Chronic Recurrent Myocardial Ischemic Injury Is Significantly Attenuated by Pre-Emptive Adeno-Associated Virus Heme Oxygenase-1 Gene Delivery

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OBJECTIVES
We assessed the hypothesis that overexpression of the antioxidant enzyme heme oxygenase (HO-1) may protect against chronic recurrent ischemia/reperfusion injury.

BACKGROUND
Multiple and recurring episodes of myocardial ischemia can result in significant myocardial damage, including myocyte death, fibrosis, and wall thinning, leading to impaired ventricular function and cardiac failure.

METHODS
In this study we used a closed-chest rodent model of chronic recurring myocardial ischemia and reperfusion to investigate the efficacy of pre-emptive gene therapy in overexpressing the antioxidant enzyme HO-1, using adeno-associated virus (AAV)-2 as the delivery vector.

RESULTS
We show that constitutive overexpression of HO-1 can prevent myocardial wall thinning, inflammation, fibrosis, and deterioration of cardiac function (as measured by echocardiography, histology, and immunohistochemistry) induced by repeated transient myocardial ischemia and reperfusion injury. With HO-1 therapy, there was a significant reduction in apoptosis as determined by levels of markers of survival proteins and terminal deoxynucleotidyltransferase dUTP nick end-labeling staining. This prevention of tissue damage was also associated with reduction in superoxide generation.

CONCLUSIONS
Taken together we provide the first evidence of the therapeutic efficacy of pre-emptive AAV–HO-1 delivery for prevention against multiple ischemic injury. This approach protects myocytes by simultaneously activating protective response and inhibiting pathological left ventricular remodeling and, therefore, may be a useful cardio-protective strategy for patients with coronary artery disease at a high risk for recurrent myocardial ischemia.

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Despite the significant advances in pharmacological and interventional therapies for acute coronary syndrome, ischemic heart disease remains the leading cause of death in the western world (1). Gene transfer strategy to overexpress a therapeutic protein for gain of function has been proposed as a strategy for the treatment of acute ischemic disease (2). Although several gene delivery studies have reported myocardial salvage against acute ischemia and reperfusion (I/R) (3–5), they have the following limitations: the therapeutic gene has to be delivered concomitant with acute I/R, the duration of gene expression is often short, and there is no evidence that gene therapy can be effective against multiple ischemic episodes over time. These problems are particularly relevant clinically because the onset of acute ischemia is unpredictable, and in high-risk patients the ischemic events are frequent, recurring, and potentially silent over a lifetime.

To address the first challenge, we have developed the concept of “pre-emptive gene therapy” by intramyocardial administration of the therapeutic gene (heme oxygenase [HO] and superoxide dismutase) in advance of the I/R using recombinant adeno-associated virus (AAV) delivery (6,7). Furthermore, we have shown that inducible gene expression using a hypoxia-responsive element to regulate therapeutic gene expression is particularly effective in protecting the myocardium from acute I/R injury (8).

In this study we addressed the second challenge, i.e., whether our approach will provide long-term protection of the myocardium against multiple, recurring episodes of ischemia over time. This issue represents a broad clinical problem in that tissue ischemia can be silent and can lead to extensive damage in many end organs (such as the heart, brain, and kidney), resulting in progressive diseases such as ischemic cardiomyopathy and vascular dementia. We chose HO-1 as the therapeutic gene on the basis of evidence that the catalytic by-products of this enzyme exert antioxidant and cytoprotective effects (9–12). However, it is not known if pre-emptive delivery of the HO-1 gene can confer myocardial protection in the context of chronic intermittent episodes of ischemia and reperfusion.

In this study we employed a rodent closed-chest model of repetitive I/R induced over multiple days to examine the effect of intramyocardial rAAV–HO-1 gene therapy. Our
data demonstrated that a single administration of AAV–HO-1 several weeks in advance of the injury protected against deleterious effects of subsequent repeated episodes of ischemia and, importantly, resulted in the activation of cellular survival processes that led to cytoprotective effects.

**METHODS**

Plasmids and HO–1 vector construction. A 986-bp fragment of HO-1 containing the open reading frame sequence was cleaved from the pBS KS cloning vector at KpnI-PstI sites and subcloned at the corresponding sites in puc 18 plasmid. The insert was cut at EcoRI sites and cloned into corresponding sites in rAAV2 backbone (pAAV–CMV–HO-1) containing the cytomegalovirus promoter and the bovine growth hormone poly-adenylation signal flanked by the AAV inverted terminal repeat sequences. Packaging, propagation, and purification of AAV-2 viral particles were carried out at the Harvard Gene Therapy Initiative Core Facility (Boston, Massachusetts) by standard procedures.

RNA extraction and reverse transcriptase-polymerase chain reaction of HO-1. For reverse transcriptase-polymerase chain reaction (RT-PCR) detection of HO-1 transcripts, 100 ng of total RNA was used for first-strand cDNA synthesis and PCR amplification with the One-Step Platinum Taq RT-PCR kit (Life Technologies, Carlsbad, California). A 185-bp fragment was amplified for 30 cycles of the reaction. cDNA synthesis and PCR amplification with the One-Step Platinum Taq RT-PCR kit (Life Technologies, Carlsbad, California) by standard procedures.

**Abbreviations and Acronyms**

- **AAV**: adeno-associated virus
- **ANOVA**: analysis of variance
- **DHE**: dihydroethidium
- **ECG**: electrocardiogram
- **HO**: heme oxygenase
- **I/R**: ischemia and reperfusion
- **LAD**: left anterior descending coronary artery
- **PBS**: phosphate-buffered saline
- **RT-PCR**: reverse transcriptase-polymerase chain reaction
- **SD**: Sprague-Dawley
- **TUNEL**: terminal deoxynucleotidyltransferase dUTP nick end-labeling

**Echocardiography.** Animals underwent echocardiography 12 days after the last I/R injury. Rats were anesthetized with isoflurane for echocardiographic examinations. Rat heart rates ranged from 350 to 400 beats/min during echocardiography. Echocardiography was performed using a 6- to 15-MHz Ultraband Intraoperative Linear Array probe and a Sonos 5500 ultrasound imaging system (Philips Ultrasound, Andover, Massachusetts). Images were obtained from both the parasternal short and long axes, saved to cine-loop utilizing native frame rates of >60 Hz, and analyzed utilizing an off-line analysis program. Endocardial borders were traced and end-systolic and end-diastolic areas and volumes were calculated. Echocardiographic acquisition and analyses were performed by a technician who was blinded to treatment groups.

**Histology.** Following the echocardiography, the hearts were harvested, washed in phosphate-buffered saline (PBS), and fixed in 10% formalin overnight at 4°C. Paraffin-embedded tissues were cut into 5-μm-thin sections and stained with hematoxylin and eosin and by Masson's Trichrome stain for collagen determination. Parallel sections were stained with mouse alpha-smooth muscle actin antibody (IMMH-2, Sigma, St. Louis, Missouri) for detection of myofibroblasts.

**Detection of superoxide ion generation.** Superoxide generation was assessed via the conversion of dihydroethidium (DHE) to ethidium as previously described (14). Dihydroethidium enters the cell and is oxidized primarily by superoxide to yield fluorescent ethidium, which intercalates into deoxyribonucleic acid and is thereby retained in the cell. Briefly, hearts were harvested and snap-frozen in embedding medium and stored at −80°C. Tissues were then sectioned in 6-micron-thin stepwise sections from the apical to the basal region of the heart. Sections were then washed once with 1X PBS and incubated with 5 μM DHE in 1X PBS for 30 min at 37°C. Sections were then cover-slipped with the aqueous media and visualized with a laser scanning confocal microscope equipped with a krypton/argon laser. Experimental and control tissues were processed and imaged in parallel by a person blind to the experiment. Laser settings were identical for acquisition of images from the specimens. Fluorescence was detected with a 585-nm long-pass filter.
Apoptosis detection by terminal deoxynucleotidyltransferase dUTP nick end TUNEL staining. For this experiment, tissues were harvested 12 hours after the last ischemic episode. Apoptosis was detected using a CardioTacs staining kit (Trevigen, Gaithersburg, Maryland) as recommended by the manufacturer. Briefly, unfixed frozen heart sections were cut into 6-μm-thin sections, thawed, dried, dehydrated, and fixed with 3% paraformaldehyde for 10 min. Sections were then permeabilized with cytonin, and endogenous peroxide was quenched with a 5% hydrogen peroxide solution. During apoptosis the chromosomal DNA is cleaved by endonucleases to generate DNA fragments with free 3′-hydroxyl residues. These 3′ ends of cleaved DNA fragments were labeled with the enzyme terminal deoxynucleotidyl transferase, which adds nucleotides at the site of DNA breaks. The incorporated biotinylated nucleotides were visualized by binding of streptavidin-horseradish peroxidase followed by reaction with TACS Blue Label to generate a dark blue precipitate. The tissues were viewed under a standard light microscope where the apoptotic cells are clearly and unequivocally distinguished by the dark blue nuclear staining. The positive control was generated by the addition of TACS-Nuclease, which generates the DNA breaks. Both TUNEL-positive and total nuclei were...
Table 1. Echocardiographic analyses after repeated I/R

<table>
<thead>
<tr>
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<th>Sham</th>
<th>HO-1</th>
<th>LacZ</th>
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<tbody>
<tr>
<td>PWT (mm)</td>
<td>0.117 ± 0.002</td>
<td>0.115 ± 0.003†‡</td>
<td>0.088 ± 0.003*</td>
</tr>
<tr>
<td>AWT (mm)</td>
<td>0.117 ± 0.002</td>
<td>0.115 ± 0.003†‡</td>
<td>0.088 ± 0.003*</td>
</tr>
<tr>
<td>LAVS (mm)</td>
<td>0.026 ± 0.005</td>
<td>0.040 ± 0.004†‡</td>
<td>0.098 ± 0.003*</td>
</tr>
<tr>
<td>LAAS (mm)</td>
<td>0.143 ± 0.013</td>
<td>0.183 ± 0.006†‡</td>
<td>0.313 ± 0.003*</td>
</tr>
<tr>
<td>LAVD (mm)</td>
<td>0.155 ± 0.006</td>
<td>0.170 ± 0.004†‡</td>
<td>0.215 ± 0.005*</td>
</tr>
<tr>
<td>LAAD (mm)</td>
<td>0.417 ± 0.006</td>
<td>0.445 ± 0.005†‡</td>
<td>0.518 ± 0.005*</td>
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Ejection fraction (%) 81 ± 3 72 ± 2†‡ 51 ± 2*

*aLacZ vs. sham, p < 0.05; †HO-1 vs. sham, not significant; ‡HO-1 vs. lacZ, p < 0.05; n = 5–6/group.

Western blots. Tissues were harvested 12 h after the last episode of I/R injury and homogenized in tris HCl buffer, and protein concentration was determined by Bradford assay. Protein (12 μg) was loaded on acrylamide gel, transferred to polyvinylidiene difluoride membrane, and probed with either bcl-xl (Cell Signaling), bax (Cell Signaling, Danvers, Massachusetts), phospho-bad (Ser112) (Cell Signaling), bcl-2 (Novus Biologicals, Littleton, Colorado), and HO-1 (Stressgen, Canada) antibodies as described previously (8).

Statistics. All results are expressed as mean ± SEM. Determination of densitometric analyses of bax, bcl-xl, and inhibitor of apoptosis protein gene expression were measured by one-way analysis of variance (ANOVA). For echocardiography, ANOVA with an i-test post-hoc analysis was performed to determine differences between the groups. A value of p ≤ 0.05 was considered to be statistically significant.

RESULTS

AAV-mediated intra-myocardial over-expression of HO-1 in rat hearts with repetitive I/R. We performed the surgical studies according to the protocol described previously (13), with slight modification as described in Methods. The rAAV-CMV-HO-1 vector was injected at five sites corresponding to the area at risk, as shown previously (6). We achieved almost 30% transduction efficiency using this protocol as demonstrated by HO-1 positive immunostaining (data not shown). Five weeks after gene delivery, the chest was reopened and a suture was placed around the left anterior descending coronary artery (LAD) (without ligation) and exteriorized underneath the skin, and the chest was closed. After a one-week recovery period, animals underwent the repetitive I/R protocol as described in the Methods section (Fig. 1). Ischemia was confirmed by the increase in ST-segment as determined by electrocardiogram (ECG), and a representative tracing is shown in Figure 1C. We determined HO-1 gene expression by semi-quantitative RT-PCR and Western blot, respectively, in the injected animals not subjected to I/R protocol. As shown in Figure 1E, the HO-1 protein levels were significantly higher in the HO-1-treated as compared to lacZ-treated animals. Moreover, the increase in HO-1 protein was present in the ischemic area corresponding to the area targeted by gene transfer, whereas in the extracts from the remote region of the left ventricle, the HO-1 protein levels were not elevated and not significantly different from the lacZ-treated ventricles.

Effect of HO-1 overexpression on ventricular function and remodeling of ischemic myocardium. We evaluated the consequence of HO-1 gene transfer after repeated ischemia and reperfusion injury over time on ventricular remodeling and function. Echocardiographic analyses performed one week after the last ischemic episode of repetitive I/R injury demonstrated significant improvement in ejection fraction in HO-1-treated animals as compared to lacZ-treated controls. (* vs. sham; ** vs. lac Z, p < 0.05, n = 5 to 6/group). (B) Representative picture of M-Mode echocardiogram showing significant increase in left ventricle cavity and decrease in wall thickness in lacZ-treated animals when compared to HO-1-treated group. Other abbreviations as in Figure 1.
Figure 3. Effect of HO-1 gene transfer on ventricular remodeling and fibrosis after repeated I/R. (A) Masson Trichrome staining shows significant anterior wall thinning and collagen deposition two weeks after the last ischemic episode in lacZ-treated animals in contrast to HO-1–treated rats with decreased collagen deposition. Quantification of the fibrotic area as a percentage of total left ventricular area shows significant attenuation of fibrosis in the HO-1–treated animals as compared to saline or lacZ-treated controls. (n = 4/group, * vs. saline and lacZ, p < 0.05) (B) Representative alpha-smooth muscle actin antibody staining demonstrates positive staining both in vessel walls as well as in the myocardium in lacZ-treated animals indicating higher phenotypic conversion to cardiac fibroblasts as compared to HO-1–treated animals, which demonstrated positive staining only in the vessel walls. Abbreviations as in Figure 1.
I/R protocol demonstrated severe wall thinning and reduced ejection fraction in the lacZ-treated animals relative to the HO-1–treated animals (Table 1, Fig. 2). Left ventricular function and chamber dimensions in the HO-1–treated animals were almost identical to sham animals (Table 1), indicating nearly complete prevention of left ventricular wall remodeling. Marked wall thinning and fibrosis were observed at one week after the last ischemic episode in the lacZ-treated controls, which was completely prevented in HO-1–treated animals (24.7 ± 5.1 mm² vs. 10.8 ± 4.3 mm²) (Fig. 3A).

Gross and microscopic histological analyses of hearts one week after the last ischemic episode in the lacZ-treated animals revealed severe wall thinning, inflammatory cell infiltration, and collagen deposition that was absent in the HO-1–treated hearts. In addition, interstitial myofibroblasts (stained with alpha-smooth muscle actin antibody) were observed in the ischemic area in the lacZ-treated animals, while HO-1–treated animals exhibited only a sporadic presence of these cells (Fig. 3B). Quantitatively, there was a significant decrease in the smooth muscle actin positive area in the HO-1–treated animals as compared to lacZ- or saline-treated controls (3,733 ± 719 mm² vs. 14,877 ± 2,800 mm² or 11,674 ± 1,572 mm²).

Figure 4. Expression of markers of apoptosis after repeated I/R. (A) Representative Western blots of two samples from each group (except in sham) from myocardial samples obtained 12 h after the last episode of intermittent I/R. (B) Bcl-2 and Bcl-xL were highly induced in lacZ-treated animals, while phospho-bad was elevated in the HO-1–treated animals. There was no change in levels of bax and alpha-sarcomeric actin levels. HO-1–injected animal without I/R injury was used as a negative control. Abbreviations as in Figure 1.
was a significant increase in levels of anti-apoptotic proteins, bcl-2 and bcl-xl, in lacZ-treated animals. Furthermore, there was a significant increase in phosphorylated bad at Ser 112, in HO-1–treated animals compared to lacZ-treated controls (Fig. 4). There was no change in the expression of bax and alpha-sarcomeric actin protein levels between the different groups. It is important to note that HO-1 overexpression alone (without myocardial I/R injury) did not induce changes in the levels of survival proteins. At the same time, apoptotic cell death in the myocardium, as measured by TUNEL staining was reduced by approximately 50% in the HO-1–treated animals when compared to lacZ–treated animals (0.31 ± 0.045 vs. 0.59 ± 0.035, p < 0.005, n = 5 to 6/group). Apoptotic index was determined as described in Methods. Other abbreviations as in Figure 1.

**DISCUSSION**

Because the occurrence of I/R is unpredictable and recurring, we hypothesize that pre-emptive administration of a therapeutic gene by a vector that enables long-term expression may provide effective long-term protection against chronic multiple episodes of I/R and may be ideal for high-risk patients who experience recurring episodes of ischemia. In this study, our data show that prior delivery of AAV–HO-1 five weeks in advance provides significant protection against recurring multiple I/R injury and the
development of myocardial damage as characterized by diffuse focal fibrosis, wall thinning, and ventricular dilation in the absence of evidence of a large infarct.

Several biological properties of AAV make it a suitable vector for long-term cardioprotective gene therapy. Adenovirus associated virus is weakly immunogenic, thereby circumventing a robust immune response by the host (15,16). Studies from our laboratory and others have demonstrated that AAV can provide stable expression of the transgene with long-term protection against the deleterious effects of irreversible cell loss. An important caveat of this study is that we injected the transgene into normal myocardium. However, in the clinical setting, it is likely that patients at high risk of recurrent ischemia may have scars or already hibernating myocardium. It remains to be determined whether overexpression of HO in already scarred tissue affects an already irreversible cell loss. An important caveat of this study is that we injected the transgene into normal myocardium. However, in the clinical setting, it is likely that patients at high risk of recurrent ischemia may have scars or already hibernating myocardium. It remains to be determined whether overexpression of HO in already scarred tissue affects an already irreversible cell loss. An important caveat of this study is that we injected the transgene into normal myocardium. However, in the clinical setting, it is likely that patients at high risk of recurrent ischemia may have scars or already hibernating myocardium. It remains to be determined whether overexpression of HO in already scarred tissue affects an already irreversible cell loss.

Our data are supported by several recent studies that have demonstrated that phosphorylation of bad is good for cell survival (23). Recently, it has been demonstrated that addition of heme results in degradation of bad and upregulation of bcl-xl expression in human neutrophils (24). Since the main function of HO is to degrade heme and if heme inactivates bad, then our data would suggest that overexpression of HO may not only prevent bad degradation but also induce its phosphorylation, which may be part of an endogenous survival gene program protecting against irreversible cell loss. An important caveat of this study is that we injected the transgene into normal myocardium. However, in the clinical setting, it is likely that patients at high risk of recurrent ischemia may have scars or already hibernating myocardium. It remains to be determined whether overexpression of HO in already scarred tissue affects an already irreversible cell loss.
The hearts from HO-1 knock-out mice have greater susceptibility to I/R injury (25), thus suggesting a protective role of HO-1 in myocardial injury. In addition, we have previously demonstrated that HO-1 markedly reduced myocardial injury after a single episode of I/R injury (6,8). The protective effect of HO-1 is, at least in part, due to the inhibition of pro-inflammatory and pro-oxidant mechanisms activated by reperfusion (6,26), which can trigger the cascade of events that leads to cell death. Thus, HO-1 overexpression may have a sparing effect on the ischemic myocardium to reduce I/R-induced injury and prevent the sequence of events leading to cell loss and subsequent ventricular dysfunction. Indeed, HO-1 knock-out mice also show significantly increased apoptosis after chronic exposure to hypoxia (27), indicating a protective role of HO-1 against the development of apoptosis and fibrosis.

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REFERENCES