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Preemptive heme oxygenase-1 gene delivery reveals reduced mortality and preservation of left ventricular function 1 yr after acute myocardial infarction

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Liu X, Simpson JA, Brunt KR, Ward CA, Hall SR, Kinobe RT, Barrette V, Tse MY, Pang SC, Pachori AS, Dzau VJ, Ogunyankin KO, Melo LG. Preemptive heme oxygenase-1 gene delivery reveals reduced mortality and preservation of left ventricular function 1 yr after acute myocardial infarction. Am J Physiol Heart Circ Physiol 293: H48–H59, 2007. First published February 23, 2007; doi:10.1152/ajpheart.00741.2006.—We reported previously that pre-delivery of heme oxygenase-1 (HO-1) gene to the heart by adeno-associated virus-2 (AAV-2) markedly reduces ischemia and reperfusion (I/R)-induced myocardial injury. However, the effect of preemptive HO-1 gene delivery on long-term survival and prevention of postinfarction heart failure has not been determined. We assessed the effect of HO-1 gene delivery on long-term survival, myocardial function, and left ventricular (LV) remodeling 1 yr after myocardial infarction (MI) using echocardiographic imaging, pressure-volume (PV) analysis, and histomorphometric approaches. Two groups of Lewis rats were injected with 2 × 1011 particles of AAV-LacZ (control) or AAV-human HO-1 (dHO-1) in the anterior-posterior apical region of the LV wall. Six weeks after gene transfer, animals were subjected to 30 min of ischemia by ligation of the left anterior descending artery followed by reperfusion. Echocardiographic measurements and PV analysis of LV function were obtained at 2 wk and 12 mo after I/R. One year after acute MI, mortality was markedly reduced in the HO-1-treated animals compared with the LacZ-treated animals. PV analysis demonstrated significantly enhanced LV developed pressure, elevated maximal dP/dt, and lower end-diastolic volume in the HO-1 animals compared with the LacZ animals. Echocardiography showed a larger apical anterior-to-posterior wall ratio in HO-1 animals compared with LacZ animals. Morphometric analysis revealed extensive myocardial scarring and fibrosis in the infarcted LV area of LacZ animals, which was reduced by 62% in HO-1 animals. These results suggest that preemptive HO-1 gene delivery may be useful as a therapeutic strategy to reduce post-MI LV remodeling and heart failure.

Adenovirus; echocardiography; fibrosis; heart failure; pressure-volume analysis; ventricular remodeling

Coronary artery disease (CAD) is the most prevalent cause of morbidity and premature death in all ages and ethnic groups, (15) often leading to myocardial infarction (MI) and sudden death (6, 16). Over time, CAD leads to progressive impairment of cardiac function in those patients that survive the initial MI, culminating in heart failure (25). Current drug therapies for MI, such as antianginal, fibrinolytic, and anti-platelet agents, although effective in ameliorating the acute symptoms of the disease and in reducing peri-MI death, have not been found to significantly improve the long-term outcome after MI (25, 59). Likewise, the success of acute revascularization procedures such as primary coronary angioplasty, stenting, or coronary artery bypass grafting is limited by restenosis and graft atherosclerosis (5, 22, 48). Ironically, the improved survival of MI patients treated with these therapies has led to a drastic increase in the number of patients suffering from chronic heart failure (15, 30, 60). The socioeconomic impact of this phenomenon imposes severe financial strain on the health care system and calls for a fundamental shift in the therapeutic approach to management of the disease to focus on prevention rather than recovery (35, 51).

Our group (1, 36) reported previously that predelivery of antioxidant genes such as heme oxygenase-1 (HO-1) or extracellular superoxide dismutase to the myocardium provides a preemptive strategy for myocardial protection in the event of MI. HO-1, in particular, may be well suited as a therapeutic agent for myocardial protection, because the catabolic byproducts of heme metabolism, carbon monoxide (CO) and bilirubin, have been reported to exert pleiotropic cytoprotective effects, including reduction of oxidative stress (4, 44, 52), inflammation (39, 57), and apoptosis (2, 14, 28, 36, 40, 54), which are the principal pathological stimuli leading to irreversible myocardial damage following ischemia and reperfusion (I/R) (7, 13, 54). In this regard, our group (36) showed that intramyocardial delivery of the HO-1 gene using adenovirus-2 (AAV-2) in advance of occlusion/reperfusion of the left anterior descending artery (LAD) markedly reduces infarct size in association with decreased levels of myocardial pro-oxidant species and inflammatory and proapoptotic proteins. More recently, our group (40) reported that this strategy can also confer protection from injury triggered by recurrent transient I/R events in a closed-chest rat model of ischemic cardiomyopathy. Subsequently, work by our group (30) showed that HO-1 gene delivery reduces fibrosis and ventric-
ular remodeling during the intervening 3 mo after acute I/R injury. On the basis of these findings, we proposed that pre-delivery of HO-1 gene by a vector system such as AAV, which is capable of sustained transgene expression, may be a useful cardioprotective strategy for patients at high risk of MI, such as those with CAD.

Although previous studies by our group have established the therapeutic potential of preemptive HO-1 gene delivery as a cardioprotective strategy for I/R injury, the benefit of this strategy in long-term survival and prevention of postinfarction heart failure has not been established. This is an essential requirement in the validation of this therapeutic strategy for prolonged myocardial protection. Thus, in the current study, we assessed the effect of HO-1 gene delivery on survival, left ventricle (LV) function, and remodeling 1 yr after MI using echocardiographic imaging, pressure-volume (PV) analysis of LV function, and histomorphometric approaches.

MATERIALS AND METHODS

Animals. Male Lewis rats weighing 200–225 g (6–8 wk) were purchased from Charles River Laboratories (Indianapolis, IN) and maintained on a 12:12-h light-dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided ad libitum.

Vector construction and virus production. The construction of the AAV-2 plasmid encoding human HO-1 (hHO-1) was described previously (30, 36) (see Fig. 1A). Packaging, propagation, and purification of AAV-2 viral particles were carried out at the Harvard Gene Therapy Initiative Core Facility (Boston, MA) using standard methods (50).

Intramyocardial gene delivery and acute I/R injury. Intramyocardial gene delivery and acute myocardial I/R injury were carried out as previously described (36). In preparation for intramyocardial gene transfer and I/R injury, animals were anesthetized with 60 mg/kg sodium pentobarbital. Two groups of animals were treated with 2 × 10^{11} genome particles of AAV-hHO-1 or AAV-LacZ in a final volume of 250 μL, injected subepicardially with a curved needle at five sites (50 μL/injection) to cover the approximate ischemic area (area at risk) after ligation of the LAD. Acute myocardial I/R injury was induced 6 wk after gene delivery by ligation of the proximal LAD for 30 min. Infarct size was calculated 24 h after reperfusion as described previously (36). Acute postsurgical mortality was <5%. All surgical and experimental procedures were approved by the Queen’s University Animal Care Committee and adhered to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Myocardial transgene expression. Exogenous human HO-1 transgene expression was detected using reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA (100 ng) extracted from the LV was used for first-strand cDNA synthesis and PCR amplification with the One-step platinum Taq RT-PCR kit (Life Technologies). A 185-bp fragment was amplified for 30 cycles with the following hHO-1-specific primers: forward, 5'-GCTTTGAGGAGTTGCAGG-3'; reverse, 5'-GTGTAAGGACCATCGGAGA-3'.

Measurement of HO enzyme activity. HO activity in rat heart microsomal fractions was determined by quantifying CO formed from the degradation of methemalbumin (heme complexed with albumin) according to the method of Vreman et al. (56). In brief, reaction mixtures (150 μL) consisting of 100 mM phosphate buffer, pH 7.4, 50 mM methemalbumin, and 0.5 mg/ml protein were preincubated for 10 min at 37°C. Reactions were initiated by adding β-NADPH at a final concentration of 1.5 mM, and incubations were carried out for an additional 25 min at 37°C. Reactions were stopped by instantly freezing the reaction mixture on pulverized dry ice, and CO formation was monitored by gas chromatography using a TA 3000R process gas analyzer (Trace Analytical, Newark, DE).

Echocardiography. Two groups of Lewis rats treated with AAV-hHO-1 or AAV-LacZ were used for echocardiographic assessment of LV function and chamber dimensions. Measurements were made 1 yr after acute I/R. Another set of animals was treated in the same fashion and used to assess echocardiographic and histomorphological changes in LV function and geometry 2 wk after I/R. In preparation for echocardiography, animals were lightly anesthetized by halothane using a nose cone, shaved, and positioned on a heated pad in a recumbent position. Measurements at the 2-wk time point were carried out at the apical to midapical level of the short-axis view. For the 1-yr time point, measurements were made at three levels (apex, middle, and base). Images were obtained from the parasternal window using a P 12–5 MHz probe on a Phillips HDI-5000 echocardiography machine. LV mass was calculated using the ASE formula [1.04 × (LVd + PWD + IVSd)^3 − LVd^3], where 1.04 is the specific gravity of muscle, LVd is LV diastolic diameter, and PWD and IVSd are end-diastolic posterior wall and interventricular septum thicknesses, respectively. Fractional shortening (FS) was calculated as (LVID − LVS)/LVID × 100, where LVID is LV end-systolic diameter. The relative wall thickness for each level of the LV was calculated as (PWD + IVSd)/LVID. For each parameter, an average of five cardiac cycles was used.

Hemodynamic measurements. One year after I/R, rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and placed in a supine position in a servo-controlled heating pad to maintain body temperature at ~37°C. Animals were tracheotomized, connected to a small rodent ventilator (Harvard Apparatus, Montreal, QC, Canada), and ventilated with room air at a tidal volume of 5 ml and 65 breaths/min. For measurements of the PV relationships, a microtipped catheter (SPR-838; Millar Instruments; Houston, TX) was inserted into the right carotid artery and advanced into the LV. All signals (LV pressure and conductance) were digitized at a sampling rate of 1,000 Hz and acquired to a computer using CED Spike 2 software (Cambridge, UK). PVAN software (Millar Instruments) was used for subsequent LV analysis and assessment of LV function, including heart rate (HR), LV systolic pressure, the first derivative of LV pressure (±dP/dt), ejection fraction (EF), cardiac output, end-systolic myocardial elastance constant (Ees), preload recruitable stroke work (PRSW), LV end-diastolic pressure (LVEDP) and volume (LVEDV), LV end-systolic pressure (LVESP) and volume (LVESV), LV pressure decay (τ; Weiss method), and slope of the end-diastolic and end-systolic PV relationships (18).

Histological and immunohistochemical analysis of myocardial fibrosis, apoptosis, and remodeling. At 1 day, 2 wk, and 1 yr after I/R, hearts were arrested in diastole with 0.2 N KCl, fixed by perfusion at constant pressure (100 cmH2O) with 10% formalin, harvested, and postfixed in the same fixative for 12 h. Paraffin sections (5 μm) from the 1-yr animals were stained with Masson’s trichrome stain (Ac-custain; Sigma Chemicals) for visualization of interstitial collagen deposition. Three sequential transverse thick sections corresponding to the apical, middle, and basal regions of the infarct were used for preparation of histological sections (5 μm) for morphometric quantification of fibrosis. Collagen-positive areas were calculated for each section using Sigma Scan Pro 5 (SPSS, Chicago, IL). Percent fibrosis was expressed as the ratio of fibrotic area to total LV area. For calculation of wall thickness, the distance between the endocardial and epicardial circumference was determined separately in each section at the anterior wall, posterior wall, and septum. A minimum of five measurements were taken and averaged for each section. The wall-thinning index was calculated as the ratio of anterior to posterior wall thickness. Apoptosis in the LV was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using the CardioTacs staining kit (Treven, Gaithersburg, MD) in sections collected from animals killed at 6–24 h after I/R. Apoptotic nuclei stain blue after incorporation of biotinylated dUTP by terminal deoxynucleotidyl transferase to 3’-OH residues in fragmented DNA and subsequent detection using streptavidin-conjugated horseradish...
peroxidase reaction with TACS blue label. The apoptotic index was calculated by dividing the number of apoptotic nuclei in the infarcted region by the infarct area. To detect HO-1 expression, we stained sections with a polyclonal HO-1 antiserum (SPA-895, diluted 1:100; StressGen Biotechnologies, Victoria, BC, Canada) at 4°C overnight. Sections were counterstained with methyl green. All morphometric measurements were performed by experimenters who were blinded to the groups and treatment conditions.

**Assay of myeloperoxidase activity.** Twenty-four hours after I/R, the hearts were perfused with phosphate-buffered saline (PBS; pH 7.4), flash frozen in liquid nitrogen, and pulverized. Myeloperoxidase (MPO) activity was determined semiquantitatively using the Cyto- store MPO assay (Cytostore, Calgary, AB, Canada) according to the manufacturer’s instructions. Briefly, 50 mg of LV tissue from the infarcted region were homogenized in a Dounce homogenizer in hexadecylltrimethylammonium bromide on ice. MPO activity was measured as the change in absorbance (ΔA) of fast blue B over 15 min at 450 nm in the presence of H2O2. The linear reaction velocity was determined, and one enzymatic unit of MPO was defined as the cleavage of 1 μmol of H2O2, which provides ΔA = 1.13 × 10⁻²/min.

**Statistical analysis.** Results are shown as means ± SE. Unpaired t-test was used to compare differences in fibrosis and echocardiographic and PV loop analysis of LV dimensions and function between the HO-1- and LacZ-treated animals. *P < 0.05* was considered to indicate statistical significance.

**RESULTS**

**AAV-mediated intramyocardial gene delivery leads to long-term transgene expression.** The strategy for intramyocardial gene transfer has been described previously (1, 30, 36). Briefly, the AAV vector constitutively expressing LacZ or HO-1 (Fig. 1A) was injected at five sites of the LV free wall to cover the area corresponding to the infarct risk area after ligation of the proximal LAD (Fig. 1B). The ischemic region was confined primarily to the apical region of the LV (Fig. 1B). The intramyocardial gene transfer strategy typically transduces 40–60% of the infarct risk area (36). Transgene mRNA expression was detected at 24 h and 1 yr after I/R in the HO-1-treated animals (Fig. 1C), thus confirming that AAV-2 is a suitable vector for sustained transgene expression in the myocardium. We also detected evidence of HO-1 protein expression using immunohistochemical staining in cross sections of LV from HO-1-treated animals at 1 day and 1 yr after I/R (Fig. 1D). HO activity in whole LV homogenates was detected in both groups of animals at 6 h and 12 mo after MI (Fig. 1E). There was a trend toward higher levels of HO activity (~22%) in the HO-1-treated animals compared with the LacZ controls, but the differences did not reach statistical significance.

**AAV-mediated HO-1 gene transfer markedly reduces myocardial injury after acute I/R.** The effect of HO-1 gene delivery in acute MI is shown in Fig. 2. Infarction was predominantly localized to the apical region of the LV. Minimal necrosis was seen in triphenyltetrazolium chloride (TTC)-stained sections from the HO-1-treated animals, compared with the LacZ-treated animals (Fig. 2A). Morphometric analysis of the TTC-stained sections 1 day after I/R showed no difference in ischemic LV area between the HO-1- and LacZ-treated groups (Fig. 2B). However, the infarct size (percentage of LV area) was reduced by ~80% in the HO-1-treated animals (Fig. 2C) (*n = 5, P < 0.05*). Consistent with the gross histological appearance, we saw marked necrosis and inflammatory cell infiltration in the LacZ-treated animals (Fig. 2D). In contrast, limited microscopic evidence of myocardial necrosis or inflammation was seen in the HO-1-treated animals 24 h after I/R (Fig. 2E). Because apoptosis plays a major role in myocardial cell loss after I/R, we determined whether the acute cardioprotective effects of HO-1 gene therapy are associated with decreased apoptosis in the infarcted region. A higher number of TUNEL-positive nuclei were seen throughout the infarcted region of the LacZ-treated animals (Fig. 2F) compared with the much reduced number of apoptotic nuclei in the HO-1-treated animals (Fig. 2G). In agreement with the histological findings, MPO activity was significantly reduced in the HO-1-treated animals.
animals (Fig. 2H), suggesting that the acute cytoprotective effect of HO-1 gene transfer in the infarcted myocardium is at least partially mediated by decreased inflammatory cell infiltration. The apoptotic index was 33 nuclei/mm² in LacZ-treated animals and 6 nuclei/mm² in the HO-1-treated animals (Fig. 2I).

**AAV-mediated HO-1 gene transfer enhances long-term survival after I/R injury.** To validate the therapeutic potential of HO-1 gene transfer in long-term myocardial protection, we examined the effect of HO-1 gene delivery on survival. Kaplan-Meier curves for both groups of animals are shown in Fig. 3. Postinfarction survival was significantly higher in HO-1-treated animals compared with LacZ-treated animals (P < 0.05, log rank test) throughout the 1-yr follow-up period (Fig. 3). By 6 mo after I/R, 60% of the LacZ-treated animals had survived compared with 85% survival for the HO-1-treated animals. One year after I/R, only 40% of the LacZ animals were still alive, compared with 79% for the HO-1-treated animals (Fig. 3).

**AAV-mediated HO-1 gene transfer preserves echocardiographic LV chamber dimensions and function and attenuates remodeling.** We used echocardiography to examine early (2 wk) and late (12 mo) changes in LV function and wall.

![Fig. 2. Myocardial injury after I/R injury. A: representative biventricular thick sections from LacZ- and HO-1-treated animals stained with triphenyltetrazolium chloride (TTC) to detect infarcted myocardium at 24 h after I/R. Infarcted tissue appears as yellowish white (delineated by dashed ellipse), and noninfarcted tissue appears red. B and C: morphometric assessment of LV area (B) and %infarct (C) in LacZ- and HO-1-treated animals 24 h after reperfusion. D and E: histological analysis of hematoxylin and eosin (H&E)-stained sections from the infarcted myocardium shows increased injury and inflammatory cell infiltration in the LacZ (D) compared with the HO-1-treated animals (E). F and G: immunohistochemical detection of apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) shows an increased number of apoptotic nuclei in the LacZ-treated animals (F) compared with the HO-1-treated animals (G). H: myeloperoxidase (MPO) activity in LV homogenates from HO-1- and LacZ-treated animals. I: apoptotic index (no. of apoptotic nuclei/mm²) in HO-1- and LacZ-treated animals 6 h after reperfusion. Infarct size was reduced by ~80% in the HO-1-treated animals compared with the LacZ control animals. Values are means ± SE (n = 3–5). *P < 0.05, HO-1 vs. LacZ.
thickness and chamber dimensions following I/R. In contrast to the permanent ligation model of MI, which induces rapid thinning of the infarcted LV wall and chamber dilatation, the I/R model of MI leads to slow-developing LV remodeling that is generally complete by 3–6 mo and recapitulates some features of LV remodeling in humans with reperfused MI (3, 11, 26). The results for echocardiographic analysis at 2 wk in the LacZ (n = 5)- and HO-1 (n = 5)-treated animals are shown in Fig. 4. Basal (pre-MI) LV dimensions did not differ significantly between the two groups of animals (Fig. 4, A–F); however, pre-MI LV function was slightly lower in the HO-1-treated animals (Fig. 4, G and H). No significant post I/R changes in wall thickness were observed at this time in either group of animals (Fig. 4, A, C, and D). However, we found histological (Fig. 4A) and echocardiographic evidence (Fig. 4, B, E, and F) evidence of cavity enlargement in the LacZ-treated animals. Two weeks after I/R, LV diastolic and systolic dimensions were significantly increased by 11 and 36%, respectively, in the LacZ animals relative to pre-I/R values, whereas no significant change in post-I/R chamber dimension...
was seen in the HO-1-treated animals (Fig. 4, E and F). In parallel with the changes in chamber dimensions, we observed a significant decrease in EF (Fig. 4G) and FS (Fig. 4H) in the LacZ-treated animals 2 wk after I/R, whereas no change in these parameters was seen in the HO-1-treated animals.

Because the infarcts generated by transient ligation of the proximal LAD are predominantly apical, we reasoned that echocardiographic analysis at the midpapillary level would underestimate the magnitude of LV chamber and wall remodeling at the 1-yr post-I/R time point. Thus we performed segmental echocardiographic analysis of LV dimensions and function at three levels, corresponding approximately to the apical, middle, and basal regions of the LV. Figure 5A shows the approximate location of the segments used for echocardiographic analysis of LV function and dimensions. Figure 5B shows representative M-mode frames of the three regions in both groups of animals. The results for the 1-yr post-I/R echocardiographic analysis are summarized in Table 1. Analysis of LV wall dimensions showed significant thinning of the anterior wall in the apical segment of the LacZ-treated animals (Table 1 and Fig. 5B). The middle segment of the LacZ animals also showed a trend toward thinning of the anterior wall, but the difference did not reach statistical significance compared with the HO-1-treated animals (Table 1 and Fig. 5B). No differences were seen between the two groups in wall thickness in the basal segment (Table 1 and Fig. 5B), indicating that the infarct is restricted to the anterior apical free wall and intraventricular septum. In concordance with the reduced thickness of the apical anterior wall, we observed significantly increased LV systolic dimension in of the LacZ animals (Table 1 and Fig. 5B). No difference in LV chamber dimensions was seen between the two groups in the middle segment. Interestingly, both systolic and diastolic dimensions were increased in the basal segment of the LacZ animals, suggesting remodeling of the noninfarcted region (Table 1 and Fig. 5B). In agreement with the differences in LV wall and chamber dimensions, echocardiographic indexes of LV function revealed significant decreases in EF and FS in the apical segment from the LacZ animals (Table 1).

The echocardiographic findings of LV wall thinning and chamber enlargement at 1 yr post-I/R were also verified by histomorphometric analysis of sections. Examination of fresh and trichrome-stained sections from the HO-1 animals showed preserved LV wall thickness and small anterior infarcts (Fig. 6, A and B) with decreased interstitial collagen deposition (Fig. 6C). In contrast, LacZ animals showed thinning of anterior wall (Fig. 6D) and large infarcts that have been replaced by a collagen scar (Fig. 6E). Examination of sections revealed marked accumulation of interstitial collagen in the infarct region (Fig. 6F). Morphometric assessment of the collagen positive area showed that ~30% of the LV area in the LacZ animals (n = 4) developed fibrosis compared with 11% for the HO-1-treated animals (n = 5) (Fig. 6G). These values correspond closely to the LV infarct area measured 1 day after I/R. In agreement with the echocardiographic measurements, the wall thinning ratio indicated that the apical anterior wall thickness was reduced by ~19% in the LacZ (n = 6) relative to the HO-1 (n = 8) (Fig. 6H). The wall thinning index in the middle and basal segments did not differ between the two groups (Fig. 6H).

AAV-mediated HO-1 gene transfer preserves long-term LV function following I/R injury. We performed PV analysis using a Millar microtipped catheter to obtain a direct assessment of LV function in both groups of animals 1 yr after I/R. The results of the PV analysis are summarized in Fig. 7. Representative PV loops for the two groups are shown in Fig. 7A. Compared with the HO-1-treated animals, the LacZ-treated animals had significantly lower systolic LV developed pressures (Fig. 7A). Furthermore, the loops of the LacZ animals showed a prominent rightward displacement along the LV
Table 1. Segmental 2-D echocardiographic analysis of LV dimensions and function 1 yr after acute MI in rats pretreated with HO-1 or LacZ gene transfer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HO-1</th>
<th>LacZ</th>
<th>P Value</th>
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<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>LV wall dimensions, cm</td>
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<tr>
<td>IVSsA</td>
<td>0.194±0.014</td>
<td>0.140±0.014</td>
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<td>0.134±0.007</td>
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<td>0.053</td>
</tr>
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<td>IVSsM</td>
<td>0.236±0.012</td>
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<td>LV function, %</td>
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<tr>
<td>EF-A</td>
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Two-dimensional (2-D) echocardiographic imaging was performed 1 yr after myocardial infarction (MI) in rats pretreated with heme oxygenase-1 (HO-1) or LacZ (control) gene transfer. Measurements were made at 3 levels: apex (A), middle (M), and base (B) of left ventricle (LV). IVs, interventricular septum thickness in systole; IVsd, interventricular septum thickness at diastole; PWs, posterior wall thickness at systole; PWd, posterior wall at diastole; LVDs, LV chamber diameter at systole; LVDd, LV chamber diameter at diastole; EF, ejection fraction; FS, fractional shortening.

To further establish whether the differences in LV hemodynamic parameters between the two groups were independent of loading conditions, we calculated the slope of the relationship between the maximal first derivative of ventricular pressure and end-diastolic volume [(dP/dtmax)/EDV] under conditions of transient occlusion of the inferior vena cava to reduce preload. The (dP/dtmax)/EDV is a measure of end-systolic elastance (Ees, mmHg/μl) and is a chamber size-dependent index of systolic function. Compared with the HO-1-treated animals, the slope [of the (dP/dtmax)/EDV relationship] was smaller in the LacZ-treated control animals (2.22 mmHg·s⁻¹·μl⁻¹) compared with the HO-1 treated animals (41.14 mmHg·s⁻¹·μl⁻¹) (Fig. 8A), providing evidence of systolic dysfunction and decreased contractility in these animals. This finding was further corroborated by the relationship between stroke work and EDV (PRSW), a chamber size-independent index of systolic function, which showed a decreased slope in the LacZ animals (30.11 mmHg) relative to the HO-1-treated animals (146 mmHg) (Fig. 8B).

DISCUSSION

Work in our laboratory (30, 36) showed recently that pre-delivery of HO-1 gene may yield therapeutic potential in long-term myocardial protection from I/R injury. Our premise was that an increase in basal HO-1 expression might potentiate the antioxidant reserve capacity and exert a preconditioning effect on the myocardium, rendering it resistant to injury by reactive oxygen species and inflammatory mediators produced during I/R injury. In support of this premise, our group showed that intramyocardial delivery of HO-1 by AAV-2 in advance of I/R markedly reduced myocardial injury in response to single (36) or recurrent (40) episodes of I/R, despite only modest (20–50%) increases in basal HO activity. In addition, transgenic mice overexpressing cardiac-specific HO-1 have shown reduced oxidative and inflammatory damage following I/R injury (61). As a further step toward validating the potential of HO-1 gene therapy as a preemptive strategy for long-term myocardial protection, we determined the effect of intramyocardial HO-1 delivery on postinfarction survival and LV function and remodeling. In the present study, we have shown that survival and LV functional recovery are markedly improved 1 yr after I/R in the animals treated with HO-1 gene, in association with reduced myocardial injury and preservation of LV dimensions. Thus the current findings further support the concept that HO-1 gene delivery is a useful strategy for long-term myocardial protection against I/R induced myocardial injury and failure.

The mechanism by which intramyocardial HO-1 gene transfer leads to long-term preservation of LV function and chamber dimensions is not known. The cytoprotective effects of HO-1 have widely been attributed to the catabolic by-products of heme degradation, particularly CO and bilirubin. Indeed, several studies have documented cardioprotective effects of these metabolites (45). For example, inhalation of physiological levels of CO for 24 h markedly reduces infarct size in rats after I/R in association with decreased apoptosis and inflammation (14). Others have reported that CO protects transplanted hearts from cold-induced I/R injury (2), and administration of a water-soluble CO-releasing molecule (CORM-3) at the time of reperfusion reduces infarct size in mice (21). Likewise, exog-
enous bilirubin administration has been reported to reduce infarct size in isolated rat hearts (9), and heme-derived iron has been reported to induce cytoprotection by promoting ferritin expression (4, 45). These findings would suggest that these by-products of heme degradation might render the myocardium resistant to I/R injury and decrease myocyte loss, resulting in reduced LV remodeling and improved function. However, these by-products are a double-edged sword that may exhibit cardiotoxic effects even at concentrations associated with moderate increase in HO-1 activity (12, 46, 53). Indeed, recent evidence suggests that heme oxygenase may exert both pro- and antioxidant effects, depending on the cellular redox potential and the level of HO-1 induction (46). Several studies have revealed detrimental effects of HO-derived by-products. For example, chronic CO inhalation at a dose mimicking levels normally registered with tobacco smoking (500 parts per million) worsens post-MI LV remodeling (37, 42). In addition, CO inhibits oxygen binding to myoglobin (19), and CO inhibits other hemoproteins, including cytochrome c (43, 45, 46) and endothelial nitric oxide synthase. These toxic effects of CO impair myocardial oxidative metabolism (19) and may be exacerbated in patients with advanced coronary artery disease. Bilirubin has also been reported to exert toxic effects on cell membrane stability at high concentration (10), and heme-derived iron may lead to increased reactive oxygen species and cellular damage (44, 46).

The potential cytotoxicity of the by-products of heme catabolism demands that any strategies aimed at increasing their concentration for therapeutic purposes, either by pharmacological induction or by genetic overexpression of HO-1, need to be carefully dosed. Suttner and Dennery (53) reported that high levels of exogenous HO-1 overexpression lead to significant oxygen cytotoxicity in hamster fibroblasts, with a threshold of a greater than fivefold increase in basal HO-1 protein expression, whereas lower levels of HO-1 expression were associated with cytoprotection. In our experiments, intramyocardial delivery of HO-1 gene using AAV typically yields modest (∼20–50%) increases in HO-1 protein expression and HO activity, well below the levels reported to exert cytotoxic effects. Clearly, this level of HO-1 overexpression is sufficient to confer marked protection against single (36, 49) or recurrent episodes of I/R injury, but the mechanism by which this occurs...
remains largely unknown. It is tempting to speculate the modest increase in basal HO activity achieved with AAV may be sufficient to induce sustained preconditioning of the myocardium so that it can withstand transient ischemic insults; however, this possibility remains to be proven. On the other hand, emerging evidence suggests that HO-1 may have other biological effects in addition to its role in the enzymatic breakdown of heme. Recently, HO-1 was reported to protect human monoblastic lymphoma cells from hydroperoxide-induced injury independently of its catalytic activity (23). HO-1 also functions as a phosphoprotein and interacts with other signaling molecules, including the survival kinase Akt (47). Our group (8) reported recently that HO-1 functions codependently with Akt to confer protection from pro-oxidant-induced injury in human aortic smooth muscle cells. Considering the importance of Akt in myocardial homeostasis (34), the possibility that Akt and HO-1 may function in a similar fashion to exert cardioprotection is intriguing and merits further investigation. In addition, HO-1 translocates to the nucleus (33, 45), raising the possibility that it may modulate nuclear events such as transcription and DNA binding. Thus the mechanism of HO-1 cardioprotection may involve both catalytic and noncatalytic processes. The extent to which these processes contribute to AAV-HO-1-mediated cardioprotection remain to be determined.

In addition to its cardioprotective effects, HO-1 may also modulate cardiac function. Recently, Segers et al. (49) showed that pharmacological inhibition of HO, although having no effect on basal function, reduced the inotropic response to β-adrenergic stimulation with isoprenaline in isolated rabbit myocardium. It is tempting to speculate that the modest increase in basal HO activity achieved with AAV may be sufficient to induce sustained preconditioning of the myocardium so that it can withstand transient ischemic insults; however, this possibility remains to be proven. On the other hand, emerging evidence suggests that HO-1 may have other biological effects in addition to its role in the enzymatic breakdown of heme. Recently, HO-1 was reported to protect human monoblastic lymphoma cells from hydroperoxide-induced injury independently of its catalytic activity (23). HO-1 also functions as a phosphoprotein and interacts with other signaling molecules, including the survival kinase Akt (47). Our group (8) reported recently that HO-1 functions codependently with Akt to confer protection from pro-oxidant-induced injury in human aortic smooth muscle cells. Considering the importance of Akt in myocardial homeostasis (34), the possibility that Akt and HO-1 may function in a similar fashion to exert cardioprotection is intriguing and merits further investigation. In addition, HO-1 translocates to the nucleus (33, 45), raising the possibility that it may modulate nuclear events such as transcription and DNA binding. Thus the mechanism of HO-1 cardioprotection may involve both catalytic and noncatalytic processes. The extent to which these processes contribute to AAV-HO-1-mediated cardioprotection remain to be determined.

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In addition to its cardioprotective effects, HO-1 may also modulate cardiac function. Recently, Segers et al. (49) showed that pharmacological inhibition of HO, although having no effect on basal function, reduced the inotropic response to β-adrenergic stimulation with isoprenaline in isolated rabbit myocardium.
In this regard, this new concept represents a significant shift in the therapeutic strategy for long-term myocardial protection. In this therapeutic strategy, must be addressed in future protocols to assess the safety of CO may impair cardiac function is a prevailing concern that infarction, whereas the LacZ-treated animals showed overt preserve LV function and dimensions for the 1 yr following animals. However, the HO-1-treated animals were able to whether this is due to the slightly elevated HO activity in these treated control animals (Fig. 4). basal EF in the HO-1-transduced animals relative to the LacZ-...cyclic stress such as I/R. The authors attributed these effects specifically to HO-1 activity, based on their findings with stannous protoporphyrin. However, this reagent is not a specific inhibitor of HO-1, and, consequently, the role of HO-1 in regulation of cardiac contractility could not be conclusively established. Interestingly, our group (31) recently reported that exogenous induction of HO-1 with hemin markedly enhances cardiac performance in the presence of pressure overload. However, the inotropic effect of hemin was not affected by inhibition of guanylate cyclase, suggesting that it is unlikely to be mediated by HO-1/CO-dependent stimulation of cGMP production (J. A. Simpson and L. G. Melo, unpublished observations). On the other hand, HO-1 could potentially maintain or improve cardiac performance by reducing coronary vascular tone. HO-derived CO activates soluble guanylate cyclase analogous to nitric oxide and has been reported to contribute to ischemia-induced vasodilation in dogs (38). Furthermore, bilirubin improved postischemic LV functional recovery in isolated rat hearts subjected to global ischemia (21). The maintenance of adequate coronary perfusion by CO during I/R may help preserve myocardial ATP and creatine phosphate levels to bring about bioenergetic balance in the ischemic myocardium (29). In contrast to these findings, Liu et al. (32) showed that CO blunts contractile performance of isolated papillary muscles from rats with cirrhotic cardiomyopathy, suggesting that increased myocardial HO activity may have adverse effects on cardiac function. In the current study, we observed a slight (~5%), albeit not significant, decrease in basal EF in the HO-1-transduced animals relative to the LacZ-treated control animals (Fig. 4G). We have not established whether this is due to the slightly elevated HO activity in these animals. However, the HO-1-treated animals were able to preserve LV function and dimensions for the 1 yr following infarction, whereas the LacZ-treated animals showed overt signs of heart failure. Notwithstanding this, the possibility that CO may impair cardiac function is a prevailing concern that must be addressed in future protocols to assess the safety of this therapeutic strategy.

The current study provides further support for a preemptive therapeutic strategy for long-term myocardial protection. In this regard, this new concept represents a significant shift in the treatment of heart disease, focusing on prevention rather than rescue. Such a strategy, if validated in humans, would remove some of the limitations of the current drug therapies used in the treatment of ischemic cardiomyopathy and MI, namely, the narrow time window for successful intervention (6, 59) and the unsuitability of these therapies for use in no-option patients with advanced CAD that are not suited for surgical revascularization (22). Given these limitations and the current ability to detect and assess asymptomatic patients at risk for myocardial damage with sensitive imaging technologies and serum biomarkers (17, 41, 51), the prospect of preventative therapy for patients at risk of myocardial injury is an attractive alternative to post-MI rescue. For these high-risk patients, sustained protection of the myocardium is ideal because of the unpredictable nature of acute coronary events (6). However, the current study does not provide any insight regarding the safety and efficacy of this strategy in the target patient population, namely, those with untreatable advanced CAD. Indeed intramyocardial injection has inherent risks, including increased probability of arrhythmias and hemorrhage. Although these problems are mitigated by the use of guiding and mapping techniques that allow precise delivery of the vector to the target areas of the myocardium, the risk of life-threatening arrhythmias cannot be overlooked. The future in this regard may rest with the use of new AAV vector serotypes with enhanced myocardial tropism that could be used for systemic gene delivery (58). Notwithstanding these limitations, we believe that our results support the feasibility of preemptive gene therapy for cardioprotection and merit further preclinical investigation using larger animal models of heart disease.

In summary, the current study shows that predelivery of HO-1 gene by AAV reduces mortality and preserves LV function and chamber dimensions 1 yr after acute MI. We conclude that preemptive HO-1 gene delivery may be useful as a protective therapy for patients at high risk of MI, such as no-option patients with CAD and patients that are refractory to risk reduction therapy.

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