Hypoxia Inducible Factor-1 Activation by Prolyl 4-Hydroxylase-2 Gene Silencing Attenuates Myocardial Ischemia Reperfusion Injury


Abstract—Hypoxia inducible factor-1 (HIF-1) regulates changes in transcription of key genes such as inducible NO synthase (iNOS) in hypoxic/ischemic environments. In normoxia, HIF-1 activation is controlled by HIF-1α-prolyl 4-hydroxylases, which target HIF-1α for ubiquitination and proteasomal degradation. We hypothesized that normoxic HIF-1 preservation could attenuate cardiac ischemia/reperfusion injury via a preconditioning effect. HIF-1 preservation was achieved by using small interfering RNA (siRNA) to silence murine HIF-1α-prolyl-4-hydroxylase-2 (PHD2). PHD2 siRNA reduced PHD2 mRNA expression 89±1.5% (P<0.001) in a time- and concentration-dependent manner in normoxic murine microvascular endothelial cells (EC). PHD2 silencing in normoxic EC stabilized HIF-1α protein levels while significantly increasing HIF-1α transcriptional activity and iNOS mRNA expression. Wild-type mice infused with PHD2 siRNA (1.5 μg/g body weight) showed a 61±2.4% (P<0.05) reduction in cardiac PHD2 mRNA within 24 hours. In addition HIF-1α protein levels and HIF-1-dependent iNOS mRNA levels were increased. PHD2 siRNA-transfected hearts from wild-type mice (n=6) subjected to 30 minutes ischemia followed by 60 minutes reperfusion exhibited reduced infarct size when compared with saline-treated controls (9.7±1.9% versus 31.6±1.8%, respectively, P<0.0001, n=6) and to control mice transfected with a nontargeting siRNA control (28.4±3.0%, P<0.0001, n=6). Hearts from iNOS knockout mice receiving PHD2 siRNA by identical injection protocol (n=6) exhibited infarct size indistinguishable from saline controls (28.7±1.3%). These results show that in vitro and in vivo, PHD2 silencing using a siRNA strategy produces transcriptionally active HIF-1. Normoxic activation of HIF-1 in hearts following in vivo PHD2 siRNA administration attenuates reperfusion injury via an iNOS-dependent pathway. (Circ Res. 2006;98:133-140.)

Key Words: small interfering RNA • hypoxia inducible factor-1α-prolyl 4-hydroxylase-2 • cardiac ischemia/reperfusion • inducible NO synthase

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric α,β transcription factor that mediates tissue responses to hypoxia.1 HIF-1 promotes transcription of more than 40 genes involved in oxygen homeostasis in response to diminished oxygen tension, including inducible NO synthase (iNOS), vascular endothelial growth factor (VEGF), and heme oxygenase-1.2 HIF-1α and β mRNAs are expressed in most human tissues,3 but HIF-1 activity is determined by expression and activity of the α subunit.4 Under normoxic conditions, HIF-1 is ubiquitinated and degraded by the 26S proteasome.5–7 Posttranslational hydroxylation of HIF-1α targets the subunit for the von Hippel Lindau tumor suppressor protein E3 ubiquitin ligase complex.8–11 Three prolyl hydroxylase isoforms have been identified and use O2 and 2-oxoglutarate as substrates to generate 4-hydroxyproline at residue 402 and/or 564 of HIF-1α.12,13 Because HIF-1α-prolyl 4-hydroxylases possess a high Michaelis constant (Km) for O2, changes in the cellular O2 concentration are transduced into changes in the rate at which HIF-1α is hydroxylated, ubiquitinated, and degraded.14 Expression of the prolyl 4-hydroxylases varies among cell types and in response to physiological stimuli.15,16 Prolyl-4 hydroxylase-2 (PHD2) is ubiquitously expressed and exhibits the highest specific activity toward HIF-1α.14 Cellular hypoxia stabilizes HIF-1α protein, ultimately permitting nuclear translocation and dimerization to the β subunit. Heterodimer binding to hypoxia response elements on key genes at the DNA consensus sequence 5′-RCGTG-3′ drives transcription.17

We recently reported that administration of the prolyl hydroxylase inhibitor, dimethylxallyl glycine (DMOG), 24 hours before the onset of ischemia significantly reduced postischemic infarct size in rabbit hearts.18 Although HIF-1 is active in hypoxic/ischemic states, our work shows that increasing transcriptional activity of HIF-1 under normoxic conditions potently preconditions hearts against ischemic stress. However, pharmacological approaches have limi-
tions such as unanticipated off-targeting effects.\textsuperscript{19,20} DMOG is a nonspecific prolyl hydroxylase inhibitor and questions remain concerning the specific mechanisms that produced the preconditioning effects we observed.

In the current study, we used a small interfering RNA (siRNA) to silence murine PHD2 expression. We show that PHD2 gene silencing significantly increased HIF-1 transcriptional activity both in vitro in murine microvascular endothelium and in intact murine hearts following in vivo administration. PHD2 gene silencing resulted in significantly reduced infarct size in wild-type mice. Finally, our results using iNOS knockout mice suggest that NO plays a key role in mediating the cardioprotection observed following HIF-1 activation.

**Materials and Methods**

**Reagents and Chemicals**

RNA isolation kits RNeasy, QiAshredder, RNAiFect, and Effectene transfection reagents were obtained from Qiagen (Valencia, Calif). NuPAGE Novex precast gel system, Thermoscript RT-PCR system, and Platinum Taq DNA polymerase were obtained from Invitrogen (Carlsbad, Calif). Mouse anti-HIF-1\textsubscript{α} monoclonal antibody (ab1) was purchased from Abcam (Cambridge, UK). The Renaissance Western Blot Chemiluminescence Reagent Plus was purchased from Perkin Elmer Life Sciences Inc (Boston, Mass). The dual luciferase assay system and pHR-L-null vector were purchased from Promega (Madison, Wis). Immobilon membranes were obtained from Millipore (Bedford, Mass). siPORT\textsuperscript{\textregistered} Amine transfection reagent was purchased from Ambion (Austin, Tex). Selective iNOS inhibitor 1400W was purchased from Cayman Chemical (Ann Arbor, Mich). Sterile tissue culture plastic ware was obtained from Corning (Corning, NY). Culture media was obtained from GIBCO-Invitrogen (Carlsbad, Calif). Specialty gases were obtained from BOC gases (Corning, NY). Culture media was obtained from GIBCO-Invitrogen (Corning, NY). Pentobarbital and all other chemicals and reagents were obtained from Sigma Chemicals (St Louis, Mo).

**Endothelial Cell Culture**

SVEC4–10 (ATCC CRL-2181) is an endothelial cell (EC) line derived by SV40 transformation of murine microvessel EC, originally isolated from the axillary lymph node vessels of an adult male C3H/HeJ mouse.\textsuperscript{21} Cells were maintained in DMEM with 4.5 g/L glucose and 10% FCS and 1% antibiotic and culture medium renewed every 48 hours.

**HIF-1\textsubscript{α} Western Blot Analysis**

Nuclear extracts were isolated from mouse hearts and SVEC4–10 cells as described previously.\textsuperscript{18} Proteins were resolved by SDS-PAGE (4% to 20%) and electrophoretically transferred to polyvinylidene fluoride membranes (0.45-μm pore size). Immunodetection for HIF-1\textsubscript{α} in SVEC4–10 cells and mouse hearts used a mouse anti-HIF-1\textsubscript{α} monoclonal antibody. All membranes were stained with Ponceau S solution (0.2% wt/vol in 1% acetic acid) to ensure equal loading and transfer of proteins.\textsuperscript{22}

**Preparation and In Vitro Transfection of Murine PHD2 siRNA**

We used PHD2 siRNAs, targeting the following regions of murine PHD2 (GenBank accession no. NM_011031): 90 to 108, 541 to 559, 782 to 800, and 1048 to 1066. The forward sequences of DNA corresponding to PHD2-duplex siRNAs were: GCACATGAC- CGATCTGATT, GAAGGAGACTATTACCATA, GAAAT- CACTGTAACATCA, and GAAGAAATCGAGAGGATCA.

SMARTpool siRNAs, individual siRNAs, siSTABLE siRNA, and nontargeting siRNAs were synthesized by Dharmacon (Lafayette, Colo). Of the four individual siRNAs belonging to the SMARTpool, the siRNA targeting the sequence 90 to 108 was most effective in transient transfection assays. The siSTABLE duplex (2′-hydroxyl, annealed, desalted, dialyzed, and sterile filtered) of this siRNA was used for in vivo studies (see below). Transient transfections in SVEC4–10 were performed using RNAiFect. RNAiFect is based on a lipid formulation and has been designed for efficient transfection of cells with siRNA. The ratio of siRNA to RNAiFect was optimized for the cell line and siRNA combination used. SiRNA studies were controlled by transfection of a nontargeting scrambled RNA duplex siRNA control (NTSC, Dharmacon) containing 21 nucleotide sequences demonstrating no homology to murine genes.

**Dual Luciferase Reporter Assay**

SVEC4–10 were first transfected with the PHD2 siRNA for 24 hours or mock transfected (no PHD2 siRNA). SVEC4–10 were then cotransfected with a hypoxia response element luciferase reporter vector pEpo3\textsubscript{\textregistered}/Glut1-Luc (containing a trimer of murine Epo 3′ enhancer and the Glut-1 promoter; a kind gift of Dr Paul Schumacker, University of Chicago, Ill) and vector pHR-L-null (Promega) containing a synthetic Renilla gene sequence (hRluc) to enable accurate control for transfection efficiency and indexing of luciferase activity.\textsuperscript{14} Transfection was achieved using Effectene optimized according to the instructions of the manufacturer. Twenty-four hours later, mock-transfected SVEC4–10 were exposed to medium alone or medium containing CoCl\textsubscript{2} (150 μmol/L) and cultures incubated for 4 hours to chemically stabilize HIF-1\textsubscript{α}. Firefly and Renilla luciferase output were quantified by luminometer and results expressed as an index of relative light units (RLU).

**RNA Preparation and RT-PCR Analysis**

Total RNA was extracted and purified using QIAshredders and RNeasy columns (Qiagen) according to the specifications of the manufacturer. Total RNA (1 μg) was reverse transcribed into cDNA using the Thermoscript RT-PCR system and Platinum Taq DNA polymerase. Primers used for PCR are shown in Table 1.

**In Vivo siRNA Administration Protocol**

The PHD2 or nontargeting siRNAs were administered to animals via IP injection as described recently.\textsuperscript{23} Animals received 1.5 μg of siRNA per gram of body weight. Before administration, PHD2 siRNA was bound to siPORT\textsuperscript{\textregistered} Amine transfection reagent according to the instructions of the manufacturer. Briefly, siPORT\textsuperscript{\textregistered} Amine was incubated for 30 minutes at 22°C in saline. The siPORT\textsuperscript{\textregistered} Amine/saline mixture was then incubated with the PHD2 siRNA in a 1:1 ratio for 30 minutes at 22°C. The siPORT\textsuperscript{\textregistered} Amine/siRNA was administered in a total volume of 200 μL.

**TABLE 1. Primer Sequences Used in RT-PCR**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>PHD1 GGAGTTAGCCGGCTACAGTACAGC</td>
<td>CTGTGTCTCTGGCAAGTGATCCACG</td>
<td>672</td>
</tr>
<tr>
<td>PHD2 GTGACTCTTCGAGAGGGCAAGG</td>
<td>CCGAGATCACTATCATGACTGCAGG</td>
<td>900</td>
</tr>
<tr>
<td>eNOS TGGAGCGGAGATATCAGTATG</td>
<td>CAGCCTGTCCTGGTATG</td>
<td>100</td>
</tr>
<tr>
<td>iNOS TCAGGCTCTGGAAATTCACA</td>
<td>ATCTGGTGCCTATGCACC</td>
<td>350</td>
</tr>
<tr>
<td>β-Actin TCTAGAGGGCTATGCTCTCC</td>
<td>TCTTGATGTGCAGAGATTTC</td>
<td>125</td>
</tr>
</tbody>
</table>
Murine Model of Global I/R Injury

The methodology for Langendorff-isolated, buffer-perfused mouse heart preparation was previously described in detail.24,25 The adult male F2 homozygous (−/−) iNOS gene knockout B6,129 mice were purchased from Jackson Laboratory (Bar Harbor, Maine).26 Wild-type mice for these studies were from the B6,129 strain. This ex vivo model enabled the measurement of ventricular contractile function as well as infarct size after 30 minutes of no-flow global ischemia and 60 minutes of reperfusion (I/R). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the NIH (Publication No. 85-23, revised 1996). We subjected hearts from 7 groups of mice for these studies were from the B6,129 strain. This ex vivo model enabled the measurement of ventricular contractile function as well as infarct size after 30 minutes of no-flow global ischemia and 60 minutes of reperfusion (I/R). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the NIH (Publication No. 85-23, revised 1996). We subjected hearts from 7 groups of mice (n=6 for each group) to global cardiac I/R. Group 1, wild-type control mice, received sterile saline by IP injection 24 hours before cardiac I/R injury. Group 2, wild-type PHD2 siRNA-treated mice, received PHD2 siRNA (1.5 μg/g) by IP injection 24 hours before cardiac I/R injury. Group 3, wild-type nontargeting control siRNA-treated mice, received the nontargeting control siRNA (1.5 μg/g) by IP injection 24 hours before cardiac I/R injury. Group 4, PHD2 siRNA-treated iNOS−/− mice, received PHD2 siRNA (1.5 μg/g) by IP injection 24 hours before cardiac I/R injury. Group 5, iNOS−/− mice, received sterile saline by IP injection 24 hours before cardiac I/R injury. Group 6, wild-type PHD2 siRNA-treated mice plus 1400W, received PHD2 siRNA (1.5 μg/g) by IP injection 24 hours before cardiac I/R injury followed by treatment with 1400W (10 mg/kg) IP 30 minutes before cardiac I/R injury. Group 7, wild-type nontargeting control siRNA-treated mice plus 1400W, received the nontargeting control siRNA (1.5 μg/g) 24 hours before cardiac I/R injury followed by treatment with 1400W (10 mg/kg) IP 30 minutes before cardiac I/R injury. All hearts were then subjected to global I/R injury and infarct size determined as described above.

Functional Recovery Assessment

Briefly, a left atrial incision was made to expose the mitral annulas through which a water-filled latex balloon was passed into the left ventricle (LV). The balloon was attached via polyethylene tubing to a Gould pressure transducer that was connected to a PowerLab acquisition system (AD Instruments, Castle Hill, NSW, Australia). The balloon was inflated to adjust the LV end-diastolic pressure (LVEDP) to ~10 mm Hg. Myocardial ischemic damage was measured using multiple, independent end points of tissue injury. These included infarct size, LV developed pressure (LVDP), rate-pressure product (RPP), heart rate, and coronary flow. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. RPP, an index of cardiac work, was calculated by multiplying LVDP by heart rate.

Statistical Analysis

All measurements of infarct size and risk areas are expressed as group means±SE. Changes in hemodynamics and infarct size variables were analyzed by 2-way repeated-measures ANOVA to determine the main effect of time, group, and time-by-group interaction. If the global tests showed major interactions, post hoc contrasts between different time points within the same group or between different groups were performed using t test. Statistical differences were considered significant if the probability value was <0.05. Molecular tests reported comprise the results of 3 separate observations.

Results

PHD2 Gene Silencing in SVEC4–10

PHD2 mRNA expression was assessed in SVEC4–10 transfected with a pooled PHD2 siRNA (SMARTpool) and compared with cells transfected with nontargeting control siRNA. Figure 1A and 1B show that the PHD2 siRNAs produced significant concentration and time-dependent reductions in PHD2 mRNA when compared with the highest concentration (100 nmol/L) of the nontargeting control siRNA. PHD2 silencing by PHD2 siRNA was long lasting as demonstrated by 54% reduction in mRNA levels after 24 hours and ~90% by 72 hours with 100 nmol/L SMARTpool siRNA (Figure
Effect of PHD2 Silencing on HIF-1α Protein Levels and HIF-1 Transcriptional Activity

In normoxic environments, PHD2 hydroxylates HIF-1α protein at proline 402 and/or 564 targeting the molecule for ubiquitination and degradation by the 26S proteasome. To study the effectiveness of PHD2 silencing in HIF-1α stabilization in normoxic murine EC, we measured HIF-1α protein levels in SVEC4–10 transfected with the PHD2 siRNA. Our results show that HIF-1α steady-state levels increase in a concentration and time-dependent fashion (Figure 2A and 2B). Steady-state levels of HIF-1α in SVEC4–10 transfected with nontargeting control siRNA were not different from normoxic, medium-exposed controls (Figure 2A).

Effect of In Vivo PHD2 Silencing on Cardiac iNOS Gene Expression and HIF-1α Stabilization

Identical concentrations (1.5 μg/g) of PHD2 siRNA or nontargeting siRNA control were administered by IP injec-
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4B). showed no change in PHD2 or iNOS mRNA (Figure 4A and 4B). Also, PHD2 silencing in heart and lungs (B) was removed and snap frozen and total RNA isolated. Semiquantitative RT-PCR was then performed using primers to the murine PHD2, iNOS, and β-actin genes. mRNA expression was assessed and compared with animals injected with nontargeting control siRNA for 48 hours. C, HIF-1 expression was assessed and compared with animals injected with wild-type nontargeting control siRNA for 48 hours. 

Figure 4. PHD2 siRNA injection silences PHD2 expression in the heart and lungs while upregulating iNOS. PHD2 siRNA or nontargeting control siRNA (1.5 μg/g) were administered by IP injection into wild-type mice for 0, 5, 24, and 48 hours. Heart (A) and lungs (B) were removed and snap frozen and total RNA isolated. Semiquantitative RT-PCR was then performed using primers to the murine PHD2, iNOS, and β-actin genes. Amplicons were resolved via polyacrylamide gel electrophoresis as described above. The siRNA administration protocol used in these studies produced significant PHD2 silencing in heart and lung tissue within 5 hours of injection with sustained silencing observed over the ensuing 48 hours (Figure 4A and 4B). Also, PHD2 silencing resulted in significant upregulation of cardiac iNOS expression by 24 hours that was also sustained for the 48-hour study period. Animals receiving nontargeting control siRNA showed no change in PHD2 or iNOS mRNA (Figure 4A and 4B).

Nuclear extracts isolated from the identical hearts reported above show that cardiac PHD2 gene silencing mediated via the PHD2 siRNA produced a corresponding significant increase in nuclear HIF-1α protein at 5 hours (Figure 4C). The sustained 48-hour PHD2 silencing observed in our studies produced significant and sustained stabilization of HIF-1α.

Effect of In Vivo PHD2 Silencing on I/R Injury

Figure 5 shows left ventricular functional recovery data from the 7 study groups. Following 60 minutes of reperfusion, the RPPs (P<0.01) were significantly higher in PHD2 siRNA-transfected animals compared with wild-type saline controls and wild-type nontargeting siRNA controls. The improved recovery of RPP was lost in iNOS−/− mice infused with PHD2 siRNA (group 4) and wild-type PHD2 siRNA treated with iNOS inhibitor 1400W (group 6). No significant changes in RPP were observed in mice from wild-type nontargeting control siRNA treated with iNOS inhibitor 1400W (group 7) and saline-infused iNOS−/− (group 5).

Figure 6 shows that PHD2 siRNA-treated animals exhibited significant reduction of infarct size (group 2, 69% reduction, P<0.0001) as compared with wild-type saline-infused (group 1) and wild-type nontargeting control siRNA treated mice (group 3). The protective effects of cardiac PHD2 silencing and HIF-1 stabilization was lost in iNOS−/− (group 4) and wild-type PHD2 siRNA mice treated with iNOS inhibitor 1400W (group 6). These results demonstrate the essential role of iNOS expression in preconditioning the myocardium against I/R injury following HIF-1 activation. Whereas coronary flow was not significantly different between the study groups, we did not observe any detrimental effects in the siRNA treated groups (data not shown). The baseline levels of heart rate, coronary flow, and LVDP were not different between the groups (Table 2).

Discussion

HIF-1 is a heterodimeric α,β transcription factor that mediates tissue responses to hypoxia. By virtue of its ability to upregulate genes intimately involved in ischemic preconditioning (eg, iNOS, VEGF, and HO-1), HIF-1 becomes an attractive molecular target to limit ischemic or posts ischemic tissue injury. HIF-1 transcriptional activity is determined by expression and stability of the α subunit. Under normoxic conditions, hydroxylation of proline residues 402 and/or 564 by the action of prolyl 4-hydroxylases continually targets the HIF-1α protein subunit for ubiquitination and proteasomal degradation, typically resulting in low to undetectable steady-state levels in cell nuclei. We recently reported that the nonspecific HIF prolyl hydroxylase inhibitor DMOG significantly increased nuclear HIF-1α levels and HIF-1 transcriptional activity in normoxic microvascular endothelium in vitro. In vivo, we showed that hearts preconditioned by prior DMOG administration exhibited significantly reduced infarct size following I/R in rabbits.

In the current study, we used a specifically designed siRNA directed against murine PHD2 in a series of in vitro and in vivo experiments. RNA interference is a coordinated, sequence-specific mechanism producing posttranscriptional gene silencing. Berra et al demonstrated that inhibition of the PHD2 isofrom with siRNA, but not inhibition of PHD1 or PHD3, was sufficient to upregulate HIF-1α in EC. These
investigators further showed that PHD2 plays a key role in regulating steady-state levels of HIF-1α in normoxia and that PHD2 silencing fully prevents HIF-1α degradation following re-oxygenation of posthypoxic EC.33

Our in vitro studies show that SVEC4–10 transfected with the PHD2 siRNA under normoxic conditions exhibited both concentration and time-dependent silencing of PHD2 mRNA (Figure 1A and 1B). PHD2 silencing under identical conditions resulted in long-lasting concentration-dependent stabilization of HIF-1α steady-state levels in SVEC4–10 nuclei (Figure 2A and 2B). HIF-1 activated under these conditions was transcriptionally active (Figure 2C), promoting expression of murine iNOS but not eNOS (Figure 3). Thus, using an RNA interference strategy in a murine cell system, we

Figure 5. PHD2 silencing improves left ventricular function recovery hearts from 7 groups: (1) wild-type saline-infused control; (2) wild-type PHD2 siRNA; (3) wild-type nontargeting siRNA control; (4) iNOS−/− infused with PHD2 siRNA; (5) saline-infused iNOS−/−; (6) wild-type PHD2 siRNA plus 1400W; (7) wild-type nontargeting control siRNA plus 1400W were subjected to 30 minutes of normothermic ischemia followed by 60 minutes of reperfusion. Significantly higher RPPs were observed only in PHD2 siRNA-transfected animals (group 2) at the conclusion of 60 minutes of reperfusion when compared with wild-type saline controls (group 1) and wild-type nontargeting siRNA controls (group 3; *P<0.01). The increase in RPP was lost in group 4 iNOS−/− mice infused with PHD2 siRNA and group 6 wild-type PHD2 siRNA plus 1400W, showing the essential role that iNOS expression plays in preconditioning the myocardium. Mice from group 7, wild-type nontargeting control siRNA plus 1400W and group 5, saline-infused iNOS−/− had RPPs similar to group 1, wild-type saline-infused control.

Figure 6. In vivo PHD2 silencing attenuates cardiac ischemia reperfusion injury hearts from 7 groups: (1) wild-type saline-infused control; (2) wild-type PHD2 siRNA; (3) wild-type nontargeting siRNA control; (4) iNOS−/− infused with PHD2 siRNA; (5) saline-infused iNOS−/−; (6) wild-type PHD2 siRNA plus 1400W; (7) wild-type nontargeting control siRNA plus 1400W were subjected to 30 minutes of normothermic ischemia followed by 60 minutes of reperfusion. Group 2 hearts (wild-type PHD2 siRNA-treated animals) exhibited significant attenuation of infarct size when compared with group 1 (wild-type saline-infused) and group 3 (wild-type nontargeting control siRNA treated mice, *P<0.001). The protective effects of cardiac PHD2 silencing and HIF-1 stabilization was lost in group 4 (iNOS−/− mice infused with PHD2 siRNA) and group 6 (wild-type PHD2 siRNA plus 1400W) showing the essential role that iNOS expression plays in preconditioning the myocardium against ischemia reperfusion injury. Expectedly, mice from group 7 (wild-type nontargeting control siRNA plus 1400W) and group 5 (saline-infused iNOS−/−) had infarct sizes similar to group 1, wild-type saline-infused control.
obtained long-lasting HIF-1 activation and demonstrated its regulatory impact on iNOS, a key gene implicated in late phase of ischemic and pharmacological preconditioning.\textsuperscript{24}

When administered in vivo into wild-type mice, the PHD2 siRNA induced significant PHD2 gene silencing within 5 hours in both heart and lung tissue that was sustained for a full 48 hours (Figure 4A and 4B). PHD2 silencing was not present in saline control mice or mice receiving nontargeting control siRNA. PHD2 silencing produced significant HIF-1α stabilization in murine myocardium that was sustained under normoxic conditions for 48 hours (Figure 4C). In addition, PHD2 siRNA in hearts from wild-type mice produced iNOS upregulation that was detectable at 5 hours and sustained for 48 hours (Figure 4A).

Our siRNA treatment strategy produced significant cardioprotection against I/R stress in the mouse heart (Figure 6). Hearts from PHD2 siRNA-treated mice showed reduction in infarct size (69%, \(P<0.0001\)) as compared with hearts from saline-treated mice or mice receiving nontargeting control siRNA. Reduced infarct size in hearts from PHD2 siRNA-treated mice was associated with significant improvements in hemodynamic performance (Figure 5).

Prior reports show that HIF-1 activation attenuates I/R injury.\textsuperscript{18,25,34,35} The current study advances existing knowledge by showing that activation of HIF-1 in normoxic myocardium via a gene specific, nonpharmacological mechanism (PHD2 silencing) produces NO-dependent cardioprotection. The iNOS\textsuperscript{−/−} mice treated by an identical PHD2 siRNA injection protocol demonstrated no cardioprotection. Postischemic infarct sizes in iNOS\textsuperscript{−/−} mice receiving the PHD2 siRNA were not different from saline-treated control mice or mice injected with nontargeting control siRNA (Figure 6). Furthermore, the rate-pressure products as well as postischemic infarct sizes in PHD2 siRNA-infused wild-type mice in which iNOS was inhibited using 1400W were not different from saline-treated control wild-type mice or wild-type mice injected with nontargeting control siRNA. In addition to using iNOS\textsuperscript{−/−} mice, the pharmacological approach of inhibiting iNOS using 1400W further supports the essential role of iNOS in preconditioning the myocardium against I/R injury. Xi et al activated HIF-1 in wild-type mice by CoCl\textsubscript{2} infusion 24 hours before I/R injury and showed significant reductions in infarct size when compared with saline-treated control mice.\textsuperscript{25} The CoCl\textsubscript{2}-induced delayed cardioprotection was absent in iNOS gene-deficient mice. Enhanced iNOS expression in myocardium resulting via HIF-1 dependent mechanisms or by other means (eg, monophosphoryl lipid A,\textsuperscript{24} adenosine A\textsubscript{3} receptor activation,\textsuperscript{36} sildenafil\textsuperscript{27}) increases myocardial NO. NO generated from iNOS activation activates guanylate cyclase resulting in enhanced formation of cGMP. cGMP may activate protein kinase G, leading to subsequent opening of mitochondrial K\textsubscript{ATP} channels.\textsuperscript{37} Current thought suggests this phenomenon promotes cardioprotection both by preventing ATP depletion and by preventing increases in intracellular calcium. Additional studies also suggest that NO signaling attenuates cell death resulting from necrosis and apoptosis, by altering Bcl2/Bax ratio.

In summary, we have used a gene silencing strategy that produced prolonged activation of HIF-1 both in murine endothelium in vitro and in murine cardiac tissue following IP treatment. Targeting HIF-1 in this fashion resulted in significant iNOS-dependent cardiac preconditioning against I/R injury. This work provides new insight into the key role that HIF-1–dependent genes such as iNOS play in cardiac preconditioning. Finally, this work indicates that RNA interference may provide a useful molecular approach for attenuation of cardiac I/R injury and identifies PHD2 as a new molecular target for therapeutic intervention. The short time frame over which cardiac I/R injury develops in the current model present limitations for extrapolating to human disease. Studies are in progress in our laboratory that will examine longer postischemic periods in animals receiving the PHD2 siRNA.

**Note Added in Proof**

Two families of “prolyl 4-hydroxylases” exist: HIF prolyl 4-hydroxylases and pro-collagen prolyl 4-hydroxylases. HIF prolyl 4-hydroxylases are highly specific for HIF-1 whereas the ability of pro-collagen prolyl 4-hydroxylases to hydroxylate HIF-1 remains unknown. At the time we designed the siRNA in early summer of 2004, the nomenclature wasn’t completely worked out for murine systems. We chose the sequence for a murine prolyl 4-hydroxylase from our study of the GenBank database and began our studies. Our dramatic results both in cultured cells and in intact mice led to the submission of the manuscript. At the author proof stage, revisions to the GenBank database showed that the siRNA we designed (from GenBank accession No. NM_011031) was to murine pro-collagen prolyl 4-hydroxylase-2 not to HIF prolyl 4-hydroxylase-2. The published literature would suggest that using an siRNA to inactivate pro-collagen prollyl

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### Table 2. Hemodynamic Data

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDP (mm Hg)</th>
<th>LVDP (mm Hg)</th>
<th>Heart Rate (bpm)</th>
<th>RPP (mm Hg/min×10\textsuperscript{−3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Wild-type saline-infused control</td>
<td>6.8±0.3</td>
<td>97.0±6.2</td>
<td>418.2±5.9</td>
<td>40.5±2.3</td>
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<tr>
<td>2  Wild-type PHD2 siRNA</td>
<td>7.4±0.2</td>
<td>102.5±3.9</td>
<td>419.7±7.5</td>
<td>42.9±0.9</td>
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<tr>
<td>3  Wild-type NTSC</td>
<td>7.0±0.7</td>
<td>101.5±2.2</td>
<td>415.8±6.5</td>
<td>42.2±1.1</td>
</tr>
<tr>
<td>4  iNOS\textsuperscript{−/−} PHD2 siRNA</td>
<td>8.2±0.2</td>
<td>98.9±4.6</td>
<td>429.2±4.7</td>
<td>42.5±2.4</td>
</tr>
<tr>
<td>5  iNOS\textsuperscript{−/−} saline-infused control</td>
<td>7.4±0.4</td>
<td>100.9±2.6</td>
<td>421.7±3.5</td>
<td>42.5±1.1</td>
</tr>
<tr>
<td>6  Wild-type+1400W PHD2 siRNA</td>
<td>6.7±0.3</td>
<td>99.3±3.9</td>
<td>424.5±4.3</td>
<td>42.7±1.8</td>
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<tr>
<td>7  Wild-type+1400W NTSC</td>
<td>7.4±0.4</td>
<td>105.8±4.0</td>
<td>430.3±3.7</td>
<td>45.5±1.6</td>
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4-hydroxylase-2 would not result in HIF-1 activation. But in every case in multiple studies over the past 18 months both in cultured cells and in mice we found very potent HIF-1 activation. Our observations are conclusive. Activation of HIF-1 in murine heart by using our siRNA exerts dramatic protective effects.

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Hypoxia Inducible Factor-1 Activation by Prolyl 4-Hydroxylase-2 Gene Silencing Attenuates Myocardial Ischemia Reperfusion Injury
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Effect of PHD2 Gene Silencing in Murine SVEC Employing PHD2 siRNA #3: SVEC4-10 were transfected with PHD2 siRNA #3 (100nM) or non-targeting siRNA control (NTSC, 100nM) for 0, 24, 48 and 72 hours. PHD2/β-actin mRNA expression was assessed and compared to cells transfected with non-targeting control siRNA. Values are presented as mean ± SEM of 3 individual experiments (*P<0.05). PHD2 mRNA levels were reduced by 50% after 24 hours and further decreased to ~60% by 72 hours using 100nM siRNA #3. Transfection with non-targeting siRNA control produced no effects on PHD2 mRNA expression when compared to non-transfected control SVEC4-10.
PHD2 SiRNA Fails To Silence Cardiac And Lung PHD1 Expression: PHD2 siRNA was administered by intraperitoneal injection into wild-type mice for 0, 24 and 48 hours. Heart and lungs were removed, snap frozen and total RNA isolated. Semi-quantitative RT-PCR was then performed employing primers to murine PHD1 and β-actin genes. PHD1 expression was unchanged in both heart and lungs over 48 hours suggesting that PHD2 siRNA mediated gene silencing was specific.