Activation of hypoxia-inducible factor-1 via prolyl-4 hydroxylase-2 gene silencing attenuates acute inflammatory responses in postischemic myocardium

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ISCHEMIA-REPERFUSION (I/R) injury results in significant cardiac dysfunction owing in part to the release of potent proinflammatory cytokines. Cytokine- and/or chemokine-mediated neutrophil recruitment to inflammatory sites of injury as part of an essential host defense mechanism results in neutrophil extravasation across endothelial barriers and significant parenchymal cell injury. Transendothelial migration in this paradigm is preceded by margination and trapping of leukocytes occurring within the first 2 h of reperfusion (12). Although neutrophils remain primarily in the border zone, monocytes migrate rapidly into the infarct zone (1). The dominant factor driving migration of neutrophils into myocardium is the reperfusion-dependent induction of interleukin-8 (IL-8) (27). IL-8 is a CXC chemokine that mediates adhesion, activation, and migration of blood neutrophils (PMN) into sites of inflammation. Several human studies highlight the consequences of unregulated IL-8 secretion in the generation of cardiac injury (40, 41, 44). Inhibition of proinflammatory cytokine production and neutrophil recruitment in postischemic myocardium may be critical for long-lasting protection from I/R injury.

Biological processes known as preconditioning enhance endogenous cellular mechanisms within the myocardium, resulting in protection against postischemic injury. Several preconditioning strategies have been reported, including sublethal ischemia and pharmacological approaches (38, 43, 45). Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric αβ transcription factor that mediates tissue responses to ischemia-hypoxia (48). HIF-1 promotes transcription of >100 genes, including inducible nitric oxide synthase (iNOS), vascular endothelial growth factor, and heme oxygenase-1 (HO-1; see Ref. 49). HO-1 is a stress-responsive protein that ameliorates cardiac damage resulting from I/R insults (7, 18). Induction of iNOS expression is critically linked to the phenomenon of delayed ischemic preconditioning (2).

Posttranslational hydroxylation of the HIF-1α subunit negatively regulates HIF-1 activity in normoxic cells by signaling ubiquitination and degradation through proteasome pathways (21, 22). Three prolyl hydroxylase isoforms have been identified that utilize O2 and 2-oxoglutarate as substrates for generating the 4-hydroxyproline at residues 402 and/or 564 of HIF-1α that initiate processes leading to degradation (4, 24).

We recently reported that administration of dimethylxallylglycine (DMOG), a nonspecific prolyl hydroxylase inhibitor, 24 h before the onset of ischemia significantly reduced postischemic infarct size in rabbit hearts (36). In that study, DMOG

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administered before I/R significantly attenuated posts ischemic serum IL-8 levels and the sequestration of PMN in myocardium. In a more recent study, we employed a small-interfering RNA (siRNA) to silence prolyl-4 hydroxylase-2 (P4HA2) expression in murine hearts, which promoted highly significant HIF-1 activation (35). Using an ex vivo Langendorff apparatus in that study, we showed that HIF-1 activation via P4HA2 gene silencing resulted in significantly reduced infarct size in posts ischemic hearts. Kido et al. (25) employed a transgenic mouse model and demonstrated that constitutive expression of cardiac HIF-1α resulted in attenuated infarct size following myocardial infarction. The authors concluded that a single gene, HIF-1α, induces therapeutic angiogenesis, limits infarct size, and improves myocardial function after acute coronary occlusion.

In the present study, we examined the role of HIF-1 in regulation of chemokine expression in vivo in posts ischemic murine myocardium and in vitro in murine cardiomyocytes. Regulation of three murine CXC chemokines [cytokine-induced neutrophil chemoattractant factor (KC), macrophage inflammatory protein-2 (MIP-2) and lipopolysaccharide-induced CXC chemokine (LIX)] were studied. We show here for the first time that HIF-1 activation employing an siRNA-mediated strategy to silence the P4HA2 gene before I/R attenuates myocardial expression of the CXC chemokines KC, MIP-2, and LIX and the CC chemokine monocyte chemoattractant protein (MCP)-1. Furthermore, myocardial PMN infiltration in P4HA2 siRNA-treated hearts was significantly attenuated and was associated with significantly reduced myocardial infarct size. These findings are further supported by the results from in vitro studies using murine cardiomyocytes showing that activated HIF-1 powerfully regulates cardiomyocyte chemokine expression.

MATERIALS AND METHODS

Reagents and chemicals. DMOG was obtained from Cayman Chemicals (Ann Arbor, MI). Recombinant human tumor necrosis factor (TNF)-α was purchased from Collaborative Biomedical Products (Bedford, MA). Pentobarbital sodium was obtained from Sigma Chemicals (St. Louis, MO). Hypoxia chambers (Modular Incubator Chamber) were obtained from Billups-Rothenberg (Del Mar, CA). Specialty gases were obtained from National Welders Supply (Charlotte, NC). Sterile tissue culture plasticware was obtained from Corning (Corning, NY). Claycomb Media and FBS were obtained from JRH Biosciences (Lenexa, KS). SiPORT-Amine transfection reagent was purchased from Ambion (Austin, TX). Tri Reagent was obtained from Molecular Research Center (Cincinnati, OH). RNA isolation kits (MRC) were obtained according to the manufacturer’s specifications (Qiagen). Murine hearts were snap-frozen in liquid nitrogen and subsequently powdered with a BioPulverizer (RPI). Total RNA was isolated from heart tissue using Tri Reagent according to the manufacturer’s specifications (MRC).

RNA isolation and real-time quantitative PCR analysis. Total RNA from HL-1 cardiomyocyte cell culture was extracted and purified using QiAshredders and RNeasy columns according to the manufacturer’s specifications (Qiagen). Murine hearts were snap-frozen in liquid nitrogen and subsequently powdered with a BioPulverizer (RPI). Total RNA was isolated from heart tissue using Tri Reagent according to the manufacturer’s specifications (MRC).

Total RNA (1 μg) was reverse transcribed into cDNA using the Thermoscript RT-PCR system. cDNA was diluted (1:500), and real-time quantitative PCR (QPCR) was performed using Brilliant SYBR Green QPCR Master Mix along with murine primers (Table 1). Primers were designed to anneal to sequences on separate exons or to span two exons. Cycling parameters were as follows: 95°C for 10 min
Table 1. Primers for murine chemokines, growth factors, myeloperoxidase, cell adhesion molecules, and β-actin

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Sequence 5' → 3'</th>
<th>Reverse Sequence 5' → 3'</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC</td>
<td>CTGGTGTGGTTGCTTTAACGC</td>
<td>CTGGTGTGGTTGCTTTAACGC</td>
<td>168</td>
</tr>
<tr>
<td>MIP-2</td>
<td>GTGGGGAGAGGTTGAGTTG</td>
<td>GTGGGGAGAGGTTGAGTTG</td>
<td>166</td>
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<tr>
<td>LIX</td>
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<td>GAGTCTGTCTAGTTCTCCTCGG</td>
<td>110</td>
</tr>
<tr>
<td>MPO</td>
<td>GTGGATAGTACGACCTGGA</td>
<td>GTGGATAGTACGACCTGGA</td>
<td>265</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CACAAGCTACTCATTGGGATCAT</td>
<td>CACAAGCTACTCATTGGGATCAT</td>
<td>239</td>
</tr>
<tr>
<td>MCP-1</td>
<td>AGATCACATTCACGGTGCTG</td>
<td>AGATCACATTCACGGTGCTG</td>
<td>125</td>
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<tr>
<td>TGF-β1</td>
<td>TCTTCAGAGGCAGGAAACAGG</td>
<td>TCTTCAGAGGCAGGAAACAGG</td>
<td>125</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TCTTGCAGTGGGATTTGAC</td>
<td>TCTTGCAGTGGGATTTGAC</td>
<td>125</td>
</tr>
</tbody>
</table>

KC, cytokine-induced neutrophil chemoattractant factor; MIP-2, macrophage inflammatory protein-2; LIX, lipopolysaccharide-induced CXC chemokine; MPO, myeloperoxidase; ICAM, intercellular adhesion molecule; MCP-1, monocyte chemotactic protein-1; TGF-β1, transforming growth factor-β1.

and 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. A dissociation profile was generated after each run to verify specificity of amplification. All PCR assays were performed in triplicate. No template controls and no RT controls were included. β-Actin was used as a housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the RT efficiency among the different cDNA reactions. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software (Stratagene) to compare the levels of a target gene in test samples relative to a sample of reference (calibrator from untreated cells).

**In vivo siRNA administration protocol.** Mice were randomly assigned to one of the following groups (n = 6 in each group): group I (sham controls) received volume-matched 0.9% saline intraperitoneal injection, underwent anesthesia, mechanical ventilation, and thoracotomy but no cardiac manipulation; group II (saline I/R controls) animals received volume-matched 0.9% saline intraperitoneal injection followed by cardiac I/R protocol 24 h later; group III (nontargeting siRNA I/R controls; NTC) animals received NTC siRNA at a dose of 1.5 μg of siRNA/μl body wt in a total volume of 0.2 ml saline followed by I/R protocol 24 h later; group IV (P4HA2 siRNA I/R) animals received P4HA2 siRNA at a dose of 1.5 μg of siRNA/μl body wt in a total volume of 0.2 ml saline followed by I/R protocol 24 h later. All animals, with the exception of the sham group, were subjected to the cardiac I/R protocol described below. The effects of P4HA2 silencing were compared with saline-treated and nontargeting siRNA-treated I/R controls.

Before administration, siRNA was bound to siPORT Amine transfection reagent as previously described (35). Briefly, siPORT Amine was incubated in saline at 30 min at 22°C, and this mixture was then incubated with the siRNA in a 1:1 ratio for an additional 30 min at 22°C. Intraperitoneal administration of the siPORT Amine-bound siRNA was performed 24 h before implementation of the cardiac I/R protocol.

In *vivo* myocardial I/R protocol. A total of 48 male B6,129 wild-type mice (body wt: 27–33 g) were used. The protocols for care and use of the animals reported in these studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were conducted in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 80-23; Office of Science and Health Reports, Bethesda, MD 20205].

Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) followed by intraperitoneal injection of 30 mg/kg every 40 min thereafter. Anesthetized animals were then orotracheally intubated and ventilated (Harvard Apparatus Rodent Ventilator model 680). Tidal volumes were set at 0.22 ml, and respiratory rate set at 133 breaths/min. A thoracotomy was then performed through the left fourth intercostal space, and hearts were exposed by opening the pericardium. The left descending coronary artery was identified, and a snare was placed around the proximal portion. Myocardial ischemia was induced for a period of 30 min by tightening the snare and watching for blanching of the myocardium. A 120-min reperfusion period was initiated by releasing the snare. After completion of the I/R protocol, hearts were removed and processed for either infarct size, histology, protein preparation, or RNA isolation.

**Determination of infarct size.** Hearts were mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM CaCl₂ and heparin. After the blood was washed out, the suture around the coronary artery was retightened, and ~0.3 ml of 10% Evans blue dye were injected as a bolus in the aorta until the heart turned blue. The heart was then perfused with saline to wash out the excess Evans blue. The heart was then removed, frozen, and cut into 6–8 transverse slices from apex to base of equal thickness (~1 mm). The slices were then incubated in a 10% triphenyltetrazolium chloride solution in an isotonic phosphate buffer (pH 7.4) at room temperature (RT) for 30 min and then fixed in 10% formalin for 2–4 h. The areas of infarcted tissue, the risk zone, and the entirety of the left ventricle were determined by computer morphometry using Bioquant imaging software. Infarct size was expressed both as a percentage of the left ventricle and ischemic risk area (37).

**Immunohistochemistry of myocardium for chemokine KC.** Hearts from sham, nontargeting control siRNA I/R, and P4HA2 I/R animals were studied. Hearts were fixed in 10% buffered formalin, embedded in paraffin, and serial sectioned at 5 μm. Sections were deparaffinized and rehydrated through graded alcohols to running tap water. The “Steamer Method” of antigen retrieval was performed using 0.01% citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were washed in PBS X3. Endogenous peroxidase activity was blocked (1% hydrogen peroxide in PBS) for 5 min. PBS containing 0.025% Triton X-100 was used for this and all subsequent washes. Sections were incubated in 1% normal horse serum for 1 h at RT. Avidin/biotin block (Vector) was performed following the manufacturer’s instructions. Polyclonal anti-mouse KC antibody (R&D Systems) was applied at 1:100 dilution (1 μg/ml) in PBS overnight at RT. After PBS washes, sections were incubated with biotinylated anti-goat IgG secondary antibody (7.5 μg/ml) in 2% normal horse serum for 30 min at RT. Slides were washed three times and then incubated with Vectastain Elite ABC reagent. Three PBS washes were followed by chromogen development with 3,3′-diaminobenzidine and hematoxylin counterstain. Slides were examined by bright-field microscopy at ×40 magnification (20).

**Statistical analysis.** Mean values were calculated from data obtained from six animal studies in each group and at least three separate in vitro experiments. Data are presented as means ± SE. Results were compared using one-way ANOVA and the post hoc Tukey’s test to identify specific differences between groups. Statistical analysis was performed using SigmaStat 3.1 (SPSS, Chicago, IL), and statistical significance was set at P < 0.05.
RESULTS

Prolyl hydroxylase inhibition and hypoxia activate HIF-1 in HL-1 cardiomyocytes. Cells were exposed to medium alone (normoxic controls), medium plus CoCl₂ (150 μM), medium containing the prolyl hydroxylase inhibitor DMOG (500 μM), or medium plus hypoxia (1% O₂) for 6 h. Nuclear extracts were isolated, and Western blot analysis was performed for detection of HIF-1α protein. As shown in Fig. 1A, robust HIF-1α stabilization was observed following exposure to hypoxia and to DMOG. Although CoCl₂ stabilizes HIF-1α in many cell types, minimal HIF-1α stabilization was observed in HL-1 cardiomyocytes.

Subconfluent HL-1 cardiomyocytes were cotransfected with the pHRE-Luc and pHRL-null vectors. After 24 h, cells were treated under the conditions stated above, and the HIF-1-dependent promoter response was assessed by luciferase reporter assay. As shown in Fig. 1B, prolyl hydroxylase inhibition following DMOG exposure and hypoxia promoted strong, functional HIF-1 activation (9-fold and 8-fold, respectively, P < 0.001 vs. control). CoCl₂ exposure failed to induce significant HIF-1 promoter activity. Thus hypoxia or DMOG exposure potently stabilizes HIF-1α and induces HIF-1-dependent transactivation in HL-1 cardiomyocytes.

HIF-1 activation promotes HO-1 and iNOS expression in HL-1 cardiomyocytes. Emerging research suggests that HIF-1 activation in a number of cell systems promotes HO-1 and iNOS expression. Under the conditions described above, we examined the impact of HIF-1 activation by hypoxia and prolyl hydroxylase inhibition on HO-1 and iNOS mRNA and protein expression in HL-1 cardiomyocytes. As seen in Fig. 2A, exposure to hypoxia resulted in 3.8-fold induction in HO-1 mRNA expression and 2.7-fold induction of iNOS mRNA as assessed by QPCR (P < 0.001 vs. control). Exposure to

DMOG also induced HO-1 and iNOS mRNA (6.9- and 6.4-fold, respectively, P < 0.001 vs. control). Western blot analysis showed significant increases in HO-1 and iNOS protein expression (Fig. 2B). In agreement with our earlier observa-

Fig. 3. Activation of HIF-1 attenuates cytokine-induced chemokine generation in HL-1 cardiomyocytes. ELISA analysis of conditioned media from HL-1 cardiomyocytes pretreated with DMOG (18 h) and treated with or without tumor necrosis factor (TNF)-α (1 ng/ml) for 4 h. Secretion of cytokine-induced neutrophil chemoattractant factor (KC) was minimal in resting cells and was not detectable in DMOG-treated media controls. Activation of HIF-1 by DMOG pretreatment attenuated the TNF-induced KC response in a dose-dependent manner. *P < 0.001 vs. TNF control. Macrophage inflammatory protein (MIP)-2 and lipopolysaccharide-induced CXC chemokine (LIX) levels in the conditioned media were below the detection limits of the assay.
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CoCl₂ exerted minimal impact on HO-1 and iNOS mRNA or protein expression. These results suggest a possible cytoprotective role for HIF-1 activation in HL-1 cardiomyocytes.

**HIF-1 activation attenuates cytokine-mediated chemokine and ICAM-1 expression in HL-1 cardiomyocytes.** We have previously demonstrated that transient HIF-1 activation suppresses cytokine-stimulated IL-8 promoter activity and protein secretion in human microvascular endothelial cells (36). We examined the impact of HIF-1 activation on TNF-mediated chemokine (murine IL-8 homologs) secretion in HL-1 cardiomyocytes. Cells were exposed to DMOG (18 h), washed, and then exposed to media alone or to medium containing TNF-α (1 ng/ml) for 4 h. Conditioned media was analyzed by ELISA for the murine chemokines KC, MIP-2, and LIX. In media control cells, the level of KC was minimal and undetectable following DMOG treatment (Fig. 3). HIF-1 activation by DMOG produced a concentration-dependent reduction in TNF-stimulated secretion of KC (P < 0.001 vs. TNF alone). TNF-α-stimulated MIP-2 and LIX secretion in HL-1 cardiomyocytes was below the detection limit of the assay; thus, the effects of HIF-1 activation on LIX and MIP-2 secretion could not be determined. The disparity we observed in the secretion of KC and MIP-2 protein by activated HL-1 cardiomyocytes is consistent with the findings of Boyd et al. (3).

HIF-1 activation was also induced in HL-1 cardiomyocytes via P4HA2 gene silencing following transfection with an siRNA to murine P4HA2 (35). After transfection, cells were washed and exposed to TNF-α (1 ng/ml, 2 h). Cells were lysed, and total RNA was isolated and analyzed for expression of KC, LIX, and MIP-2. As shown in Fig. 4A, TNF-induced significant upregulation of KC and LIX mRNA (2- to 3-fold, P < 0.001 vs. respective controls). Activation of HIF-1 via P4HA2 siRNA attenuated the cytokine-induced expression of KC and LIX (P < 0.001 vs. respective TNF controls). MIP-2 mRNA was undetectable by QPCR in HL-1 cardiomyocytes. In addition, cell adhesion molecule mRNA expression was examined in HL-1 cardiomyocytes exposed to TNF-α (1 ng/ml, 4 h). As seen in Fig. 4B, HIF-1 activation induced by P4HA2 gene silencing significantly attenuated TNF-induced ICAM-1 mRNA expression (P < 0.005 vs. TNF controls).

**HIF-1 activation via P4HA2 gene silencing attenuates infarct size in postischemic murine hearts in vivo.** HIF-1 activation in reperfusing myocardium resulted in tissue preservation, as demonstrated by significant reductions in infarct size (Fig. 5A). The infarct size decreased from 40.8 ± 1.5 in the saline-treated I/R control group to 14.8 ± 1.6 in the P4HA2 siRNA-treated I/R control group to 14.8 ± 1.6 in the P4HA2 siRNA-treated I/R control group.
treated group (% of risk area, mean ± SE, *P < 0.001, P4HA2 I/R vs. I/R). In contrast, treatment with the NTC siRNA failed to mimic the protective effect of P4HA2 silencing, as indicated by an infarct size of 43.7 ± 1.6. The risk areas expressed as a percentage of the left ventricle were not statistically different between the groups (44.7 ± 3.2, 50.3 ± 1.4, and 50.6 ± 4.8 for saline I/R, NTC I/R, and P4HA2 I/R, respectively; Fig. 5B).

**HIF-1 activation via P4HA2 gene silencing reduces cardiac chemokine and ICAM-1 transcription and neutrophil sequestration in postischemic hearts.** We previously reported that HIF-1 activation by DMOG attenuated plasma IL-8 levels when compared with I/R controls in rabbits. In the current study, we examined the impact of HIF-1 activation by P4HA2 siRNA infusion on levels of cardiac chemokines following I/R. As shown in Fig. 6, A–D, the expression of the cardiac chemokines KC (>25-fold), MIP-2 (>60-fold), LIX (7-fold), and MCP-1 (>5-fold) was elevated in “reperfusng” myocardium (*P < 0.001, I/R vs. sham controls). HIF-1 activation by P4HA2 gene silencing significantly attenuated expression of all cardiac chemokines examined in postischemic hearts (*P < 0.02, P4HA2 I/R vs. I/R controls).

Furthermore, HIF-1 activation significantly attenuated cardiac ICAM-1 expression following I/R, as shown in Fig. 7A (0.001, P4HA2 I/R vs. I/R controls). Diminished chemokine and ICAM-1 expression in reperfusing, postischemic myocardium was accompanied by reductions in the extent of neutrophil infiltration as determined by QPCR for ICAM-1 expression following I/R, as shown in Fig. 7A. Figure 7B, *P < 0.002, P4HA2 I/R vs. I/R controls).

**Chemokine KC localizes to cardiomyocytes in postischemic murine heart: Attenuation of expression by P4HA2 gene silencing.** Figure 8, A–C, shows the results of immunohistochemical studies, staining for the KC chemokine protein. In our studies, we found that sham heart (no ischemia) exhibited no significant signal for KC protein (Fig. 8A). However, postischemic heart subjected to treatment with NTC siRNA 24 h before 30 min of ischemia and 2 h of reperfusion exhibited dramatic KC signal that was very homogenously located within cardiomyocytes (Fig. 8B). Figure 8C shows the effects of administering a P4HA2 siRNA (HIF-1 activation) 24 h before 30 min of ischemia followed by 2 h of reperfusion. In P4HA2 siRNA-treated postischemic heart, we found that, although KC protein is still present, the extent of expression is dramatically reduced. These findings are in agreement with the QPCR data reported above (Fig. 6A).

**DISCUSSION**

Reperfusion of working myocardium following ischemic events rapidly induces the onset of acute inflammatory responses. In immediate postischemic periods, complement activation and generation of reactive oxygen species trigger a cytokine/chemokine cascade initiated by TNF-α release, originating from within cardiac mast cells (11, 16). Cytokines and CXC chemokines generated during postischemic periods play crucial roles in upregulating adhesion receptor expression on coronary microvascular endothelial cells and cardiomyocytes (17), critical events that lead to neutrophil (PMN) recruitment and amplification of inflammatory responses in the myocardium (5, 6, 23, 46). Prior research has revealed that PMN recruitment in postischemic myocardium plays a key role in producing cardiac injury. Multiple studies show that attenuated postischemic cardiac PMN sequestration significantly reduces infarct size and improves cardiac contractile function (26, 29, 30, 39). Rui and colleagues (42) presented important evidence recently that suggests that cardiomyocytes in reperfusing myocardium may be an important source of peptides that generate interstitial-to-blood chemotactic gradients that promote trans-
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HIF-1, a potent αβ transcription factor, mediates tissue responses to hypoxia. The activated heterodimer binds to the consensus sequence 5′-RCGTTG-3′, which drives transcription of genes involved in oxygen homeostasis, iNOS, vascular endothelial growth factor, and HO-1. HIF-1 activity is dependent upon expression and activity of the α-subunit that is regulated by posttranslational hydroxylation of proline residues mediated by prolyl hydroxylases. Proline hydroxylation targets HIF-1α for proteosomal degradation following binding by the von Hippel Lindau tumor suppressor protein E3 ubiquitin ligase complex. Cellular hypoxia produced by exposure to diminished environmental oxygen tensions or by “chemical hypoxia” mediated through prolyl hydroxylase inhibition (e.g., DMOG) stabilizes HIF-1α, producing αβ heterodimerization and activation. Emerging knowledge suggests that HIF-1 activation regulates genes that mediate inflammatory responses that occur following cytokine release (e.g., TNF) and I/R injury.

In the current report, we employed the murine cardiomyocyte cell line HL-1, which has been previously used for the study of cardiac muscle cell structure and function (50). Our studies show that HL-1 exposed to DMOG and environmental hypoxia exhibit robust HIF-1α stabilization (Fig. 1A). HIF-1α stabilization was accompanied by significant increases in the activity of the HIF-1 reporter vector pEpo3/Glut-1 Luc (Fig. 1B).

A significant body of evidence now suggests that enhanced transcription of the HIF-1-driven genes HO-1 and iNOS in murine myocardium significantly reduce postischemic cardiac injury (28, 31, 43, 47). Our results show that HIF-1 activation significantly increased HO-1 and iNOS gene and protein expression in HL-1 cardiomyocytes (Fig. 2, A and B).

Current research suggests that the oxidant-sensitive cytokine TNF-α plays a crucial role in initiating postischemic cardiac inflammatory events (14, 15). A recent report from this laboratory shows that activation of cardiac HIF-1 by systemic administration of DMOG attenuates postischemic infarct size, serum chemokine surges, and myocardial PMN sequestration in a rabbit model of cardiac I/R injury (36). From these studies, we hypothesized that HIF-1 activation may modulate TNF-α-induced secretion of CXC chemokines from murine cardiomyocytes. We found that TNF-α exposure induced substantial secretion of the chemokine KC in HL-1 cardiomyocytes (Fig. 3). Interestingly, however, neither LIX nor MIP-2 protein was detectable in conditioned medium from TNF-α-exposed HL-1 with the ELISA assays used in this study. When HIF-1 was activated in cardiomyocytes by DMOG exposure, concentration-dependent reductions in TNF-α-stimulated KC secretion were observed. We recently reported that P4HA2 siRNA-mediated gene silencing in murine hearts induced robust HIF-1 activation (35). HIF-1 activation in HL-1 cardiomyocytes via P4HA2 siRNA produced significant reductions in TNF-α-induced KC and LIX mRNA (Fig. 4A). HL-1 transfected with a NTC siRNA and subsequently exposed to TNF-α were indistinguishable from HL-1 exposed to TNF-α alone (Fig. 4A). No MIP-2 mRNA was detectable by QPCR in TNF-α-exposed HL-1. Thus our studies show that chemokine transcription and secretion in cytokine-exposed HL-1 cardiomyocytes were significantly attenuated by HIF-1 activation. Although LIX mRNA was induced by TNF-α exposure in HL-1 cardiomyocytes, LIX protein was undetectable given the limits of our assay. Of major importance was our finding that HIF-1 activation by P4HA2 gene silencing in HL-1 cardiomyocytes significantly reduced cytokine-induced ICAM-1 mRNA expression by 50% (Fig. 4B). Thus a cytokine-induced proinflammatory phenotype characterized by chemokine and cell adhesion molecule expression is significantly downregulated by HIF-1 activation in murine cardiomyocytes.

We next sought to translate our in vitro observations in murine cardiomyocytes into intact hearts. To accomplish this, we utilized an in vivo model of myocardial I/R injury produced by occlusion of the left anterior descending coronary artery for 30 min, followed by reperfusion for 120 min. Before I/R injury, cardiac HIF-1 activation was produced by siRNA-mediated P4HA2 gene silencing, as previously reported by this laboratory (35). When compared with saline or nontargeting siRNA-treated I/R controls, our results show that HIF-1-activated hearts exhibited highly significant reductions in infarct size (Fig. 5A).

In this in vivo model, I/R induced striking elevations in KC, MIP-2, and LIX transcription in the saline and NTC siRNA hearts when compared with sham controls (i.e., anesthesia, open chest, no cardiac manipulation). In contrast, HIF-1 activation by P4HA2 gene silencing greatly reduced transcription of these CXC chemokines (Fig. 6, A–C). Immunohistochemi-
cal studies of hearts from sham, NTC siRNA-treated I/R, and P4HA2-treated I/R confirmed our QPCR data for the chemokine KC. Figure 8, A–C, shows that no KC signal was observed in sham-treated hearts, dramatic KC signal was present in cardiomyocytes in NTC siRNA-treated I/R heart, and diminished KC signal was present in P4HA2 siRNA-treated myo-
cardium. The KC signal observed in the two siRNA-treated hearts subjected to I/R was localized to cardiomyocytes.

Our data also reveal new findings with respect to regulation of the CC chemokine MCP-1. As previously reported by Frangogiannis et al. (17), we found that MCP-1 expression increased significantly in postischemic myocardium (Fig. 6D). As with the CXC chemokines, HIF-1 activation significantly downregulated the CC chemokine MCP-1 in postischemic myocardium (Fig. 6D). The significance of this finding at present is unclear. Emerging data support a key biological role for MCP-1 in postischemic myocardium. Dewald and colleagues (10) found delayed removal of dead cardiomyocytes and diminished myofibroblast accumulation in postischemic myocardium from MCP-1 knockout mice. Work by Hayashidani et al. (19) suggests that MCP-1 promotes left ventricular remodeling and failure following myocardial infarction (19). The significance of attenuated but not completely blocked MCP-1 expression at 2 h following onset of reperfusion in the current study is unknown given prior work which shows that peak postischemic MCP-1 expression occurs at 6 h (9).

Equally as important, HIF-1 activation resulted in reduced ICAM-1 expression in postischemic hearts (Fig. 7A). Prior research has revealed that cardiomyocyte ICAM-1 binding to activated neutrophils via β2-integrin receptors mediates cardiomyocyte cell death (13). Thus HIF-1 activation via P4HA2 gene silencing attenuated both cardiac chemokine and ICAM-1 expression following I/R. Although MIP-2 mRNA was minimally expressed in sham-treated hearts and undetectable in HL-1 cardiomyocytes, it was significantly upregulated in hearts following I/R injury, suggesting that MIP-2 in the intact myocardium originates from a cell type other than the cardiomyocyte. Attenuation of myocardial inflammation was associated with concomitant reductions in neutrophil infiltration as demonstrated by QPCR for the PMN marker gene myeloperoxidase (Fig. 7B).

In conclusion, this study supports a growing body of evidence that I/R injury induces substantial cardiac inflammatory responses that are characterized by the rapid transcription of proinflammatory chemokines. Chemokine production in postischemic hearts precipitates rapid sequestration of activated PMN, which are implicated in cardiac contractile dysfunction and cardiomyocyte injury. HIF-1 activation downregulates postischemic chemokine and ICAM-1 transcription, thereby attenuating PMN sequestration and reducing myocardial injury. However, causative relationships between HIF-1-mediated inhibition of inflammation and diminished cardiomyocyte viability have yet to be fully established. Emerging data sug-

Fig. 8. Chemokine KC localizes to cardiomyocytes in postischemic murine heart: Attenuation of expression by P4HA2 gene silencing. A: heart taken from a sham animal (anesthesia, open chest, no ischemia, 2 h mechanical ventilation) stained for murine chemokine KC (5 μm section, ×40 magnification). Image shows intact cardiac histology with no evidence of KC expression. B: heart obtained from an animal treated with a nontargeting siRNA 24 h before 30 min ischemia, 2 h reperfusion (5 μm section, ×40 magnification). Disruption of the contractile architecture is evident, i.e., stretching and waviness of myocardial fibers characteristic of the early stages of myocardial infarction injury. Section shows dramatic KC protein staining localized to cardiomyocytes. C: heart obtained from an animal treated with a P4HA2 siRNA 24 h before 30 min ischemia, 2 h reperfusion (5 μm section, ×40 magnification). Section shows dramatic attenuation of KC protein staining. Although attenuated, KC signal present in this image is still localized to cardiomyocytes.
gest that HIF-1 regulates cardiac metabolism in multiple ways. Furthermore, reductions in postischemic inflammatory reactions that we observed in ischemic myocardium following HIF-1 activation may have resulted from reductions in infarct size because of direct protective actions on cardiomyocytes. Future research is needed to address these important issues.

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