Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury

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Ischemia and reperfusion represent major mechanisms of tissue injury and organ failure. The timing of administration and the duration of action limit current treatment approaches using pharmacological agents. In this study, we have successfully developed a preemptive strategy for tissue protection using an adenoassociated vector system containing erythropoietin hypoxia response elements for ischemia-regulated expression of the therapeutic gene human heme-oxygenase-1 (hHO-1). We demonstrate that a single administration of this vector several weeks in advance of ischemia/reperfusion injury to multiple tissues such as heart, liver, and skeletal muscle yields rapid and timely induction of hHO-1 during ischemia that resulted in dramatic reduction in tissue damage. In addition, overexpression of therapeutic transgene prevented long-term pathological tissue remodeling and normalized tissue function. Application of this regulatable system using an endogenous physiological stimulus for expression of a therapeutic gene may be a feasible strategy for protecting tissues at risk of ischemia/reperfusion injury.

Ischemia and reperfusion (I/R)-induced tissue injury are major causes of mortality and morbidity in the civilized world (1). I/R injury can develop as a consequence of hypotension, shock, or bypass surgery leading to end-organ failure such as acute renal tubular necrosis, liver failure, and bowel infarct. I/R injury can also develop as a result of complications of vascular disease such as stroke and myocardial infarction. In addition, multiple subclinical I/R incidents can induce cumulative tissue injury leading to chronic degenerative diseases such as vascular dementia, ischemic cardiomyopathy, and renal insufficiency. Cytoprotective strategies using pharmacological agents have yielded limited success in the prevention of I/R injury (2–6). Practical difficulties with timing of administration of the therapy, achieving adequate tissue levels of therapeutic product, and unregulated transgene expression pose significant clinical challenges. Several drug-inducible gene expression systems have been developed (7–10). However, these systems require the exogenous administration of a ligand to induce expression of the therapeutic product and usually yield high basal levels of gene expression, thus reducing the efficacy of regulation.

One of the major mechanisms by which the cells control gene expression during low oxygen tension involves the activation of transcription factor hypoxia-inducible factor 1α (HIF1α), which is quickly degraded during normoxic conditions by ubiquitination mechanisms (11, 12) that include proline hydroxylation and acetylation (13, 14). Activation of HIF1α leads to transcription of several target genes such as vascular endothelial growth factor (15), erythropoietin, nitric oxide synthase, and several antioxidant enzyme systems such as superoxide dismutase, and heme-oxygenase-1 (HO-1), which may provide protection against I/R injury. We have previously shown marked reduction of postinfarction myocardial injury with constitutive HO-1 overexpression (16). However, constitutive HO-1 expression could potentially lead to long-term tissue toxicity and cellular damage (17, 18).

Therefore, an ideal strategy for tissue protection against I/R injury would be a single administration of a therapeutic gene using a vector that will provide regulated transgene expression in response to an endogenous pathophysiological stimulus such as hypoxia. Accordingly, in this study, we have developed an adenoassociated vector (AAV) containing tandem repeats of erythropoietin hypoxia response elements (HREs) for hypoxic/ischemic regulation of human HO-1 (hHO-1) expression. Our data demonstrate that a single administration of this vector before injury into rat skeletal muscle, liver, and heart yields low basal expression during normoxic conditions but is readily induced to express high levels of therapeutic gene in response to acute I/R *in vivo*, providing protection against such injury.

Materials and Methods

Cell Culture, Transfection, and Hypoxic Treatment. HEK 293 cells were plated under normal conditions. At 60–80% confluency, the cells were transfected with AAV-4HRE-mSV40-GFP, AAV-4HRE-mSV40-luciferase, or AAV-mSV40-lucifersase (no HREs). After 48 h of transfection, cells were switched to serum-free media. After 12 h of incubation under serum-free media, cells were either incubated under normoxic conditions or switched to hypoxic conditions for further 16 h. After this incubation period, cells were either visualized for GFP expression or harvested for luciferase assay.

***In Vivo* Gene Delivery.** Male Sprague–Dawley rats weighing 175–200 g were purchased from Charles River Laboratories and were 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. Myocardium gene delivery was performed on normal Sprague–Dawley rats 5 weeks in advance of I/R injury as described (16). For direct gene delivery to the tibialis anterior skeletal muscle of the left hind limb, a small incision was made in the skin and a total of 4×10^{11} infectious particles of either recombinant AAV-4EpoHRE-mSV40-hHO-1 or the control vector AAV-4EpoHRE-mSV40-lacZ were injected in five sites. The incision was closed, and animals were allowed to recover. For the liver gene delivery, a midline incision was made in the abdomen. Animals were injected with similar concentration of the virus described above via the portal vein slowly over a period of 5 min. After the injection, the incision was closed, and animals were returned to their cages. All surgical and experimental procedures were approved by the Harvard Medical Area Standing Committee on Animals.

Abbreviations: I/R, ischemia and reperfusion; HO-1, heme-oxygenase-1; hHO-1, human HO-1; AAV, adenoassociated vector; HRE, hypoxia response element.

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I/R Models of Heart, Liver, and Skeletal Muscle. Five weeks after gene delivery, acute I/R was conducted. Myocardial I/R injury was performed as described (16). For liver I/R, portal vein and hepatic artery were identified and clamped for a period of 1 h. Ischemia was confirmed by discoloration of the hepatic lobes. At the end of ischemic period, vessels were unclamped and reperfusion was achieved. Animals were killed at 24 h after reperfusion. For hind limb I/R, a tourniquet method was used as described (19).

Morphometric Determination of Myocardial Infarct Size. Twenty-four hours after reperfusion, the heart was excised and rinsed in ice-cold PBS. Five to six biventricular sections of equivalent thickness were made perpendicular to the long axis of the heart, incubated in 1% triphenyl tetrazolium chloride (Sigma) in PBS (pH 7.4) for 15 min at 37°C, and photographed on both sides. Infarct size was calculated as described (16).

Statistical Analysis. All results are expressed as mean ± SEM. Determination of HO-1 protein content, activity assay and densitometric analyses of tumor necrosis factor α (TNF-α) and IL-6 gene expression were measured by one-way ANOVA. $P \leq 0.05$ was considered to be statistically significant.

Results

Hypoxic Induction of GFP and Luciferase in HEK 293 Cells. To test the inducibility of gene expression under hypoxia, we transfected HEK 293 cells with plasmids AAV-4EpoHRE-mSV40-GFP and AAV-4EpoHRE-mSV40-luciferase constructs that contain four tandem repeats of erythropoietin HREs and a minimal simian virus 40 (SV40) promoter (see Fig. 1 and supporting information, which is published on the PNAS web site). Cells under hypoxia demonstrated very high levels of GFP expression compared with cells incubated under normoxic conditions transfected with the same plasmid (Fig. 1*a*).

To quantitate the level of reporter gene induction, we transfected HEK293 cells with AAV-4EpoHRE-mSV40-luc and incubated them under same conditions as described above. Under hypoxic condition, HEK 293 cells expressed luciferase at ≈8 times higher levels than cells transfected with the same plasmid incubated under normoxic conditions (Fig. 1*b*). To determine that the inducibility was mainly caused by the HRE tandem repeats, we transfected HEK 293 cells with AAV-mSV40-luciferase plasmid lacking the HREs and incubated these cells under the same conditions as above. Exposure of these cells to hypoxia did not lead to any significant induction of luciferase activity, thereby demonstrating the specificity of the HREs in regulating gene expression under hypoxic conditions.

Ischemic Induction of hHO-1 Prevents I/R-Mediated Injury in Skeletal Muscle, Liver, and Myocardium. To determine whether our composite vector is responsive to hypoxia *in vivo* in multiple tissues, we injected 6- to 8-week-old Sprague–Dawley rats with 4×10^{11} viral infectious particles of either AAV-4EpoHRE-mSV40-LacZ or AAV-4EpoHRE-mSV40-HO-1 via direct injection into the skeletal muscle and heart and via the portal vein to the liver. Five weeks after therapeutic gene delivery into the tibialis anterior muscle of the left limb, I/R was induced by the tourniquet method (19). In the animals injected with the vector but without I/R injury, we could not detect any transgene expression by semiquantitative RT-PCR up to 38 cycles, indicating little or negligible gene expression. Hind limb ischemia resulted in significant induction of the transgene (Fig. 2*a*), whereas hHO-1 mRNA was not detected in the lacZ-transduced rats, indicating the specificity of the primers to the transgene. We assessed the functional effect of transgene induction by determining the extent of tissue injury by using histological methods and biochemical markers of injury such as serum creatine kinase (20) and tissue myeloperoxidase (MPO) (21) enzyme activity. The animals treated with the inducible vector showed attenuation of skeletal muscle injury, as demonstrated by reductions in serum creatine kinase (CK) levels compared with lacZ-treated controls (373 ± 2 vs. 597 ± 40 units/liter, respectively; $P < 0.05$; n = 3–4 per group), and trend toward decreased muscle MPO activities (165 ± 3.3 vs. 285 ± 99 OD per 0.5 g of tissue; $P < 0.06$; n = 3–4 per group) as well as a reduction in tissue necrosis and inflammation as detected by hematoxylin and eosin staining 4 h after reperfusion (Fig. 2*b*).

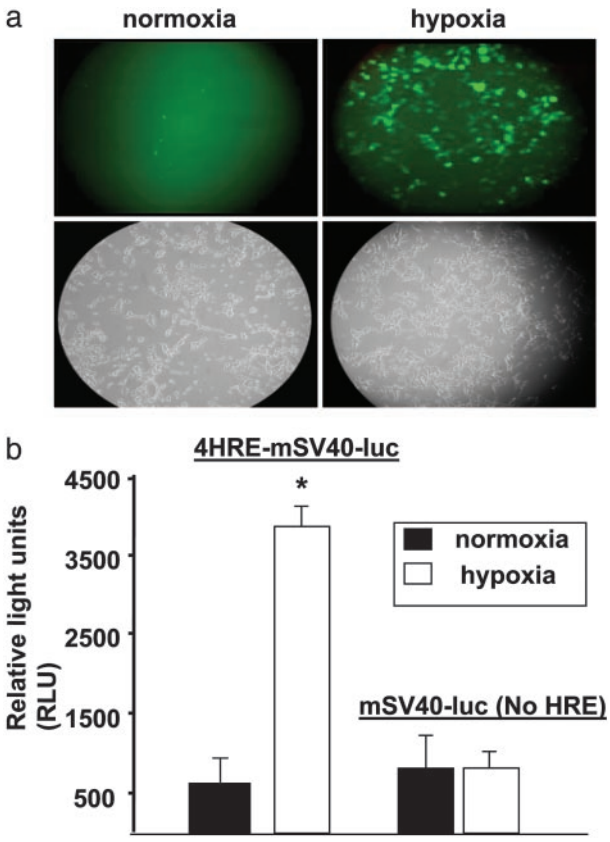

Fig. 1. Hypoxia induces reporter gene expression in HEK 293 cells. (*a*) Cells were transfected with plasmid AAV-4EpoHRE-mSV40-GFP, incubated under normoxic or hypoxic conditions, and visualized for GFP expression. (*b*) To quantitate induction of gene expression by hypoxia, HEK 293 cells were transfected with the plasmids containing the luciferase gene, incubated in hypoxia, and assayed for luciferase activity (mean of three separate experiments, $P < 0.05$).

As in skeletal muscle, liver ischemia resulted in transgene induction, which was not detectable at baseline or after 24 h of reperfusion (Fig. 2*a*). The brief induction of transgene expression resulted in significant attenuation of liver injury, as indicated by decrease in markers of liver injury such as serum alanine aminotransferase (ALT) (20) enzyme levels (204 ± 89 vs. 604 ± 156 units/liter; $P < 0.05$; n = 3–5 per group) and lung myeloperoxidase (MPO) enzyme activities (0.27 ± 0.06. vs. 0.57 ± 0.09 mean OD per 0.5 g of tissue; $P < 0.05$; n = 3–4 per group) 24 h after reperfusion. Similarly, significant increase in transgene mRNA expression was observed in the myocardium after 1-h ischemia (Fig. 2*a*). The level of hHO-1 transgene mRNA then returned to baseline when measured at 12 h after reperfusion and was barely detectable by 24 h. Positive HO-1 immunostaining was observed in hHO-1-treated animals after 1 h of ischemia but was absent at baseline and in lacZ-treated controls (see Fig. 2 and supporting information). Planar morphometric analyses of the triphenyl tetrazolium chloride-stained tissue sections (Fig. 3*a*) revealed a significant 65% decrease in the infarct size in the hHO-1-treated groups as compared with the lacZ control group

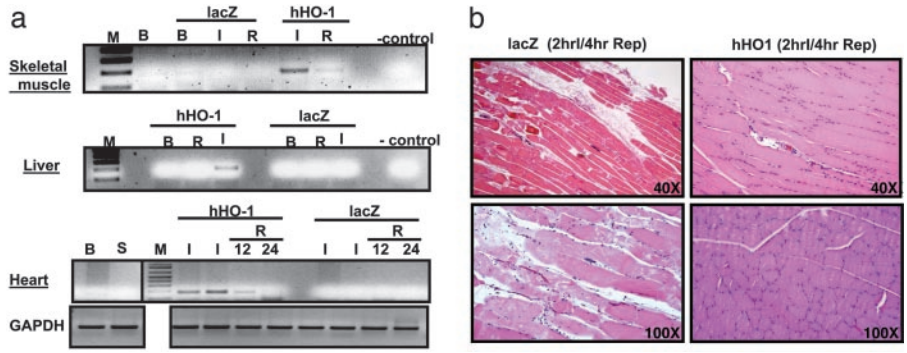

Fig. 2. Ischemic induction of hHO-1 prevents I/R-mediated injury in skeletal muscle, liver, and myocardium. (*a*) Semiquantitative RT-PCR analysis demonstrated significant increase in hHO-1 mRNA expression in hHO-1-treated tissues during ischemia, which was undetectable after 4 h of reperfusion in skeletal muscle and 24 h of reperfusion in liver and heart and in lacZ-treated controls (M, marker; I, ischemia; R, reperfusion; B, basal; S, sham). (*b*) Skeletal muscle tissue harvested from animals subjected to I/R were processed and subjected to hematoxylin and eosin staining. Representative sections from two lacZ- and hHO-1-treated rats demonstrated reduced skeletal muscle inflammation and preserved structure in hHO-1-treated rats.

(31.0 ± 5.58 vs. 12.1 ± 2.15; $P < 0.01$; $n = 5–6$ per group). Histopathological examination of sections stained with hematoxylin and eosin revealed significant reduction in necrosis in the HO-1-treated animals, whereas no protection was seen in the animals treated with control vector without the HREs (AAV-mSV40-HO-1) (Fig. 3*b*).

Ischemia Induces Temporal Differences in Transgene and Endogenous HO-1 Expression. To study the mechanisms and consequences of ischemia induction of transgene expression, we examined the differences in temporal patterns of expression between the transgene and the endogenous HO-1 gene during both ischemia and reperfusion in the myocardium. There was a significant increase in

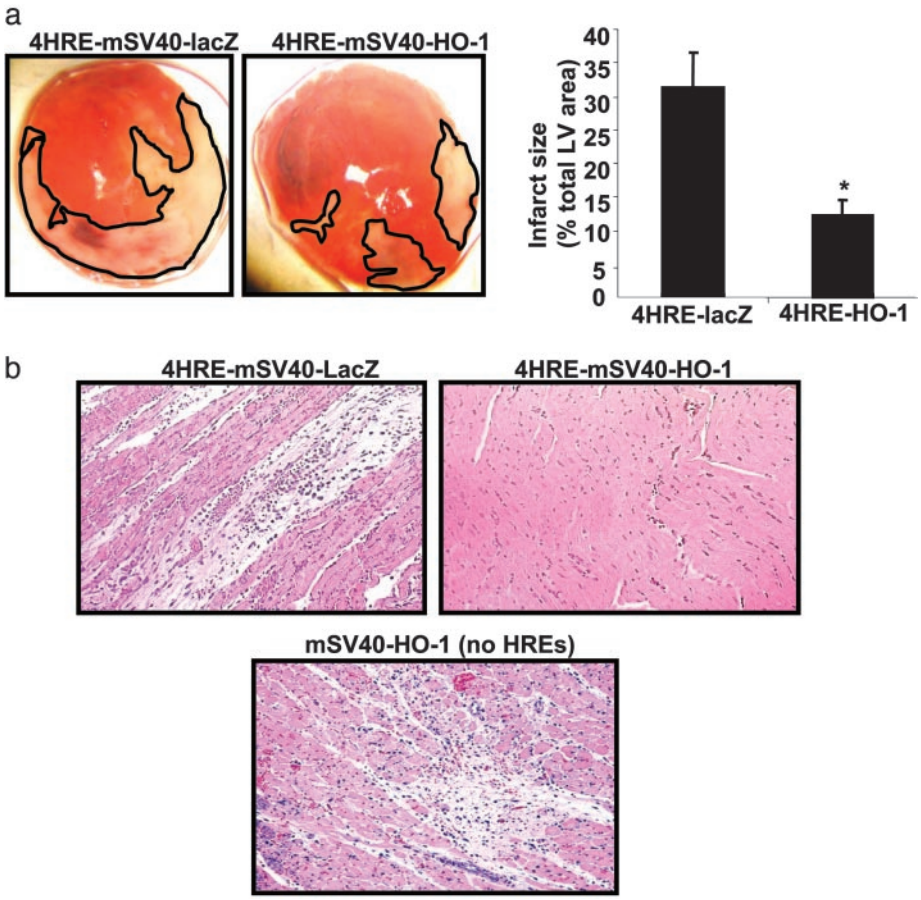

Fig. 3. Induction of transgene during ischemia protects the myocardium and reduces the infarct size. (*a*) Triphenyl tetrazolium chloride staining was performed to assess infarct size. In animals transduced with 4HRE-mSV40-lacZ, the infarct area was ≈30% of the total LV area, whereas in the presence of 4HRE-mSV40-HO-1, there was a significant decrease ($n = 5–6$ animals per group, $P < 0.05$) in the infarct size. (*b*) Hearts from animals transduced with 4HRE-mSV40-lacZ or 4HRE-mSV40-hHO-1 were harvested 24 h after I/R injury. Hematoxylin and eosin staining from hHO-1-transduced animals after I/R showed reduced inflammation and necrosis compared with large neutrophil-laden areas of necrosis in the LacZ-transduced group. Animals treated with vector without the HREs were not protected.

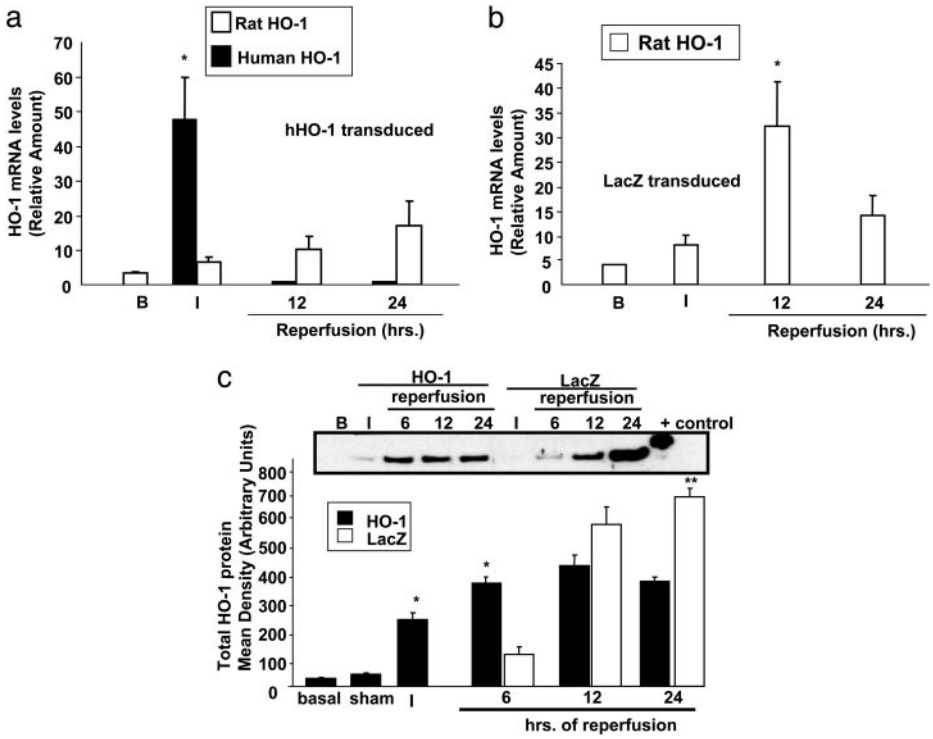

Fig. 4. Ischemia induces temporal difference in endogenous and exogenous HO-1 gene expression. (*a*) Real-time RT-PCR analyses demonstrated immediate increase in human HO-1 mRNA levels during ischemia; levels decreased by 12 h after ischemia and became undetectable by 24 h of reperfusion ($n = 5–6$ per group; * vs. basal, $P < 0.01$). (*b*) Real-time RT-PCR analysis demonstrated that endogenous HO-1 mRNA levels in lacZ-treated animals do not increase until 12 h of reperfusion ($n = 5–6$ per group; *, compared with ischemia alone, $P < 0.01$). (*c*) Western blot performed on microsomal fractions demonstrated a significant increase in HO-1 protein levels in response to ischemia and maximized up to 6 h of reperfusion in hHO-1 transduced animals when compared with baseline and lacZ controls ($n = 5–6$ per group; *, vs. basal or sham, $P < 0.05$; **, vs. HO-1 at 24 h, $P < 0.05$). Thereafter, there was an increase in HO-1 protein levels in lacZ-treated animals, but not in the hHO-1-treated animals.

exogenous human HO-1 mRNA expression within 1 h of ischemia (Fig. 4*a*). The level of hHO-1 transgene mRNA returned to baseline within 12 h after reperfusion and was barely detectable by 24 h. In contrast, endogenous rat HO-1 mRNA did not increase until 12 h after reperfusion in both control and AAV-4EpoHRE-mSV40-hHO-1-treated animals (Fig. 4 *a* and *b*). Interestingly, the endog-

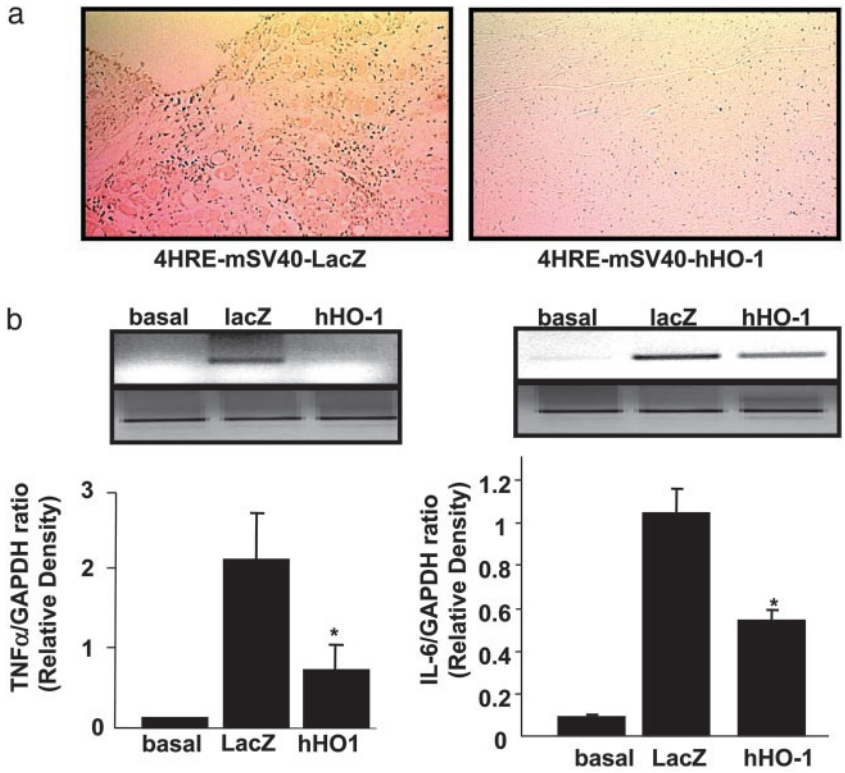

Fig. 5. Ischemia overexpression of transgene attenuates proinflammatory cytokine mRNA levels. (*a*) CD45 staining demonstrated massive infiltration of neutrophils in the infarcted area of the lacZ-treated animals, which was nearly absent in the hHO-1-treated animals. (*b*) Semiquantitative RT-PCR analyses demonstrated that, during ischemia, TNF-α and IL-6 mRNA levels were significantly attenuated in the 4HRE-mSV40-hHO-1-treated cells (0.73 ± 0.38 vs. 2.04 ± 0.61, $n = 5–6$ per group, $P < 0.05$) vs. the lacZ-treated controls (0.58 ± 0.09 vs. 1.05 ± 0.04, $n = 5–6$ per group, *, vs. lacZ group, $P < 0.05$).

Table 1. Echocardiographic analyses of rat myocardium 1 month after I/R injury

	Sham	hHO-1	LacZ
SWD, mm	0.2 ± 0.001	0.16 ± 0.003*	0.08 ± 0.002†
PWD, mm	0.2 ± 0.001	0.16 ± 0.002*	0.08 ± 0.002†
LAVD, mm	0.3 ± 0.01	0.37 ± 0.009*	0.50 ± 0.01†
LAVS, mm	0.12 ± 0.002	0.15 ± 0.006*	0.3 ± 0.008†
LAAD, mm	0.6 ± 0.02	0.73 ± 0.01*	0.87 ± 0.007†
LAAS, mm	0.39 ± 0.02	0.42 ± 0.01*	0.62 ± 0.007†
EF, %	69 ± 1.5	61 ± 1.06*	41 ± 2.18†
LALD, mm	1.13 ± 0.01	1.17 ± 0.01	1.24 ± 0.03

SWD, septal wall thickness (diastole); PWD, posterior wall thickness (diastole); LAVD, long axis volume (diastole); LAVS, long axis volume (systole); LAAD, long axis area (diastole); LAAS, Long axis area (systole); EF, ejection fraction; LALD, long axis length (diastole).
*HO-1 vs. lacZ, $P < 0.05$.
†lacZ vs. sham, $P < 0.05$.

enous rHO-1 mRNA levels were attenuated in the hHO-1-treated animals, suggesting that exogenous hHO-1 transgene overexpression exerted negative feedback on endogenous rHO-1 expression.

We determined HO-1 protein levels in the myocardial tissues obtained from both hHO-1- and lacZ-treated animals killed at various time points. At baseline, there was no difference in the HO-1 protein between sham-treated or uninjured animals, indicating low levels of basal expression that were not affected by the sham surgery (Fig. 4*c*). In the lacZ-transduced animals, HO-1 protein was not detectable until 12 h of reperfusion and showed a time-dependent increase. In contrast, there was an immediate 5-fold increase in total HO-1 protein levels in hHO-1-transduced animals (relative to lacZ controls) after 1 h of ischemia. The levels of total HO-1 protein were sustained for the 24-h reperfusion period in the hHO-1-treated animals. At 24 h of reperfusion, HO-1 protein levels in the lacZ-treated animals were higher than those in the hHO-1-treated animals, reflecting the endogenous HO-1 response in the absence of early tissue protection by therapeutic transgene expression. The time-dependent changes in total HO-1 protein levels were mirrored by parallel changes in total heme-oxygenase activity measured as the rate of bilirubin appearance (see Fig. 3 and supporting information).

hHO-1-Mediated Protection from I/R Injury Is Associated with Decreased Proinflammatory Cytokine Expression. Proinflammatory mediators such as TNF-α and IL-6 have been demonstrated to play an important role in I/R-mediated injury. We examined the mRNA levels of these genes in the infarcted myocardial tissues in both hHO-1- and lacZ-transduced animals by using semi-quantitative RT-PCR. Our results demonstrated that there was significant up-regulation of TNF-α and IL-6 gene expression in lacZ-treated control animals during ischemia (Fig. 5 *b* and *c*), whereas the up-regulation of these genes was attenuated in the hHO-1-treated animals. The cytokine gene expression coincided with reduced macrophage and neutrophil infiltration as demonstrated by the absence of CD45-positive cells in the hHO-1-treated tissues (Fig. 5*a*).

We evaluated the consequence of this rapid and transient therapeutic transgene expression in the long-term functional recovery and remodeling of the myocardium after I/R injury. Echocardiographic analyses performed 1 month after injury demonstrated severe wall thinning and reduced ejection fraction in the lacZ-treated animals relative to the HO-1-treated animals (Table 1). Left ventricular function and chamber dimensions in the HO-1-treated animals were almost identical to sham animals, indicating nearly complete prevention of left ventricular wall remodeling. Marked wall thinning, fibrosis, and cell loss were observed at 4 months after injury in the lacZ-treated controls, which were completely prevented in HO-1-treated animals (Fig. 6). These results demonstrate that a timely induction of HO-1 can result in both short- and long-term protection against acute myocardial injury.

Discussion

Hypoxia and oxidative stress associated with I/R are common causes of tissue injury accounting for organ damage in stroke, myocardial infarction, ischemic bowel disease, and kidney and liver failure. Although the molecular mechanisms underlying I/R-induced cellular damage have been characterized (22), the narrow time window for successful therapeutic intervention limits the efficacy of current drug and gene therapy strategies for I/R injury. Furthermore, reperfusion, although essential for tissue salvage, may exacerbate tissue damage initiated during ischemia, because of increased production of reactive oxygen species (ROS). Because the pathophysiological events leading to I/R injury are unpredictable and recurring, we postulate that a preemptive gene therapy strategy in which the therapeutic gene is administered in advance of I/R, and its expression is under the regulation of a pathophysiological stimulus such as hypoxia, may be ideal for high-risk patients such as those with advanced coronary artery disease, or those susceptible to hypoperfusion such as with sepsis or undergoing risky surgery or hypotension. Thus, although the therapeutic gene is continuously present in the target tissues, its expression is activated only in the presence of ischemia, and is quickly deactivated upon reperfusion of the ischemic tissue. Here we show that incorporation of hypoxia responsive elements in the gene delivery vector provides an on–off physiological switch, which renders transcription of the therapeutic gene completely subservient to the hypoxic stimulus triggered by ischemia. Such a level of endogenous

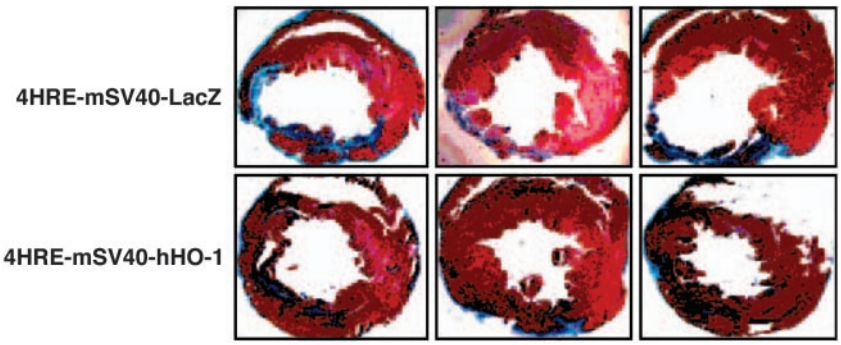

Fig. 6. Ischemic induction of the transgene inhibits long-term ventricular remodeling and protects against tissue injury. Masson trichrome staining shows significant anterior wall thinning and collagen deposition 4 months after injury in 4HRE-mSV40-lacZ-treated animals in contrast to 4HRE-mSV40-hHO-1-treated rats, where the myocardium appeared normal with decreased collagen deposition.

regulation of transgene expression is a desirable feature for safe and efficacious expression of therapeutic genes in humans.

Our approach provides a systematic characterization of the physiological behavior of the composite vector and its therapeutic potential and contributes crucial data toward validating the use of hypoxia sensitive vectors as a tool for delivery of physiologically regulated therapeutic genes for protection from I/R-induced tissue injury. In addition, given the widespread tropism of AAV (23) and its capability for long-term expression of therapeutic genes, the prevalence of hypoxia as a principal trigger of injury in diverse tissues, the ubiquitous distribution of hypoxia-inducible factor 1α (24, 25), and the effectiveness of HO-1 in mediating tissue protection from I/R injury, this approach is generally applicable to a variety of tissues that may undergo I/R injury, such as the kidneys, lungs, liver, and brain. The selection of HO-1 as a therapeutic target from among many other potential therapeutic genes, such as superoxide dismutase (26), nitric oxide synthase (27, 28), and vascular endothelial growth factor (29), was made on the basis of its documented cytoprotective effects (30, 31) for the purpose of demonstrating the feasibility and therapeutic potential of this strategy with a validated cardioprotective gene. However, we emphasize that this strategy can be extended to the expression of any gene or combination of gene(s) with therapeutic value for protection from ischemia-induced injury.

The lack of protection in the control animals despite the presence of endogenous HO-1 defense mechanism may be related to differences in expression patterns of endogenous and the exogenous HO-1 in myocardial I/R injury model. For example, endogenous HO-1 expression in the heart was up-regulated 6–12 h after I/R in the control lacZ-treated rats. This native response is likely caused by I/R-mediated activation of the inflammatory cascade and the consequent release of cytokines. Indeed, cytokines have been reported to induce HO-1 expression both at the transcriptional and posttranscriptional levels (32). Despite HO-1 protein levels that equaled or surpassed those in the hHO-1-treated rats, this "late" response was clearly not enough to provide significant myocardial protection in the lacZ-treated animals. In contrast, the immediate induction of hHO-1 transgene expression by ischemia in the rats treated with the inducible vector led to rapid accumulation of HO-1 protein, which may exert significant cytoprotective effects by down-regulating cytokine gene expression (33) during reperfusion phase. Taken together, our data indicate that the prompt overexpression of hHO-1 transgene during ischemia by an hypoxia-sensitive vector provides essential cytoprotection from I/R injury during the critical window for protection, which is not adequately compensated by the delayed increase in endogenous HO-1. Hence, these data support the concept of "early intervention" in the prevention of I/R injury.

In summary, we report the proof of concept of a preemptive gene therapy strategy that may be useful for long-term protection of a variety of tissues against ischemic and oxidative injury. This strategy involves the hypoxia-inducible expression of a therapeutic gene (such as HO-1) to provide immediate production of cytoprotective gene product at the onset of ischemia. From a practical standpoint, this approach may be used for patients at high risk for acute coronary ischemia, and the treatment may be administered in cardiac catheterization laboratory at the time of elective or emergency cardiac interventions or in the operating room during cardiac surgery. This approach may also be used by direct injection into tissues that are susceptible to I/R in high-risk patients in intensive care units, those undergoing complicated surgery or for patients with shock, trauma, or sepsis.

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