Cardiac-Specific Expression of Heme Oxygenase-1 Protects Against Ischemia and Reperfusion Injury in Transgenic Mice

Shaw-Fang Yet, Rong Tian, Matthew D. Layne, Zhi Yuan Wang, Koji Maemura, Maria Solovyeva, Bonna Ith, Luis G. Melo, Lunan Zhang, Joanne S. Ingwall, Victor J. Dzau, Mu-En Lee,† Mark A. Perrella

Abstract—Heme oxygenase (HO)-1 degrades the pro-oxidant heme and generates carbon monoxide and antioxidant bilirubin. We have previously shown that in response to hypoxia, HO-1–null mice develop infarcts in the right ventricle of their hearts and that their cardiomyocytes are damaged by oxidative stress. To test whether HO-1 protects against oxidative injury in the heart, we generated cardiac-specific transgenic mice overexpressing different levels of HO-1. By use of a Langendorff preparation, hearts from transgenic mice showed improved recovery of contractile performance during reperfusion after ischemia in an HO-1 dose–dependent manner. In vivo, myocardial ischemia and reperfusion experiments showed that infarct size was only 14.7% of the area at risk in transgenic mice compared with 56.5% in wild-type mice. Hearts from these transgenic animals had reduced inflammatory cell infiltration and oxidative damage. Our data demonstrate that overexpression of HO-1 in the cardiomyocyte protects against ischemia and reperfusion injury, thus improving the recovery of cardiac function. (Circ Res. 2001;89:168-173.)

Key Words: heart • infarction • Langendorff preparation • cytoprotection • inflammation

O xidative stress in the heart caused by ischemia and reperfusion leads to cardiomyocyte death.1–3 Several studies have shown that increased expression of myocardial stress proteins and/or antioxidant enzymes protects against postischemic injury.4–6 In response to stress, elevated expression of heat shock proteins may protect the myocardium.7 These heat shock proteins are thought to mediate cardioprotection through their biological functions as molecular chaperones by preventing protein denaturation.7 Heme oxygenase (HO)-1, a stress response and cytoprotective protein, also known as hsp32, protects cells from death due to pathophysiological stress.8–12 By degrading the pro-oxidant heme and generating the antioxidant bilirubin,13,14 HO-1 may protect cells against oxidative injury. In addition, carbon monoxide (CO), another HO-1 reaction product, contributes to the regulation of vascular tone and is reported to have anti-inflammatory properties, which may contribute to the cytoprotective action of HO-1.15,16

HO-1 is upregulated in the heart and blood vessels in response to hemodynamic stress in rats17,18 and ischemia/reperfusion injury in pigs,19,20 implicating an important role for HO-1 in cardiovascular homeostasis. We have recently shown that in response to hypoxia, HO-1–null mice develop right ventricular infarcts with organized mural thrombi. Furthermore, increased lipid peroxidation and oxidative damage occur in right ventricular cardiomyocytes from HO-1–null but not wild-type mice.12 Thus, we hypothesized that HO-1 may play a central role in cardiac homeostasis by protecting cardiomyocytes from ischemia/reperfusion-induced injury and secondary oxidative damage. To gain insight into the cardioprotective role of HO-1 in vivo, we generated transgenic mice overexpressing HO-1 specifically in the heart. We measured cardiac performance during reperfusion in an isolated perfused heart preparation and assessed infarct size and tissue injury in an in vivo myocardial infarction model to examine whether HO-1 protects against ischemia/reperfusion-induced myocardial injury.

Materials and Methods
Generation of Transgenic Mice
To generate a transgenic construct (Figure 1A, top) expressing the human HO-1 (hHO-1) cDNA under the control of cardiac-specific mouse α-myosin heavy chain (αMHC) promoter,21 a 300-bp DNA fragment containing bovine growth hormone polyadenylation sequences (bGHpA) was ligated 3’ to the 1-kb hHO-1 cDNA open reading frame. The fragment containing hHO-1/bG HpA was then ligated downstream from the 5.5 kb of the mouse cardiac-specific

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†Deceased.
Correspondence to Shaw-Fang Yet, PhD, Cardiovascular Division, Brigham and Women’s Hospital, 75 Francis St, Thorn 1127, Boston, MA 02115. E-mail syet@rics.bwh.harvard.edu.
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Figure 1. Characterization of cardiac-specific HO-1 transgenic mice. A, At the top, a diagram of the cardiac αMHC promoter/hHO-1 transgenic construct is shown. Cardiac αMHC promoter indicates 5.5-kb cardiac-specific mouse αMHC promoter; HO-1, 1-kb human HO-1 cDNA; and pA, 300-bp bovine growth hormone polyadenylation sequences. The position of the Southern probe is shown above the construct; the positions of PCR primers used for genotyping are indicated below the construct. At the bottom, a Southern blot analysis of EcoRi-digested genomic DNA from wild-type (WT) and TG.L, TG.M, and TG.H transgenic lines is shown. The Southern probe was hybridized to a 3-kb transgenic fragment (Tg) and a 2-kb endogenous fragment (En). B, Northern blot analysis of RNA isolated from WT and three independent founder lines of transgenic mice (TG.L, TG.M, and TG.H) is shown. The blots were hybridized with a random-primed 32P-labeled hHO-1 cDNA probe, which hybridizes to a 1.8-kb endogenous mHO-1 message and a 1.5-kb message derived from the hHO-1 transgene. To visualize the mHO-1 RNA, the exposure time of spleen samples was longer than that of the heart samples. The blots were hybridized with a 32P-labeled 28S oligonucleotide to verify equivalent loading. C, HO-1 transgene is expressed specifically in the heart but not in other organs. Northern blot analysis of RNA isolated from several tissues of the transgenic TG.H line is shown. Northern analysis was performed as described in panel B. D, Western blot analysis of total protein extracted from WT, TG.L, TG.M, and TG.H mice is shown. Aliquots (25 μg) of spleen or heart protein were subjected to Western blotting with a polyclonal HO-1 antiserum, which recognizes both endogenous mouse HO-1 (mHO-1) proteins, diluted 1:1000 (SPA 895, StressGen), as described. The immunoblot bands were measured by densitometric analysis of the film with the use of NIH image software.

Isolated Perfused Heart Preparations

Mice were heparinized (100 U) by intraperitoneal injection 30 minutes before the experiments and were killed by cervical dislocation. Hearts were rapidly excised, arrested in ice-cold buffer, and connected via the aorta to the perfusion cannula as described. Retrograde perfusion was maintained at a constant pressure of 70 mm Hg by gravity. The flow of thehebian veins was drained by a thin polyethylene tube (PE-10) through the apex of the left ventricle (LV). A water-filled balloon was inserted into the LV for recording ventricular pressure and heart rate with the use of a commercially available data acquisition system (MacLab ADInstruments). Balloon volume was adjusted to achieve an end-diastolic pressure of 5 to 10 mm Hg. Coronary flow was monitored by collecting coronary sinus effluent. Hearts were perfused with phosphate-free Krebs-Henseleit buffer containing (mmol/L) NaCl 118, NaHCO3 25, KCl 5.3, CaCl2 2, MgSO4 1.2, EDTA 0.5, glucose 11, and pyruvate 0.5. The perfusate was equilibrated with 95% O2/5% CO2 (pH 7.4). Temperature was maintained at 37°C by water jacket. After stabilization for 30 minutes, the hearts were subjected to global ischemia by clamping the perfusion line. During ischemia, the hearts were surrounded by the perfusate, and the temperature was maintained at 37°C. After 30 minutes of ischemia, the perfusion line was released, and hearts were reperfused for 40 minutes. Cardiac contractile performance and coronary flow were recorded during stabilization, ischemia, and reperfusion.

In Vivo Ischemia and Reperfusion

Mice were subjected to a myocardial ischemia and reperfusion model as described. Mice were anesthetized by intraperitoneal injection of pentobarbitonal sodium (60 mg/kg body wt). Additional doses were given during the procedure as needed to maintain anesthesia. A rodent ventilator (model 683, Harvard Apparatus) was used with 100% oxygen during the surgical procedure. The skin on the neck was opened to guide the placement of the needle. Ventilation was provided by a blunt-ended 22-gauge catheter into the trachea via the mouth. During the operation, the animals were kept warm by using heat lamps. The chest was opened by a horizontal incision through the skin and muscle layers. An incision was made in the muscle between the ribs, and they were separated with a retractor to expose the heart. Ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) by using a 8-0...
Assessment of Area at Risk and Infarct Size
After 1 hour of ischemia and 24 hours of reperfusion, the LAD was occluded with a suture at the same site of the initial ligation. To demarcate the ischemic area at risk, Evans blue dye (1%) was perfused into the aorta and coronary arteries with distribution throughout the ventricular wall proximal to the site of coronary artery ligation.23,24 The nonischemic area was stained blue. Hearts were excised and sliced into five (≈1-mm) cross sections below the ligature. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 minutes. Viable myocardium stained red, and the infarct appeared pale. The infarct area (pale), the area at risk (not blue), and the total LV area from both sides of each section were measured by using NIH Image software, and the values obtained were averaged. The thickness of each section was measured by using a dial caliper. The individuals conducting the measurements were blinded to the experimental groups. The LV area at risk, and infarct area of each section were multiplied by the thickness of the section and then totaled from all five sections. The ratio of area at risk/LV and the ratio of infarct area/area at risk were calculated and expressed as a percentage as described.25-27

Histological Analysis and Immunohistochemistry
Ventricles from wild-type and transgenic mice were fixed in 10% formalin overnight at 4°C or fixed in methyl Carnoy’s solution at 4°C for 5 hours and then in 70% ethanol overnight and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). To detect hHO-1 transgene expression, we stained heart sections with an antibody specific to human hHO-1 transgene mRNA (obtained at ×100 magnification) were measured by computerized planimetry, and the areas staining positive for CD45 and MAL-2 were divided by colorimetric analysis.27 The respective areas staining positive for CD45 and MAL-2 were divided by the ischemic myocardial areas and multiplied by 100.

Results
Generation and Characterization of Cardiac-Specific HO-1 Transgenic Mice
To determine whether overexpression of HO-1 protects against oxidative injury in vivo, we used the cardiac-specific αMHC promoter to direct HO-1 expression to cardiomyocytes. By Southern blot analysis, three indepen-
systolic functional recovery, as demonstrated by higher LVDevP compared with that in hearts from wild-type mice (Figure 2B). Compared with hearts from wild-type mice, hearts from all three lines of HO-1 transgenic mice showed improved recovery. Postischemic recovery of cardiac function in the transgenic hearts improved in an HO-1 dose–dependent manner: TG.H transgenic hearts had the best recovery, followed by TG.M hearts, and then by TG.L hearts. It is noteworthy that LVDevP from TG.H hearts recovered almost immediately on reperfusion. This may be due to a more rapid return of heartbeat after reperfusion (data not shown). Cardiac contractile performance was also estimated by RPP. Transgenic hearts again showed better recovery than did wild-type hearts in an HO-1 dose–dependent manner (Figure 2C).

Transgenic Mice Overexpressing HO-1 Have Reduced Infarct Size After Ischemia and Reperfusion In Vivo
To test the hypothesis that cardiac-specific overexpression of HO-1 may protect against ischemia and reperfusion injury of the heart in vivo, we experimentally induced myocardial infarction in mice by ligating the LAD for 1 hour. To assess the myocardial infarct after 24 hours of reperfusion, total LV area, area at risk, and infarct area were measured. Before harvest, the ligature was retired at the previous ligation site to briefly occlude the LAD; Evans blue was then perfused into the aorta and coronary arteries to demarcate the nonischemic area (blue) and the ischemic area, which is the area at risk (not blue). Hearts were then excised and sliced into five cross sections below the ligature, followed by TTC staining. Viable myocardium stained red, and the infarct appeared pale.23,24 Large infarcts were present in wild-type mouse hearts (Figure 3A); in contrast, HO-1 transgenic mouse hearts showed small infarcts (Figure 3B). Despite a similar percentage of LV at risk (risk area/LV) between wild-type and transgenic mouse hearts (P=0.53, Figure 3C), the infarct size (infarct/risk area) was significantly reduced in transgenic mice compared with wild-type mice (P=0.001, Figure 3C).

To assess the myocardial injury after ischemia and reperfusion, histological sections of the ventricles were stained with H&E for analysis. The cardiomyocyte cytoarchitecture was disrupted, and cell degeneration and death were apparent within the infarcts of wild-type hearts (Figure 4A). In contrast, cardiomyocytes in similar ischemic areas were intact in transgenic mice (Figure 4B). In addition, ventricular sections from wild-type mice, subjected to ischemia and reperfusion injury, showed marked inflammatory cell infiltration, as demonstrated by CD45 immunostaining (Figures 4C and 4E). However, HO-1 transgenic mice had minimal inflammatory cell infiltration (Figures 4D and 4F). In similar ischemic areas, the CD45-positive area was ~15-fold higher in wild-type hearts (1.73±0.04%) compared with transgenic hearts (0.11±0.02%). Immunostaining with the neutrophil-specific Ly-6G or macrophage-specific MOMA-2 antibodies revealed that the inflammatory cells were predominantly neutrophils (data not shown). To assess oxidative damage in the infarcted myocardium, we stained ventricular sections with an antibody (MAL-2) that recognizes oxidation-specific lipid-protein adducts. Intense MAL-2 staining was observed in the ischemic myocardium near the infarct site in wild-type

### Baseline Function in WT and HO-1 Transgenic Mouse Hearts

<table>
<thead>
<tr>
<th></th>
<th>WT (n=12)</th>
<th>TG.L (n=12)</th>
<th>TG.M (n=5)</th>
<th>TG.H (n=9)</th>
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</thead>
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<tr>
<td>Body weight, g</td>
<td>26.4±0.7</td>
<td>29.0±0.9</td>
<td>26.5±0.9</td>
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<td>Heart weight, mg</td>
<td>157±4</td>
<td>166±9</td>
<td>145±3</td>
<td>163±8</td>
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<tr>
<td>LVEDP, mm Hg</td>
<td>7.6±0.4</td>
<td>7.0±0.2</td>
<td>6.6±0.2</td>
<td>7.2±0.4</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>132±3</td>
<td>135±4</td>
<td>128±2</td>
<td>129±7</td>
</tr>
<tr>
<td>LVDevP, mm Hg</td>
<td>125±3</td>
<td>128±4</td>
<td>128±2</td>
<td>122±7</td>
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<tr>
<td>RPP, 10³ mm Hg/min</td>
<td>39.8±1.3</td>
<td>36.8±1.5</td>
<td>37.8±2.7</td>
<td>32.9±1.5</td>
</tr>
<tr>
<td>Coronary flow, mL/min</td>
<td>2.2±0.1</td>
<td>2.2±0.2</td>
<td>2.0±0.1</td>
<td>2.1±0.1</td>
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</tbody>
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LVSP indicates LV systolic pressure. Values are mean±SE.
mice (Figure 4G), whereas the staining was minimal in hearts from transgenic mice (Figure 4H). MAL-2–positive staining in wild-type hearts (2.91±0.26%) was 12-fold higher than that from hearts of transgenic mice (0.21±0.04%). Histological analysis of other organs (kidney, liver, and intestine) was also performed and indicated no damage and no difference between wild-type and transgenic mice (data not shown).

**Discussion**

In the present study, we show that HO-1 plays an important role in myocardial homeostasis by protecting cardiomyocytes from ischemia/reperfusion-induced injury and secondary oxidative damage. HO-1 dose-dependently improved the recovery of postischemic contractile performance in a Langendorff preparation, demonstrating a positive correlation between HO-1 expression levels and it conferred protection in the heart.

One of the effects of reperfusion on the ischemic myocardium is accelerated inflammation and oxidative stress, which may cause further injury.28,29 The importance of neutrophil recruitment in the pathophysiology of myocardial reperfusion injury was demonstrated in CD18-null mice.30 These mice had reduced infarct size after myocardial ischemia and reperfusion.30 Indeed, after ischemia and reperfusion, there was extensive inflammatory cell infiltration, predominately neutrophils, and increased oxidative damage in wild-type hearts compared with transgenic hearts (Figure 4). Our results suggest that HO-1 not only protects against postischemic injury in the absence of inflammatory cells (in isolated perfused heart studies) but also protects against reperfusion injury and inflammation in vivo.

It has recently been shown that upregulation of endogenous HO-1 in rat hearts by treating animals with hemin ameliorates postischemic myocardial dysfunction in isolated perfused hearts31 and decreases the infarct area.32 However, hemin treatment may also affect other systemic proteins and effectors.33–36 In the present study, the specific HO-1 expression in the cardiomyocyte was sufficient to protect against ischemia and reperfusion injury.

The molecular mechanisms by which HO-1 confers myocardial protection are still under investigation. Bilirubin protects cultured cardiomyocytes against oxidative damage37 and improves postischemic cardiac function in isolated perfused rat hearts.31 In addition, higher serum bilirubin concentration is associated with decreased risk for early familial coronary artery disease.38 It is likely that the antioxidant effects of bilirubin contribute to the protection in the heart, which has a relatively weak endogenous antioxidant defense compared with that in other organs, such as the liver and intestines.39 Recently, it has been suggested that CO prevents an inflammatory response in a rat model of hyperoxic injury, thus decreasing oxidative damage.15,16 The anti-inflammatory properties of CO may reduce the reperfusion injury in vivo. This is consistent with the decreased inflammatory cell

**Figure 3.** HO-1 protects against myocardial infarction in transgenic mice. A and B, Myocardial infarcts from WT mice (n=6) (A) and TG.H transgenic mice (TG, n=8) (B) were assessed by Evans blue and TTC staining after 1 hour of ischemia and 24 hours of reperfusion. The Evans blue–perfused area, which is not at risk, was stained blue; viable myocardium was stained red; and infarcted myocardium appeared pale. Representative WT and TG heart sections were shown and oriented anterior side up in panels A and B, respectively. Original magnification ×15. C, Myocardial infarcts are reduced in HO-1 TG mice. WT mice (open bars, n=6) and TG mice (filled bars, n=8) were subjected to 1 hour of ischemia and 24 hours of reperfusion, as in panel A. Risk area/LV indicates percentage of LV at risk; infarct/risk area, infarcted area as percentage of risk area. Error bars indicate SE. *P=0.001 vs infarct/risk area of WT mice.

**Figure 4.** Reduced cardiomyocyte injury, inflammatory cell infiltration, and oxidative damage in TG mouse hearts after ischemia and reperfusion. Representative histological analysis of WT heart sections (n=4) and TG heart sections (n=4) after 1 hour of ischemia and 24 hours of reperfusion. A, H&E-stained LVs from the infarcted area of WT mice. B, H&E-stained LVs from similar ischemic areas of TG mice. C through F, CD45 stained LVs from WT (G and E) and TG (H and F) mice. Original magnification ×400 (A and B), ×100 (C and D), ×400 (E and F), and ×200 (G and H).
infiltration in transgenic hearts. Another well-known property of CO is its vasodilatory effect, which could improve reperfusion blood flow. It is possible that both the anti-inflammatory and vasodilatory effects of CO contribute to the cardioprotection of HO-1. Taken together, our findings may lead to novel strategies for preventing cardiac injury due to ischemia and reperfusion.

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