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 adeno-associated 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.

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 × 1011 infectious particles of either recombinant AAV-4EpoHRE-mSV40-hHO-1 or the control vector AAV-4EpoHRE-mSV40-lacZ were injected into 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.

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 adeno-associated 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-luciferase (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.

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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 (TNF-α) and IL-6 gene expression were measured by one-way ANOVA. *P* ≤ 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. 1a).

To quantitate the level of reporter gene induction, we transfected HEK 293 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. 1b). 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 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. 2a), 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. 2b).

As in skeletal muscle, liver ischemia resulted in transgene induction, which was not detectable at baseline or after 24 h of reperfusion (Fig. 2a). 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. 2a). 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. 3a) revealed a significant 65% decrease in the infarct size in the hHO-1-treated groups as compared with the lacZ control group.
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
exogenous human HO-1 mRNA expression within 1 h of ischemia (Fig. 4a). 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. 4a 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-HO-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).

Pachori et al. PNAS | August 17, 2004 | vol. 101 | no. 33 | 12285
enous rHO-1 mRNA levels were increased 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. 4c). 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 semiquantitative 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. 5b 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. 5a).

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 gene expression is ideal for I/R injury as a pathophysiological stimulus, which is present only in the ischemic zone, and is quickly deactivated as soon as the reperfusion is achieved.

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

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

SWD, septal wall thickness (diastole); PWD, posterior wall thickness (diastole); LAVD, long axis volume (diastole); LVAS, long axis volume (ystole); LAAD, long axis area (diastole); LAAS, Long axis area (ystole); EF, ejection fraction; LALD, long axis length (diastole).

*HO-1 vs. lacZ, P < 0.05.
†lacZ vs. sham, P < 0.05.

![Fig. 6.](image-url) 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 (26), 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 regulating therapeutic genes for protection from I/R injury, this 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.

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 a precharacterization 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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