ( Santa Cruz, Calif ), and anti-HO-1 from Stressgen.

Measurement of Free Malondialdehyde by Gas Chromatography–Negative Ionization Chemical Ionization–Mass Spectrometry

Tissue malondialdehyde concentration was measured by gas chromatography–negative ionization chemical ionization–mass spectrometry ( GC-NICI-MS ) as described previously.21 Heart tissue homogenate was derivatized with pentfluorobenzylhydroxylamine, and the pentfluorobenzyl-oxime derivatives of malondialdehyde were measured by GC-NICI-MS with the select ion monitoring mode. Benzoaldehyde ring d₄ was used as the internal standard. The following ions were monitored for the indicated aldehyde: benzaldehyde d₄-m/z 286 ( M⁺–HF ) and malondialdehyde m/z 204 ( M⁺–C₇H₅F₅–HFNO–C₇H₅ ).

Mitochondrial Membrane Permeability Transition

In adult cardiomyocytes and H9c2 cells, changes in mitochondrial membrane potential ( Δψm ) were assessed with tetramethylrhodamine methyl ester ( TMRM ). TMRM fluorescence was measured at 5-minute intervals for 30 minutes in live cells with a 585-nm
long-pass filter and laser-scanning confocal microscopy. TMRM is a cationic dye that accumulates in mitochondria in proportion to Δψm. The TMRM concentration chosen (100 nmol/L) does not suppress mitochondrial respiration23 and is nonquenching so that mitochondrial depolarization is accompanied by a decrease in cellular TMRM fluorescence over time followed by cell shortening due to ATP depletion.24 The time required for a 2-fold decrease in TMRM fluorescence was taken as an end point for membrane permeability transition (MPT).

Mitochondrial MPT Pore Opening
Mitochondria were isolated from adult mouse hearts, and MPT pore opening in isolated mitochondria was induced by Ca2+ as described previously.24 Isolated mitochondria were resuspended in swelling buffer (containing the following [in mmol/L]: 225 mannitol, 70 sucrose, 10 KH2PO4, 1 EGTA. A Clark electrode was used to measure the oxygen content of the mitochondrial suspension with 10 mmol/L pyruvate and 5 mmol/L malate. State 4 respiration was measured at baseline; 330 μmol/L ADP was added to stimulate state 3 respiration. The respiratory control ratio was defined as the state 3 to state 4 respiration ratio. The ADP to O ratio was calculated with the use of total oxygen consumption during state 3 respiration.

Mitochondrial Respiration
Mitochondria were resuspended in respiration buffer (pH 7.2) containing the following (in mmol/L): 225 mannitol, 70 sucrose, 10 KH2PO4, 1 EGTA. A Clark electrode was used to measure the oxygen content of the mitochondrial suspension with 10 mmol/L pyruvate and 5 mmol/L malate. State 4 respiration was measured at baseline; 330 μmol/L ADP was added to stimulate state 3 respiration. The respiratory control ratio was defined as the state 3 to state 4 respiration ratio. The ADP to O ratio was calculated with the use of total oxygen consumption during state 3 respiration.

Preparation of the CO Donor CORM-3
CORM-3, a water-soluble transition metal carbonyl drug that stably releases CO, was synthesized following a published protocol,25 and its structure was confirmed by infrared spectroscopy and nuclear magnetic resonance. The synthesized drug was stored at −20°C and fresh solution was made just before use. For control experiments, CORM-3 was inactivated by dissolving it in Krebs-Henseleit buffer and allowing CO liberation overnight at room temperature.

Statistical Analysis
Several statistical techniques were employed. For 2-group comparisons, we used the unpaired 2-sample t test. For comparisons of ≥ 2 groups, we used 1-way ANOVA if there was 1 independent variable, 2-way ANOVA if there were 2 independent variables (eg, genotype and ligation status), and 2-way repeated-measures ANOVA for matched observations over time with 2 independent variables. To adjust for multiple comparisons, we performed Student-Newman-Keuls posttest for 1-way ANOVA and Bonferroni posttest for 2-way ANOVA. Pairwise comparisons were made between sham groups across genotypes, sham versus HF within each genotype, and HF groups across genotypes. Animal survival was evaluated by the Kaplan–Meier method, and the log-rank test was used to compare survival curves between nontransgenic sham and HF, HO-1 transgenic sham and HF, and nontransgenic and HO-1 transgenic HF. A value of P<0.05 was considered significant. Continuous data are summarized as mean±SD.

Results
HO-1 Is Upregulated in Murine Postinfarction HF
Ten-week-old C57BL/6 mice were evaluated 4 weeks after coronary ligation or sham operation. Figure IA in the online-only Data Supplement shows short-axis LV sections from a sham and failing heart and M-mode echocardiograms from a mouse at baseline and 4 weeks after myocardial infarction. In this example, a large infarct is present along the anterior and lateral aspects of the LV. Echocardiography revealed marked chamber remodeling with increased LV dimensions and reduced fractional shortening. As shown in Figure IB in the online-only Data Supplement, there was a 2-fold increase in cardiac HO-1 mRNA and protein levels in HF over sham groups, without changes in HO-1. HO-1 immunofluorescent staining from wild-type (WT) sham and HF hearts also indicated increased myocyte HO-1 expression in HF compared with sham groups (Figure IC in the online-only Data Supplement). For comparison, staining from nontransgenic and HO-1 transgenic hearts is also shown, indicating robust HO-1 expression in myocytes but not in the large vessels in HO-1 transgenic hearts.

HO-1 Overexpression Improves Postinfarction Survival and LV Remodeling
Baseline echocardiography confirmed the absence of a cardiac phenotype in HO-1 transgenic mice (Table I in the online-only Data Supplement). Kaplan–Meier survival curves after coronary ligation or sham operation in nontransgenic and HO-1 transgenic mice (Figure 1A) revealed that whereas mortality in nontransgenic HF was markedly higher than that in nontransgenic sham, myocyte-specific HO-1 overexpression imparted a survival benefit at 28 days after infarction. Figure 1B and 1C show short-axis LV sections, M-mode echocardiograms, and group data from nontransgenic and HO-1 transgenic sham and failing hearts. There was significant chamber dilatation (increased LV end-diastolic volume and LV end-systolic volume) and systolic dysfunction (reduced LV ejection fraction) in both HF groups over sham. However, in comparison with nontransgenic HF, LV dilatation and dysfunction were attenuated in HO-1 transgenic HF. Indeed, in the example in Figure 1B, this occurred despite larger overall infarct size in the HO-1 transgenic mouse. The LV/tibia length ratio showed a similar response pattern (Figure 1D), indicating less hypertrophy in HO-1 transgenic HF. Hemodynamic recordings (Figure 1F) and pressure-volume loops (Figure 1G) indicated improved contractility, LV dilatation, LV filling pressure, and diastolic function in HO-1 transgenic HF compared with nontransgenic HF, without appreciable differences between sham groups (Table). Notably, overall group infarct size was similar in nontransgenic and HO-1 transgenic HF (Figure 1E), suggesting that differences in remodeling and survival were independent of the degree of initial injury. Collectively, the data show that HO-1 overexpression alleviates postinfarction LV remodeling and improves mechanical performance.

HO-1 Overexpression Attenuates Hypertrophy, Fibrosis, and Oxidant Stress in the Failing Heart
Cell membrane staining with rhodamine wheat germ agglutinin revealed myofiber hypertrophy in both nontransgenic and HO-1 transgenic HF compared with sham-operated hearts (Figure 2A). The degree of hypertrophy, however, was attenuated in HO-1 transgenic HF hearts, confirming the gravimetric data (Figure 1D). To evaluate myocardial oxidative stress, we evaluated both protein-bound malondialdehyde by immunostaining and free malondialdehyde by GC-NICI-
Figure 1. Transgenic expression of HO-1 improves postinfarction LV remodeling. A, Kaplan–Meier curves after coronary ligation or sham operation in nontransgenic (NTG) and HO-1 transgenic (TG) mice. B and C, Short-axis LV sections and M-mode echocardiograms and group echocardiographic data from nontransgenic and HO-1 transgenic sham and HF hearts. D, LV/tibia length (TL) ratio from nontransgenic and HO-1 transgenic sham and HF hearts. E, Mean infarct size in nontransgenic and HO-1 transgenic HF hearts. F and G, Representative tracings of LV pressure and dP/dt max and pressure-volume loops measured in nontransgenic and HO-1 transgenic sham and HF hearts. AW indicates anterior wall; PW, posterior wall; EDD, end-diastolic dimension; ESD, end-systolic dimension; LVEDV, LV end-diastolic volume; LVESD, LV end-systolic volume; and LVEF, LV ejection fraction. *P<0.05 vs sham; #P<0.05 vs nontransgenic HF. Sample size is as noted in A.
HO-1 Suppresses Mitochondrial Respiration, MPT, and Cardiomyocyte Apoptosis in a CO-Dependent Manner

We next evaluated the effects of HO-1 on mitochondrial respiration, MPT, and cardiomyocyte apoptosis. In HO-1 transgenic hearts, robust levels of HO-1 were associated with the mitochondrial fraction (~40% of total), indicating physical proximity of HO-1 to the mitochondrial pore in these hearts but not in WT hearts (Figure 4A). Respiration studies were performed in freshly isolated mitochondria from nontransgenic and HO-1 transgenic hearts in the presence or absence of the CO donor CORM-3 (iCORM-3 control) or the CO scavenger hemoglobin. ADP to O ratios were similar among all experimental groups, ranging from 2.1±0.05 to 2.8±0.22, indicating a relatively constant relationship between ATP synthesis and oxygen consumption (data not shown). In contrast, the ratio of state 3 to state 4 respiration (respiratory control ratio) was suppressed in both HO-1 transgenic mitochondria as well as nontransgenic mitochondria incubated with CORM-3 but not inactive iCORM-3 (Figure 4B). Both HO-1 transgenic mitochondria and nontransgenic mitochondria treated with CORM-3 showed a general increase in absolute state 3 and state 4 respiration (Figure II in the online-only Data Supplement), although, overall, respiration was suppressed. The increase in CO did not change coupling because the ADP to O ratio was not affected (data not shown). Moreover, the suppressed state 3 to state 4 ratio in HO-1 transgenic mitochondria was normalized on incubation with the CO scavenger hemoglobin, indicating CO dependence of this effect. Ca2+-induced mitochondrial swelling assays were then performed to assess MPT (Figure 4C). Robust swelling was induced by Ca2+ in nontransgenic cardiac mitochondria; this was prevented by the MPT pore inhibitor cyclosporine A as well as by CORM-3 (but not iCORM-3). HO-1 transgenic cardiac mitochondria were resistant to swelling, a response that was reversed by coinubcation with hemoglobin. Nontransgenic mitochondria pre-treated with recombinant HO-1 protein were also less

**Table. LV Hemodynamics in Nontransgenic and HO-1 Transgenic Sham and HF Mice**

<table>
<thead>
<tr>
<th></th>
<th>Nontransgenic</th>
<th>HO-1 Transgenic</th>
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<tr>
<td>Heart rate, bpm</td>
<td>502±30</td>
<td>500±46</td>
</tr>
<tr>
<td>LVEDV, µL</td>
<td>25±7</td>
<td>30±9</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>98±17</td>
<td>93±11</td>
</tr>
<tr>
<td>dP/dt\text{max/IP}</td>
<td>8248±888</td>
<td>8230±2107</td>
</tr>
<tr>
<td>mm Hg/s</td>
<td></td>
<td>6215±777†</td>
</tr>
<tr>
<td>dP/dt\text{max/EDV}</td>
<td>169±56</td>
<td>159±32</td>
</tr>
<tr>
<td>mm Hg/s</td>
<td>361±125</td>
<td>292±81</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>6±5</td>
<td>6±4</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>5.6±2.1</td>
<td>6.4±1.8</td>
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Values are mean±SD. LVEDV indicates LV end-diastolic volume; LVSP, LV peak systolic pressure; dP/dt\text{max} maximal rate of change in LV pressure; dP/dt\text{max/IP}, dP/dt\text{max} normalized for instantaneous LV pressure; dP/dt\text{max/EDV}, dP/dt\text{max} normalized for end-diastolic volume; LVEDP, LV end-diastolic pressure; and tau, time constant of LV relaxation.

*p<0.05 vs respective sham; †P<0.05 vs nontransgenic HF.

MS. As seen by the immunostains in Figure 2B, malondialdehyde-modified proteins were increased in both HF groups over sham but significantly less so in HO-1 transgenic HF. Figure 2C shows representative GC chromatograms (left), the m/z values of ions 286 (benzaldehyde d5) and 204 (malondialdehyde) (center), and the GC-NICI-MS quantitative group data (right). HF in nontransgenic mice increased tissue malondialdehyde levels 2-fold over those of nontransgenic sham mice. In contrast, myocyte-specific HO-1 overexpression completely prevented the HF-associated increase in tissue malondialdehyde. These data indicate a potent antioxidant effect of HO-1 in the remodeling heart. Additionally, Masson’s trichrome staining (Figure 2D) revealed less collagen deposition in the remote myocardium of HO-1 transgenic HF hearts, suggesting an antifibrotic effect of HO-1.

**HO-1 Is Antiapoptotic and Promotes Neovascularization in the Failing Heart**

Figure 3A shows representative TUNEL stains (with troponin I costaining) from the remote and border zone of a nontransgenic HF heart, illustrating TUNEL-positive nuclei. Quantitative data demonstrated that nontransgenic and HO-1 transgenic HF groups exhibited significantly greater apoptosis of both myocytes and nonmyocytes (differentiated on the basis of attendant troponin I costaining) than their respective sham.

The apoptotic rate we observed in the failing heart (~0.5%) was both consistent with that reported for human HF (~0.12% to 0.70%) and physiologically significant because sustained apoptotic rates as low as 0.23% induce dilated cardiomyopathy in mice. However, HO-1 transgenic HF hearts exhibited a significant reduction in myocyte and nonmyocyte apoptotic rate versus nontransgenic HF. As a second measure of apoptosis, we determined myocardial levels of cleaved and uncleaved PARP protein. As seen in Figure 3B, the ratio of cleaved to uncleaved PARP increased in nontransgenic HF over sham (indicating enhanced apoptosis) but did not change appreciably between HO-1 transgenic sham and HO-1 transgenic HF, consistent with the TUNEL stains. Moreover, the protein level of p53, a central transcriptional activator of multiple proapoptotic genes, was increased 2-fold in nontransgenic HF. This increase was suppressed in HO-1 transgenic HF (with p53 levels ~50% lower than in nontransgenic mice) as well as in HO-1 transgenic sham hearts (Figure 3B). Because p53 also has prominent antiangiogenic effects, we also determined the effects of cardiac HO-1 overexpression on tissue neovascularization after infarction. As shown in Figure 3C, isolectin staining revealed that LV capillary density (excluding LV scar) was markedly decreased in nontransgenic HF in comparison with sham-operated hearts but was maintained in HO-1 transgenic HF. Moreover, among the HF groups, border zone capillary density was also significantly higher in HO-1 transgenic hearts. These results establish that HO-1 exerts important antiapoptotic effects and promotes neovascularization in the failing heart; potentially related in part to p53.
susceptible to Ca\(^{2+}\)-induced MPT (Figure III in the online-only Data Supplement).

We next measured \(\Delta\Psi\)m and MPT in living adult cardiomyocytes or H9c2 cells. WT or HO-1 transgenic cardiomyocytes were loaded with TMRM, and phenylarsine oxide (PAO) (20 \(\mu\)mol/L) was used to induce MPT. The time required for a 2-fold decrease in TMRM fluorescence was taken as an end point for MPT. TMRM mitochondrial labeling revealed the typical cardiomyocyte pattern of fluorescent bands oriented along the longitudinal axis of adult cardiomyocytes or a striking punctuate pattern in H9c2 cells (Figure IV in the online-only Data Supplement). In WT cardiomyocytes, PAO induced MPT within 15 minutes, as indicated by the decline in TMRM fluorescence (Figure 5A). The fluorescence decline was largely prevented by cyclosporine A, showing that the change was specifically a consequence of MPT. In comparison to WT myocytes, MPT induction was significantly delayed in HO-1 transgenic cardiomyocytes, analogous to the results of the mitochondrial swelling studies. Moreover, pretreatment of H9c2 cardiomyocytes with CORM-3, but not iCORM-3, attenuated PAO-induced MPT (Figure V in the online-only Data Supplement), and H9c2 cells transfected with HO-1 adenovirus, which augmented HO-1 colocalization with mitochondria (Figure VI in the online-only Data Supplement), were more resistant to \(\text{H}_2\text{O}_2\)-induced MPT, as indexed by TMRM labeling (Figure VII in the online-only Data Supplement). Taken together, these results suggest that HO-1, in large part via CO, inhibits both mitochondrial respiration and MPT in cardiomyocytes.

Figure 2. Constitutive expression of HO-1 attenuates hypertrophy, fibrosis, and oxidative stress in HF. A, Myocyte cell membrane staining with rhodamine wheat germ agglutinin. B, Immunostaining for malondialdehyde-adducted proteins in myocardium. C, Myocardial free malondialdehyde levels by GC-NICI-MS. The left panel shows representative gas chromatograms of the malondialdehyde spectrum from nontransgenic (NTG) sham (i), nontransgenic HF (ii), HO-1 transgenic (TG) sham (iii), and HO-1 TG HF (iv) hearts. The center panel shows typical spectra of ions with an m/z value of 204 (M\(^{-}\)-C\(_7\)H\(_2\)F\(_5\)-HFNO-C\(_2\)H\(_3\)) monitored for malondialdehyde (MDA) quantification in nontransgenic sham (v), nontransgenic HF (vi), HO-1 transgenic sham (vii), and HO-1 HF (viii) hearts. Benzaldehyde ring \(d_3\) ion with m/z value of 286 (M\(^{-}\)-HF) was used as an internal standard. The right panel shows the quantitative group data. D, Masson’s trichrome staining in HO-1 transgenic and nontransgenic sham and HF hearts. The black scale bar (magnification \(\times2\)) is 1 mm, and the white scale bar (magnification \(\times10\)) is 125 \(\mu\)m. *\(P<0.05\) vs sham; #\(P<0.05\) vs nontransgenic HF (n=4 to 6 per group).
Figure 3. HO-1 modulates apoptosis and neovascularization in the failing heart. A, Representative TUNEL stains from a nontransgenic sham and failing heart, and myocyte and nonmyocyte apoptosis quantification from nontransgenic (NTG) and HO-1 transgenic (TG) sham and HF hearts. Myocytes were identified by immunostaining with anti-troponin I antibody and Texas red–conjugated secondary antibody (red fluorescence), and nuclei were counterstained with DAPI (blue). TUNEL-positive nuclei appear green/cyan in the overlaid images. Bar=225 μm. B, Expression of cleaved and uncleaved PARP protein and the proapoptotic transcriptional activator p53 (Western blotting) in nontransgenic and HO-1 transgenic sham and HF hearts. C, Representative fluorescein isothiocyanate–conjugated isolectin staining of myocardial capillaries (green fluorescence) and quantification of capillary density from nontransgenic and HO-1 transgenic sham and HF hearts. *P<0.05 vs sham; #P<0.05 vs nontransgenic HF (n=4 to 9 per group).
Figure 4. HO-1 and CO suppress MPT and respiration. A, Western blots, developed with the use of anti-HO-1 antibody, of whole cell cardiac lysates and mitochondrial fractions. B, Top, Representative Clark electrode recordings from isolated cardiac mitochondria. State 4 respiration was measured after the addition of complex I respiratory substrates (10 mmol/L pyruvate and 5 mmol/L malate), and state 3 respiration was measured after the addition of 0.33 mmol/L ADP. Oxygen consumption was calculated from the slope of tracing as indicated. Bottom, Group data for state 3 to state 4 ratio in mitochondria isolated from nontransgenic (NTG) and HO-1 transgenic (TG) hearts with or without the indicated cotreatments (50 μmol/L CORM-3, iCORM, or hemoglobin [Hgb]). A.U. indicates arbitrary units. C, Ca²⁺-induced mitochondrial swelling (ie, decline in absorbance at 520 nm, A₅₂₀, an index of MPT) in cardiac mitochondria isolated from nontransgenic or HO-1 transgenic hearts with or without the indicated pretreatments: CORM-3, iCORM, hemoglobin (each at 50 μmol/L), and cyclosporine A (CsA) (30 mmol/L, an inhibitor of MTP pore opening). Samples were pretreated 5 minutes before Ca²⁺ was added. *P<0.05 vs nontransgenic; #P<0.05 vs HO-1 transgenic (n=4 to 7 per group).
Figure 5. HO-1 prevents MPT and apoptosis in cardiomyocytes in a CO-dependent manner. A, Mitochondrial membrane potential indexed by TMRM labeling (100 nmol/L for 30 minutes) in adult cardiomyocytes isolated from nontransgenic (NTG) or HO-1 transgenic (TG) hearts. PAO (20 μmol/L) was used to induce MPT, and cyclosporine A (CsA) (100 nmol/L, introduced 5 minutes before PAO) was used to inhibit MPT. The time needed for a 2-fold decrease in TMRM fluorescence was used to quantify the development of MPT. *P<0.05 vs nontransgenic; n=3 to 4 per condition. Bar=10 μm. B, Adult nontransgenic or HO-1 transgenic cardiomyocytes were exposed to 500 μmol/L H2O2 for 1 hour or media alone (control), with or without coincubation with hemoglobin (Hgb) (50 μmol/L) or desferoxamine (DFO) (100 μmol/L) starting 30 minutes before H2O2. Shown for each condition (clockwise from top left) are representative confocal images of TUNEL staining (green fluorescence 520 nm), nuclear staining with DAPI (blue fluorescence 460 nm), overlaid images (TUNEL-positive nuclei appear cyan), and phase contrast images. Quantification of the apoptotic rate is shown in the graph. P<0.005 vs nontransgenic control, #nontransgenic H2O2, $HO-1 transgenic H2O2, &HO-1 transgenic hemoglobin, @HO-1 transgenic desferoxamine; n=3 to 4 per group. C, Overall cell survival measured by MTT assay under similar conditions as in B. ^P<0.005 vs HO-1 transgenic control; n=3 to 4 per group.
We further evaluated whether the products of HO-1 catalysis had analogous protective effects on cardiomyocyte apoptosis and cell death. H$_2$O$_2$-induced apoptosis was evaluated by MTT assay, and cell death was evaluated by Propidium Iodide staining and TUNEL assay. H$_2$O$_2$ (100 μmol/L) induced robust apoptosis (Figure 5B) and cell death (Figure 5C) in WT myocytes but not in HO-1 transgenic myocytes. The resistance of HO-1 transgenic myocytes to H$_2$O$_2$-induced cell death was abrogated on cotreatment with hemoglobin and, to a lesser extent, with the iron chelator desferoxamine. However, the interpretation of the latter was confounded by the finding that desferoxamine alone also induced cell death (albeit to a lesser degree) in both WT and HO-1 transgenic myocytes. Taken together, analogous to its effect on MPT, HO-1 is antiapoptotic in cardiomyocytes, an effect in large part a consequence of its product CO (and perhaps ferrous iron). Collectively, these data suggest that CO-mediated prevention of MPT may be an important mechanism underlying the antiapoptotic effects of HO-1 in HF.

CORM-3 Treatment In Vivo Alleviates Postinfarction LV Remodeling

Having found that CO is a key cytoprotective mediator of the antiapoptotic effects of HO-1, we tested whether CORM-3 treatment would attenuate LV remodeling after myocardial infarction in vivo. C57BL/6 mice were subjected to coronary ligation and, starting 4 days later, were administered 40 mg/kg IP CORM-3 daily for 24 days, after which LV remodeling and function were assessed with echocardiography and tissue morphology. The biological activity of this dose of CORM-3 was tested by measuring blood carboxyhemoglobin. Before drug administration, carboxyhemoglobin in the blood was approximately 1%, whereas 2 to 6 hours after injection, carboxyhemoglobin increased to 5.9±0.6% and remained at this level for 24 hours, indicating long-lasting systemic delivery of CO. Figure 6A shows representative LV sections from a sham animal, a HF animal without CORM-3, and a HF animal with CORM-3 treatment. In comparison with untreated HF, the CORM-3-treated heart had less LV dilatation and remodeling. Group echocardiographic and gravimetric data 4 weeks after infarction revealed that sustained CORM-3 administration attenuated LV dilatation, improved systolic function, and reduced LV hypertrophy despite equivalent infarct size between untreated and treated groups (Figure 6B and 6C). Hemodynamic data also indicated better LV mechanical performance with CORM-3, with improvements in dP/dtmax normalized for instantaneous LV pressure, time constant of LV relaxation, and LV end-diastolic pressure over untreated HF. The CORM-3–mediated benefits occurred despite equivalent afterload in the treated and untreated groups (LV peak systolic pressure, 84±4 versus 84±10 mm Hg, untreated versus CORM-3–treated HF; P=NS) (Figure 6D). Moreover, as was the case with HO-1 transgenic mice, infarcted mice treated with CORM-3 also exhibited markedly reduced p53 expression (Figure 6E) and less myocardial apoptosis by TUNEL staining (Figure 6F) than untreated HF mice. Figure 6G depicts expression of Bax, a proapoptotic protein that can be induced by p53, and Bcl-2, an antiapoptotic protein that can be repressed by p53. Compared with untreated HF, CORM-3–treated HF hearts exhibited significantly augmented Bcl-2 levels and a trend (although not statistically significant) toward reduced Bax expression. These results are consistent with a p53-mediated effect in CORM-3–treated HF hearts and are also in agreement with the observed reduction in apoptotic rate. These data demonstrate that exogenous CO administration by CORM-3 alleviates postinfarction LV remodeling and apoptosis and that, in the failing heart, exogenous CO augments the cardioprotective effects of HO-1 upregulation.

Discussion

In this study, we show for the first time that HO-1 induction in the failing heart is a cardioprotective adaptation that alleviates postinfarction pathological LV remodeling, an effect mediated in part by CO-dependent antiapoptotic actions that are associated with, and likely due to, prevention of MPT. Several lines of evidence support these conclusions. First, HO-1 expression was upregulated 2-fold in chronically remodeled failing myocardium remote from the infarct and well after the formation of a stable scar. Second, HO-1 overexpression and gain of function in the heart ameliorates LV dilatation and dysfunction, hypertrophy, interstitial fibrosis, and oxidative stress and improved tissue neovascularization. These results indicate both a cardio-protective (anti-hypertrophic, antioxidant, anti-fibrotic, and proangiogenic) role of HO-1 in HF and a potential therapeutic effect of enhancing HO-1 function over and above the 2-fold upregulation seen in HF. Third, our observations in HO-1 transgenic mice establish an in vivo antiapoptotic effect of HO-1 in the failing heart that mitigates cell loss, p53 expression, and pathological remodeling. Fourth, in isolated mitochondria and cardiomyocytes, we found that HO-1, primarily via CO, suppresses the mitochondrial respiratory control ratio and MPT and inhibits apoptosis. Finally, sustained CO donor treatment in vivo alleviated postinfarction remodeling and attenuated apoptosis in a manner similar to HO-1 overexpression, further underscoring the potential utility of enhancing the HO-1 axis in chronic HF. Taken together, these findings indicate that augmentation of HO-1 and/or its product, CO, may represent a novel and beneficial therapeutic approach in HF.

HO-1 Upregulation Is a Cardioprotective Adaptation in the Failing Heart

The cytoprotective effects of HO-1 are mediated by its product CO (which is generated exclusively by the HO system), the production of the antioxidants biliverdin and bilirubin, and the degradation of excess amounts of the pro-oxidant heme. Previous studies have shown cardioprotective actions of HO-1 in models of acute stress (eg, ischemia/reperfusion injury). However, the role of HO-1 in chronic cardiac remodeling and HF is virtually unknown. Although prior studies have reported HO-1 upregulation in remodeled myocardium resulting from mechanical overload and neurohormonal stimulation, as well as in experimental and human HF, its significance is poorly understood. Specifically, no information is available on whether HO-1 is an important modulator of pathological remodeling and, if so,
Figure 6. CORM-3 treatment alleviates postinfarction LV remodeling. A, Representative LV sections from a sham mouse, a control HF mouse, and a HF mouse given CORM-3 40 mg/kg per day IP for 24 days starting 4 days after ligation. B, Infarct size in untreated and CORM-3–treated HF hearts. C, Echocardiographic and gravimetric assessment of LV dilatation (LV end-diastolic volume [LVEDV] and LV end-systolic volume [LVESV]), LV systolic function (LV ejection fraction [LVEF]), and LV hypertrophy (LV/tibia length). D, Mechanical assessment of LV systolic function (dP/dt max normalized for instantaneous LV pressure [dP/dt max/IP]) and diastolic performance (LV end-diastolic pressure [LVEDP] and time constant of LV relaxation [tau]) in untreated and CORM-3–treated HF hearts. E, Western blotting for p53. F, Quantification of apoptotic rate by TUNEL staining. G, Western blotting for Bax and Bcl-2 in sham, untreated HF, and CORM-3–treated HF hearts. *P<0.05 vs sham; #P<0.05 vs untreated HF (n=6 to 12 per group).
which mechanisms underlie such an effect. These questions are particularly important because chronic upregulation of a compensatory system may not necessarily induce the same effects seen during the acute stress response (for example, proinflammatory cytokines are acutely protective but detrimental over the long term); hence, the short-term actions of HO-1 cannot necessarily be extrapolated to a chronic setting. Moreover, the same catalytic products that are responsible for HO-1-mediated short-term cardioprotection (bilirubin, free iron, or CO) can potentially induce toxicity at inordinately high (or inappropriately sustained) levels.

Our results establish that the upregulation of HO-1 in the failing heart ameliorates detrimental remodeling. Using genetically engineered mice, we found that sustained HO-1 expression in failing myocardium promotes neovascularization and limits oxidative stress, myofiber hypertrophy, interstitial fibrosis, apoptosis, and p53 expression. These responses were associated with improved chamber remodeling and systolic and diastolic function. The central importance of HO-1 in HF is highlighted by its ability to favorably modulate oxidative stress, neovascularization, and apoptosis, 3 key events that are known to independently influence remodeling. Our studies in HO-1 transgenic mice suggest that further enhancement of HO-1 activity beyond the level that is normally achieved in the failing heart may be a novel therapeutic strategy to prevent progression of disease. One important, although not exclusive, mechanism of these beneficial effects appears to be the moderation of apoptosis.

**HO-1 Modulation of Apoptosis and Role of CO**

Although HO-1 is known to inhibit apoptosis,1–3 the precise mechanisms by which this occurs remain obscure. Apoptosis in the failing heart involves multiple mechanisms that produce heightened activity of extrinsic death-receptor pathways or intrinsic mitochondrial and/or endoplasmic reticulum–linked pathways.31 Emerging evidence supports an important role for mitochondria as arbiters of cell fate in the failing heart. Intrinsic pathways converge on the mitochondria to induce mitochondrial remodeling and dysfunction, which in turn leads to the release of apoptogenic proteins such as cytochrome c into the cytosol and the activation of terminal caspase cascades.31 A central event that is thought to trigger mitochondrial dysfunction and cytochrome c release is MPT, with subsequent opening of the mitochondrial pore and mitochondrial swelling.31,32 Although in early stages, brief MPT could be protective, prolonged opening of the pore is usually considered detrimental because it triggers both necrosis and apoptosis.32 Indeed, MPT contributes to the loss of mitochondrial function observed in the failing heart,31 and LV remodeling is associated with an increase in mitochondrial pore opening in hearts both 12 and 18 weeks after coronary ligation.34 In addition, cyclosporine A, a potent blocker of MPT, improves mitochondrial function in failing cardiomyocytes.33

A key finding of our study is that HO-1 reduces the respiratory control ratio in cardiac mitochondria and confers increased resistance to prolonged MPT triggered by a variety of inducers including PAO, calcium overload, and oxidative stress (H₂O₂), suggesting that reduced respiration/phosphorylation coupling and improved mitochondrial membrane stability in the face of cellular stress may underlie the cardioprotective and antiapoptotic effects of HO-1 in HF. HO-1 also produced greater resistance to apoptosis and cell death in cardiomyocytes, akin to the in vivo findings in HO-1 transgenic HF. Importantly, the beneficial effects of HO-1 on respiration, MPT, and apoptosis were associated with physical approximation of HO-1 with the mitochondria and were prevented on scavenging of CO but recapitulated by CORM-3, indicating that CO plays a primary role in the modulation of mitochondrial function and apoptosis. The in vivo observations that cardiac myocyte–restricted overexpression of HO-1 also reduced nonmyocyte apoptosis in HO-1 transgenic HF further support the notion that a small, freely diffusible molecule such as CO mediates these effects. Moreover, our results in cardiomyocytes are consistent with previous studies demonstrating that CO inhibits p53 expression and mitochondrial cytochrome c release in vascular smooth muscle cells exposed to proinflammatory cytokines,35 suppresses free radical production in activated macrophages,36 and protects PC12 cells from peroxynitrite-induced apoptosis by preventing the depolarization of mitochondrial transmembrane potential.37 It is possible that HO-1–mediated inhibition of apoptosis may be related to other mechanisms in addition to CO because iron chelation with desferoxamine also mitigated the antiapoptotic effect of HO-1 to some degree. Regardless, our observations indicate that CO–dependent inhibition of MPT, attendant mitochondrial remodeling, and apoptosis can contribute to the potent antiapoptotic effect of HO-1 in the failing heart.

If CO-mediated MPT prevention is primarily responsible for the antiapoptotic and cardioprotective effect of HO-1 in HF, then bypassing the HO-1 system with pharmacological delivery of CO should achieve similar benefits. Our studies with CORM-3 support this concept because sustained delivery of exogenous CO (CORM-3) recapitulated the effects of HO-1 transgenesis on pathological LV remodeling and dysfunction, apoptosis, and p53 expression. Importantly, the CORM-3–induced remodeling responses occurred without appreciable changes in systolic pressure, indicating a direct effect of CO on the myocardium rather than an indirect effect due to changes in afterload. Many prior studies of CO–mediated effects in vivo have used CO inhalation as a means of delivery, an approach that is quantitatively difficult to standardize with regard to dosing and therefore prone to potential toxicity. Moreover, CO inhalation exerts many nonspecific and potentially untoward effects. For these reasons, we used CORM-3, a carrier of CO that releases CO in a predictable and stable manner.25 The dose that we used increased carboxyhemoglobin to ~6% long term and was identical to that used by Motterlini and colleagues25 in a murine cardiac transplant rejection model. Further studies will be required to optimize the dose of CORM-3 for sustained CO delivery to the heart and to comprehensively determine the mechanisms underlying CORM-3–mediated improvements in LV remodeling. Nevertheless, our present findings suggest that pharmacological CO delivery may be of potential therapeutic value in HF and that feasibility of this approach should be explored in the clinical setting.
In summary, the studies reported herein reveal a novel pathophysiological role of HO-1 that was heretofore unrecognized. Our results establish that sustained HO-1 upregulation in the failing heart is an important beneficial adaptation that serves to counteract detrimental LV remodeling via antioxidant, anti hypertrophic, antifibrotic, and proangiogenic effects. We have also identified an in vivo antiapoptotic action of HO-1 in the failing heart that mitigates progressive cell loss. Our in vitro analyses support the concept that this effect is related, at least in part, to CO-mediated stabilization of mitochondrial pore opening. Consistent with a central role for CO in HO-1–dependent long-term cardioprotection, exogenous CO delivery in vivo with the use of CORM-3 also improved postinfarction LV remodeling and dysfunction and reduced myocardial apoptosis. These findings have both conceptual and practical implications. From a conceptual standpoint, they reveal a new facet of the pathophysiology of HF, that is, the cardioprotective role of HO-1. From a practical standpoint, augmentation of the HO-1 axis and/or ambient CO levels should be explored as a therapeutic approach to limit pathological LV remodeling in HF.

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Disclosures
None.

References


33. Sharov VG, Todd A, Khanal S, Imai M, Sabbah HN. Cyclopamine A attenuates mitochondrial permeability transition and improves mito-
Heme oxygenase-1 (HO-1), which degrades heme to biliverdin, ferrous iron, and carbon monoxide (CO), is rapidly inducible and cardioprotective during acute stress. However, its pathophysiological role in chronic heart failure (HF) is unknown. Using myocyte-restricted HO-1 transgenic mice, we evaluated whether HO-1 upregulation in the failing heart is a beneficial adaptation that alleviates pathological remodeling. Nontransgenic and HO-1 transgenic mice underwent either sham operation or permanent left coronary ligation to induce HF. After 4 weeks, compared with nontransgenic mice with HF, HO-1 transgenic mice with HF exhibited improved postinfarction survival and significantly less left ventricular dilatation and dysfunction, cardiac hypertrophy, fibrosis, and oxidative stress, together with improved tissue neovascularization and reduced myocardial p53 expression and apoptosis. In isolated mitochondria, mitochondrial permeability transition was inhibited by HO-1 in a CO-dependent manner and was recapitulated by the CO donor tricarbonylchloro-glycinato)ruthenium(II) (CORM-3). HO-1–derived CO also prevented H2O2-induced cardiomyocyte apoptosis and cell death. Finally, sustained in vivo treatment with CORM-3 alleviated postinfarction left ventricular remodeling, p53 expression, and apoptosis in wild-type mice. Our results establish that sustained HO-1 upregulation in HF is an important beneficial adaptation that serves to counteract detrimental left ventricular remodeling via antioxidant, antihypertrophic, antifibrotic, and proangiogenic effects. We have also identified an in vivo antiapoptotic action of HO-1 in the failing heart that is related, at least in part, to CO-mediated stabilization of mitochondrial pore opening. Therefore, augmentation of HO-1 or its product, CO, should be explored as a therapeutic approach to limit pathological left ventricular remodeling and myocyte loss in HF.
Cardioprotective and Antiapoptotic Effects of Heme Oxygenase-1 in the Failing Heart

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EXPANDED METHODS

All studies were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85-23, revised 1996).

Mouse models. Male mice (10-16 weeks of age, weighing 25-30 g) were used. Cardiac-specific HO-1 transgenic (TG) mice were obtained from Dr. Shaw-Fang Yet (Harvard Medical School). These mice have been well-characterized previously [1]. We used HO-1 TG mice expressing 4 copies of a human HO-1 transgene under the control of the α-MyHC promoter [1]. Originally bred on a FVB background, HO-1 TG mice lines were backcrossed for a minimum of 7 generations into C57BL/6. NTG littermates were used as controls. For in vivo CORM-3 supplementation studies, C57BL/6 mice were used.

Coronary ligation. Permanent coronary ligation was performed as previously described [2]. After induction of anesthesia with tribromoethanol (0.25 mg/g i.p.), mice were intubated and supported with a MiniVent Mouse Ventilator (Type 845, Harvard Apparatus) at 125-150 breaths/minute depending on body weight (tidal volume 6.4 μL/g, PEEP 5-7 cm H2O). Anesthesia was maintained with 1% isoflurane. Heat lamps and heating pads were used to maintain body temperature at 37°C. Under sterile conditions, a left thoracotomy was performed in the 4th intercostal space, the heart exposed, and the pericardium opened. An 8.0 prolene ligature was passed and tied around the proximal left coronary artery, 1 mm distal to the left atrial appendage border. Successful occlusion was confirmed by the production of pallor and dyskinesia in the distal myocardium. In sham animals, the suture was passed but not tied. The chest was then closed in layers using 5.0 silk, and the mice were allowed to recover and followed for 4 weeks.

Echocardiography. Under tribromoethanol sedation (0.25 mg/g i.p.), echocardiography (M-mode, 2D, and Doppler) was performed at baseline and 4 weeks post-operatively using a Philips Sonos 5500, 15 MHz linear array transducer, and 120 Hz frame rate. Measured parameters included the short-axis end-diastolic (ED) and end-systolic (ES) diameter (D) and
wall thickness (WT), and long-axis end-diastolic and end-systolic volume (EDV and ESV) using the modified Simpson’s method. LV systolic function was indexed by single plane planimetered LV ejection fraction (EF = EDV-ESV/EDV).

**LV pressure measurement.** In a subset of animals, LV catheterization was performed 4 weeks after coronary ligation or sham operation as previously described [3]. Mice were anesthetized with tribromoethanol (0.25 mg/g i.p.), intubated and ventilated with anesthesia maintained using 1% isoflurane. Body temperature was maintained at 37°C using a heating pad and heat lamps. The left jugular vein was cannulated for fluid/drug administration. A Millar 1.4 Fr conductance catheter (Model SPR-719) was inserted via the right carotid artery into the ascending aorta and subsequently advanced retrogradely into the LV for pressure measurement using a Powerlab/Chart system (ADInstruments). Correct catheter positioning was confirmed by on-line visualization of the pressure and conductance signals. LV pressure (P) and conductance were A:D converted at 500 Hz and recorded at steady-state after equilibration for at least 10-15 minutes. At the end of the study, IV hypertonic saline (0.5-1 μL/g) was given to determine parallel conductance. LV volume (μL) was derived from the measured conductance, parallel conductance, and *ex vivo* cuvette calibration with heparinized, warm blood. Systolic function was indexed by dP/dt_{max}, and dP/dt_{max} normalized for either instantaneous LVP (IP) or EDV as relatively load-independent indices. Diastolic function was assessed by LV end-diastolic pressure (LVEDP) and tau, the time constant of LV relaxation, as determined from the regression of dP/dt versus LVP.

**Tissue harvest.** Following the final echocardiographic or hemodynamic study, mice were given additional anesthesia with sodium pentobarbital (50 mg/kg i.p.). The heart was arrested in diastole with IV KCl and rapidly excised and rinsed in ice-cold physiological saline. The ventricles were dissected and weighed separately. A short-axis slice of the LV was fixed in formalin for 16 h, dehydrated in ethanol, and paraffin-embedded for subsequent histological
studies. The remaining LV tissue was separated into infarcted (scar) and non-infarcted regions, snap-frozen in liquid nitrogen, and stored at -80°C for biochemical and molecular studies. Unless otherwise specified, non-infarcted tissue was used for molecular analyses.

**Isolation of mouse cardiomyocytes.** Mice were deeply anesthetized with pentobarbital (80 mg/kg IM) and given heparin 10 U/g i.p. The heart was rapidly excised, and Ca^{2+}-tolerant mouse ventricular myocytes were isolated by modified Langendorff perfusion and collagenase digestion as previously described [3]. Cell viability was typically 75-80%, as assessed by trypan blue exclusion. Cells were maintained at a density of 10^4 rod-shaped cells/cm^2 in serum-free supplemented DMEM medium (with albumin 0.2%, L-carnitine 2mM, creatine 5mM, taurine 5mM, L-glutamine 1.3 mM, insulin 0.1 mM, triiodothyronine 0.1 nM, pyruvate 2.5 mM, BDM 10 mM, and penicillin/streptomycin 0.1%) at 37°C in 5% CO_2 until experimentation.

**MTT assay.** Myocyte cell death was determined using the MTT assay. Myocytes were incubated with 500 µM H_2O_2 for 1 h. Some groups were pre-incubated and co-incubated with 50 µM hemoglobin (Hgb, CO scavenger) or 100 µM desferoxamine (DFO, iron chelator) starting 30 minutes before H_2O_2. MTT solution (5 mg/ml) was added to the media 30 minutes before the end of incubation. The cells were centrifuged for 5 minutes and the media removed. Any crystals were dissolved by adding 200 µL DMSO and pipette mixing, and the plate was incubated at 37°C for 5 minutes to dissolve air bubbles. Absorbance was read at 550 nm. Cell viability was calculated as the percent ratio of absorbance of the sample to the reference control.

**Culture of H9c2 cardiomyocytes and HO-1 transfection.** H9c2 cells (rat embryonic cardiomyoblasts) were obtained from ATCC and cultured in DMEM (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin for 7-10 passages. For transfection studies, Ad5/HO-1, the replication-deficient adenoviral vector containing the entire coding region of rat HO-1 cDNA, was generated in 293 cells by homologous recombination of pCMV/HO-1 and pJM17, a circularized adenovirus genome lacking its E1 region and a portion of the E3 region. Plaque-
isolated viral clones were propagated in 293 cells and then purified over two CsCl gradients and titered by plaque assay as previously described [4]. Ad5/LacZ with the same adenoviral backbone containing the β-galactosidase gene driven by CMV promoter was used as a control vector. For transient HO-1 transfections, cells were seeded in 100 mm tissue culture dishes and transfected with Ad5/HO-1 or Ad5/LacZ for 45 min at an MOI of 10 for 72 h prior to treatments.

**Histomorphometry, immunohistochemistry, and apoptosis assessment.** Paraffin-embedded tissue sections (5 μm) were de-paraffinized, rehydrated and stained with Masson Trichrome (for collagen) using standard histologic techniques [2]. To determine myocyte cross-sectional area, rhodamine-conjugated wheat germ agglutinin (Molecular Probes) staining was performed at 1:1000 dilution for 45 min at room temperature (RT). The sections were then rinsed with PBS and mounted with SlowFade Gold antifade reagent with DAPI (Molecular Probes). Myocyte area (average of ~100 cross sectional cells with centrally located round nuclei) and the degree of tissue fibrosis (blue-green stain) were assessed using Metamorph Imaging Software. Oxidative stress was indexed by immunostaining for malondialdehyde (MDA)-adducted proteins as previously described, using IgG-purified anti-MDA primary antibody (Academy Bio-Med) [5]. For quantitation of protein-MDA adducts by immunostaining, 4-6 mice per group were used. For each heart, using the short axis section of the LV, digital images were acquired from six different fields at standard distance intervals in the remote zone and analyzed by MetaMorph 4.5 image quantitation software. The threshold for protein-MDA adduct staining was predetermined, and held constant for all sections analyzed. For immunohistological analyses, we typically analyzed 6 fields/heart with an area of 66 mm²/field (total area 396 mm²/heart).

For HO-1 immunofluorescent staining, tissue sections were blocked in PBS containing 0.1% Triton X-100, 7% goat serum, and 5% nonfat dry milk for 10 min at 37°C and then again with mouse IgG Blocking Reagent (FMK-2201, Vector Laboratories) for an additional 1 h at RT. Anti-mouse HO-1 antibody (Stressgen) was used as the primary antibody at a 1:100 dilution,
and TRITC-conjugated goat anti-mouse IgG was used as the secondary antibody at 1:200 dilution. Negative controls were performed by omission of primary antibody. Isolectin staining was performed to identify capillaries; FITC-conjugated isolectin B4 (Vector Labs) was used as primary antibody without secondary antibody. Apoptosis in tissue and cells was determined using the DeadEnd Fluorometric TUNEL System (Promega), which catalytically incorporates fluorescein-12-dUTP at DNA strand breaks. Tissue sections were further incubated with antitroponin I antibody (Santa Cruz Biotechnology) 1:1000 dilution and Texas Red-conjugated secondary antibody at 1:10,000 (Molecular Probes) to identify cardiomyocytes. All sections were counterstained with DAPI (Molecular Probes) at a final concentration of 2 µM. Optical sections were obtained with a Zeiss LSM510 inverted confocal scanning laser microscope equipped with Enterprise/argon/HeNe lasers and excitation wavelengths appropriate for multi-channel scanning in the individual tracks to allow co-localization. Images were recorded within 24 h and analyzed with Adobe PhotoShop 5.5 software.

**HO-1 gene expression.** Total RNA was isolated from cardiac tissue as previously described [2,5]. HO-1 mRNA was quantified and normalized to mouse beta-actin mRNA by real-time PCR with LUX gene-specific primers (Invitrogen) after reverse transcription (Superscript III, Invitrogen) of 250 ng total RNA purified from sham and failing hearts (TRIzol method). The forward labeled primer was CACGATCCAAGTTCAAACAGCTCTATCG, and reverse unlabeled primer was CTGTCACCCTGTGCTTGACCTC.

**Western immunoblotting.** Protein extraction, Western immunoblotting, and densitometry were performed as previously described [2,3,5]. Protein (50 µg) was denatured with SDS sample buffer (2% SDS, 10% glycerol, 5 mM DTT in Tris buffer, pH 6.8) at 90 ºC for 5 min and separated on 12% polyacrylamide gel with a 4% stacking gel in SDS-Tris Glycine running buffer. The proteins then were electro-transferred to a PVDF membrane using a Bio-Rad Mini Trans-Blot Cell in Tris-glycine-methanol buffer at 350 mA for 90 min. Immunodetection then followed the Amersham ECL Western Blotting Protocols. Primary antibodies used included
anti-p53, anti-poly-ADP ribose polymerase (PARP), anti-Bax, anti-Bcl-2, anti-β-actin, and anti-α-tubulin from Santa Cruz Biotechnology, and anti-HO-1 from StressGen.

**Measurement of free MDA by gas chromatography-negative ionization chemical ionization-mass spectrometry (GC-NICI-MS).** Tissue MDA concentration was measured by GC-NICI-MS as previously described [6]. Heart tissue homogenate was derivatized with pentfluorobenzylhydroxylamine (PFBHA) and the PFB-oxime derivatives of MDA were measured by GC-NICI-MS using select ion monitoring mode. Benzaldehyde ring $d_5$ was used as the internal standard. The following ions were monitored for the indicated aldehyde: benzaldehyde $d_5$ - m/z 286 (M$^+$-HF) and MDA m/z 204 (M$^+$-C$_7$H$_2$F$_5$-HFNO-C$_2$H$_3$).

**Mitochondrial membrane permeability transition (MPT).** In adult cardiomyocytes and H9c2 cells, changes in mitochondrial membrane potential ($\Delta \psi_m$) were assessed with tetramethylrhodamine methyl ester (TMRM). TMRM fluorescence was measured at 5-minute intervals for 30 minutes in live cells using a 585-nm long pass filter and laser-scanning confocal microscopy (LSM 510). TMRM is a cationic dye that accumulates in mitochondria in proportion to $\Delta \psi_m$. The TMRM concentration chosen (100 nM) does not suppress mitochondrial respiration [7] and is nonquenching so that mitochondrial depolarization is accompanied by a decrease in cellular TMRM fluorescence over time followed by cell shortening due to ATP depletion [8]. The time required to cause at least a 2-fold decrease in TMRM fluorescence was taken as an endpoint for the development of MPT.

**Mitochondrial isolation.** Mitochondria were isolated from adult mouse hearts by differential centrifugation as described previously [9]. Isolated mitochondria were resuspended in EGTA-free homogenization buffer (250 mM sucrose, 10 mM HEPES, pH 7.4 with Tris-HCl) to yield 3–5 mg/ml of mitochondrial protein. Mitochondria were kept on ice and used within 4 h.

**Mitochondrial MPT pore opening.** MPT pore (MPTP) opening in isolated cardiac mitochondria was induced by Ca$^{2+}$ as previously described [9]. Isolated cardiac mitochondria
were resuspended in swelling buffer (containing in mmol/L: 120 KCl, 10 Tris·HCl (pH 7.4), 20 MOPS, and 5 KH₂PO₄) to a final protein concentration of 0.25 mg/ml. Mitochondrial swelling induced by pore opening was measured spectrophotometrically as a reduction in absorbance at 520 nm (A₅₂₀).

**Mitochondrial respiration.** Freshly isolated mitochondria were resuspended in respiration buffer (pH 7.2) containing in mmol/L: 225 mannitol, 70 sucrose, 10 KH₂PO₄, 1 EGTA. A Clark electrode was used to measure the oxygen content of mitochondrial suspension with 10 mM pyruvate and 5 mM malate. State 4 respiration was measured at baseline. 330μM ADP was added to stimulate state 3 respiration. The respiratory control ratio (RCR) was defined as the ratio of state 3:state 4 respiration. The ADP:O ratio was calculated using total oxygen consumption during state 3 respiration.

**Preparation of the CO-donor tricarbonylchloro(glycinato)ruthenium(II) (CORM-3).** CORM-3, a water-soluble transition metal carbonyl drug that stably releases CO, was synthesized following a published protocol [10]. The structure of CORM-3 was confirmed by infrared spectroscopy and NMR. To detect CO release, 25 μM CORM-3 was added to PBS containing myoglobin (50 μM) and the shift in the absorbance spectrum of deoxymyoglobin to carbonmonoxy myoglobin was recorded. The synthesized drug was stored at -20°C and fresh solution was made just before use. For control experiments, CORM-3 was inactivated by dissolving it in Krebs-Henseleit buffer and allowing CO liberation overnight at RT.

**Statistical analysis.** Several statistical techniques were employed. For two-group comparisons, we used the unpaired two sample t test. For comparisons of more than two groups, we used one-way ANOVA if there was one independent variable, two-way ANOVA if there were two independent variables (e.g., genotype and ligation status), and two-way repeated measures ANOVA for matched observations over time with two independent variables. To adjust for multiple comparisons, we performed Student-Newman-Keuls post-test for one-way
ANOVA and Bonferroni post-test for two-way ANOVA. Pair-wise comparisons were made between sham groups across genotypes, sham versus HF within each genotype, and HF groups across genotypes. Animal survival was evaluated by the Kaplan-Meier method, and the log-rank test was used to compare survival curves between NTG sham and HF, HO-1 TG sham and HF, and NTG and HO-1 TG HF. A p value of < 0.05 was considered significant. Continuous data are summarized as mean ± SD.

REFERENCES


### Supplemental Table 1. Baseline Echocardiography in WT and HO-1 TG mice

<table>
<thead>
<tr>
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<th>NTG mice</th>
<th>HO-1 TG mice</th>
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<tbody>
<tr>
<td>HR (bpm)</td>
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<td>542 ± 55</td>
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<td>LVEDD (mm)</td>
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<td>27.8 ± 2.3</td>
<td>26.0 ± 2.2</td>
</tr>
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</table>

HR, heart rate; LV, left ventricular; EDD and ESD, end-diastolic and end-systolic diameter; FS, fractional shortening; EDV and ESV, end-diastolic and end-systolic volume; EF, ejection fraction; AWT, PWT, and RWT, anterior, posterior, and relative wall thickness. All values mean ± SD. n = 10 per group.
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. HO-1 is upregulated in the failing heart. A, Representative mouse LV sections 4 weeks after sham operation or coronary ligation and myocardial infarction (MI) and M-mode echocardiograms at baseline and 4 weeks post-MI. Large infarction is demonstrated with marked remodeling and systolic dysfunction. B, HO-1 mRNA analysis (real-time PCR) and HO-1 and HO-2 protein analysis (Western blotting) showing two-fold upregulation of HO-1 in heart failure (HF) (n = 4-6/group). C, Representative HO-1 immunostaining in sham and HF hearts using anti-HO-1 antibody and TRITC-conjugated secondary antibody (red immunofluorescence) indicating increased myocyte expression of HO-1 in HF (scale bar 20 µm). Examples of immunostaining from non-transgenic (NTG) and HO-1 transgenic (TG) hearts are shown for comparison (scale bar 50 µm).

Figure S2. Group data for absolute values for state 3 and state 4 respiration measured in mitochondria isolated from NTG and HO-1-TG hearts. Recordings in NTG mitochondria were performed in the absence or presence of co-treatment with 50 µmol/L CORM-3. *p < 0.05 vs. NTG; n = 3 per group.

Figure S3. Ca^{2+}-induced mitochondrial swelling (i.e., decline in A_{520}, an index of MPT) in mitochondria isolated from non-transgenic (NTG) hearts treated with or without cyclosporine A (CsA, 30 nM, 5 minutes before Ca^{2+} was added) or recombinant HO-1 protein (rHO-1, 2 µg given 15 min before Ca^{2+}).

Figure S4. Representative high magnification confocal images of TMRM-loaded adult cardiomyocyte (Left) and H9c2 cells (Right). Note clearly delineated punctuate mitochondrial staining at baseline in H9c2 cells and the typical linear organization of mitochondria along the longitudinal axis in the adult cardiomyocyte example.

Figure S5. TMRM fluorescence in H9c2 cells 30 min after exposure to PAO alone (control) or after pre-treatment with CsA (100 nM, given 5 min prior to PAO), CORM-3 or
iCORM-3 (50 μM, given 30 min prior to PAO). The panels are representative of 3-4 experiments per condition. The scale bar is 10 μm.

**Figure S6.** H9c2 cells were incubated with Ad5/HO-1 or Ad5/LacZ (control) for 45 min at an MOI of 10; 72 h later, cells were loaded with Cationic Dye to localize mitochondria (MITO, red fluorescence) 30 min prior to fixation and immunostaining with anti-HO-1 antibody and FITC-conjugated secondary antibody (green fluorescence). Nuclei were counterstained with DAPI (blue). The panels are representative of 3-4 experiments per condition. The scale bar is 20 μm.

**Figure S7.** Ad5/HO-1 and Ad5/LacZ transfected H9c2 cells were labeled with TMRM as above and then exposed to H$_2$O$_2$ (100 μM) for 60 min and observed under confocal microscopy. The top and bottom panels are low magnification (scale bar 20 μm) and high magnification (scale bar 5 μm), respectively. The panels are representative of 3-4 experiments per condition.
Figure S1
Figure S2
Figure S3

A5200.05
1 min
NTG + CsA
NTG + rHO-1
NTG
n = 3/group

Ca^{2+} 200 \mu M

\begin{align*}
\text{A}_{520} & \quad 0.05 \\
\text{1 min} & \\
n & = 3/\text{group}
\end{align*}
Figure S4

TMRM LABELING

Adult Cardiomyocyte

H9c2 Cells
Figure S7