Background—Preconditioning phenomena provide evidence for adaptive responses to ischemia that have important implications for treatment/prevention of myocardial infarction. Hypoxia-inducible factor 1 (HIF-1) mediates adaptive transcriptional responses to hypoxia/ischemia.

Methods and Results—Exposure of wild-type mice to intermittent hypoxia resulted in protection of isolated hearts against ischemia-reperfusion injury 24 hours later. Cardiac protection induced by intermittent hypoxia was lost in Hif1a\textsuperscript{-/-} mice heterozygous for a knockout allele at the locus encoding HIF-1. Erythropoietin (EPO) mRNA expression was induced in kidneys of wild-type mice subjected to intermittent hypoxia, resulting in increased plasma EPO levels. EPO mRNA expression was not induced in Hif1a\textsuperscript{-/-}\textsuperscript{-/-} mice. EPO administration to rats increased functional recovery and decreased apoptosis in isolated hearts subjected to ischemia-reperfusion 24 hours later.

Conclusions—Hearts isolated from rodents subjected to intermittent hypoxia or EPO administration are protected against postischemic injury. Cardiac protection induced by intermittent hypoxia is critically dependent on Hif1a gene dosage. Our data suggest that additional studies to evaluate therapeutic applications of EPO administration are warranted.

Key Words: hypoxia ■ ischemia ■ myocardial infarction

The survival of myocardial tissue subjected to ischemia can be increased by prior exposure to repeated brief periods of sublethal ischemia induced by transient coronary artery occlusion and reperfusion, a phenomenon known as preconditioning.\textsuperscript{1} After the preconditioning stimulus, there is an early window of acute protection that lasts 1 to 2 hours, followed by a late window of delayed protection that lasts from approximately 12 to 72 hours.\textsuperscript{2,3} The molecular mechanisms underlying delayed ischemic preconditioning have been investigated in detail but remain incompletely understood.\textsuperscript{4} Exposure of animals to chronic systemic hypoxia also induces protection against myocardial ischemia, although the mechanisms are poorly characterized.\textsuperscript{5-7}

Hypoxia-inducible factor 1 (HIF-1) activates the transcription of genes whose protein products mediate adaptive responses to hypoxia/ischemia, including erythropoietin (EPO), glucose transporter 1 (GLUT1), nitric oxide synthase 2 (NOS2), and vascular endothelial growth factor (VEGF).\textsuperscript{8} HIF-1 consists of a constitutively expressed HIF-1\textsubscript{a} subunit and an O\textsubscript{2}-regulated HIF-1\textsubscript{a} subunit.\textsuperscript{9} Peak HIF-1\textsubscript{a} expression is observed after 1, 2, and 4 to 6 hours of continuous hypoxia in mouse kidney, liver, and brain, respectively.\textsuperscript{10} In this study we report that subjecting rodents to brief periods of systemic hypoxia and reoxygenation (intermittent hypoxia) induces delayed protection against myocardial injury after ischemia-reperfusion (I-R) and that this response is critically dependent on HIF-1\textsubscript{a} expression. We also show that prior EPO administration protects the isolated perfused heart against I-R injury.

Methods

Animals

Experiments used adult male Sprague-Dawley rats (\textsim 250 g; Harlan, Indianapolis, Ind) or adult male littermate mice (\textsim 25 g) arising from brother-sister matings of wild-type and Hif1a\textsuperscript{-/-} mice that have been interbred since their derivation.\textsuperscript{11}

Langendorff Preparation

Isolated heart perfusion was performed as described.\textsuperscript{12} Sodium pentobarbital (45 mg/kg) was administered by intraperitoneal injection. The heart was excised, and the ascending aorta was cannulated.
and perfused at a constant pressure of 100 cm H₂O with Krebs-Henseleit buffer (in mmol/L, glucose 11, NaCl 118, NaHCO₃ 25, CaCl₂ 1.2, KCl 4.7, KH₂PO₄ 1.2, and MgSO₄ 1.2), which was maintained at 37°C and bubbled continuously with a mixture of 95% O₂ and 5% CO₂. A fluid-filled latex balloon was inserted into the left ventricle and inflated to an initial end-diastolic pressure of 4 to 8 mm Hg. Left ventricular pressure, heart rate (HR), and coronary flow rate (CFR) were recorded continuously. Left ventricular developed pressure (LVDP) was calculated as the difference between the systolic and end-diastolic pressures.

**Triphenyltetrazolium Chloride Staining**
Hearts were sliced into 5 transverse sections and each was weighed, incubated for 15 minutes at 37°C in 1% triphenyltetrazolium chloride (TTC), and photographed, and the area of infarcted (unstained) and viable (stained) tissue was measured by computed planimetry. The mass-weighted average of the ratio of infarct area to total cross-sectional area of the ventricle from each slice was determined (percent infarction).

**Reverse Transcriptase–Polymerase Chain Reaction**
Total RNA was extracted from mouse heart and kidney using TRIZOL (Life Technologies Inc) and reverse transcribed into cDNA using Superscribe First-Strand Synthesis System (Invitrogen) for polymerase chain reaction (PCR) analysis of EPO, EPOR, GLUT1, NOS2, and VEGF mRNA and 18S rRNA. The ratio of mRNA to 18S rRNA was normalized to the mean value for control samples.

**Plasma EPO Levels**
An ELISA for human EPO (R&D Systems) was used according to the manufacturer’s instructions. A standard curve was generated with recombinant human EPO. Results are presented as relative values that have been normalized to the mean EPO level in untreated mice.

**TUNEL Assay**
Rat hearts were formalin-fixed, paraffin-embedded, and sectioned. The TUNEL assay was performed using the ApopTag Apoptosis Detection Kit (Intergen) followed by methyl green counterstaining. For each heart, ~70 fields encompassing an entire section of the left ventricle at its greatest diameter were photographed. The total number of TUNEL-positive cells was determined in a blinded manner.

**Activated Caspase 3 Assays**
Hearts were lysed in 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, and 1 mmol/L EGTA. Aliquots (15 µg of protein) were incubated for 5 minutes at room temperature in a 100-µL reaction containing 30 µmol/L Ac-Asp-Glu-Val-Asp-amino methyl coumarin (Upstate Biotechnology), and fluorescence intensity was measured. A standard curve was generated using purified activated caspase 3 (Upstate Biotechnology). Lysate aliquots (150 µg) were fractionated by SDS-12% polyacrylamide gel electrophoresis, and immunoblots were analyzed using antibodies specific for activated caspase 3 (Cell Signaling Technology).

**Figure 1.** Effect of intermittent hypoxia on the functional recovery of mouse hearts subjected to ischemia and reperfusion. A, WT and HET littermate mice (n=9 for each group) were untreated or subjected to 5 cycles of intermittent hypoxia either 0.5 hours (IHA) or 24 hours (IHD) before isolation. Hearts were perfused in Langendorff mode for 15 minutes to establish equilibration (Eq), and then (at time 0) perfusion was stopped for 30 minutes, followed by reperfusion. B, Analysis of LVDP. *P<0.05 vs WT. C, Hearts were subjected to 30 minutes of ischemia followed by 2 hours of reperfusion, and ventricular sections were incubated with TTC. For each experimental condition, representative sections (top) and mean % infarction (bar graphs) are shown. *P<0.0001 vs WT.
Statistical Analysis
Data are presented as mean±SEM. Differences between experimental groups were analyzed for statistical significance (P<0.05) by ANOVA or paired t test.

Results
Exposure of Mice to Intermittent Hypoxia Induces Delayed Cardiac Protection
Ischemia resulting from impaired tissue perfusion has multiple components, including hypoxia, nutrient deprivation, and metabolic waste accumulation. We hypothesized that protection induced by brief coronary artery occlusion-reperfusion may be the result of adaptive responses induced specifically by hypoxia-reoxygenation. We therefore investigated whether exposure of mice to cycles of hypoxia-reoxygenation would induce a protective response similar to the effect of occlusion-reperfusion. Mice were placed in a closed chamber in which the O₂ concentration was rapidly reduced to 6% for 6 minutes, after which the chamber was vented to room air for 6 minutes. The process was repeated for 5 cycles over 1 hour. Control mice were placed in a chamber flushed with room air at 6-minute intervals. Hearts were isolated for 30 minutes or 24 hours after exposure to intermittent hypoxia (IH) (Figure 1A).

For all hearts, HR and LVDP dropped to zero within 2 minutes after cessation of perfusion. At 60 minutes, ie, 30 minutes of reperfusion, the LVDP of hearts from control wild-type (WT) mice was 12.0±2.8 mm Hg, a recovery of 23.7% (Figure 1B). Hearts from WT mice subjected to IH 24 hours before ischemia (WT-IHD) exhibited a mean LVDP of 27.9±4.4 mm Hg at 60 minutes (50.7% recovery), which was significantly increased relative to control WT mice. In contrast, acute exposure of WT mice to IH 30 minutes before ischemia (WT-IHA) had no protective effect, because the LVDP was 9.9±2.3 mm Hg at 60 minutes (19.4% recovery). Mean HR did not differ significantly between groups. The significant differences in LVDP (Figure 1B) resulted in significant differences in the rate-pressure product (LVDP×HR) between groups.

To demonstrate an effect of IH on myocardial survival, hearts were subjected to I-R and incubated in TTC, which stains viable myocardium red. Infarct size in hearts from WT mice exposed to IH 24 hours before ischemia (WT-IHA) had no protective effect, because the LVDP was 9.9±2.3 mm Hg at 60 minutes (19.4% recovery). Mean HR did not differ significantly between groups. The significant differences in LVDP (Figure 1B) resulted in significant differences in the rate-pressure product (LVDP×HR) between groups.

Loss of Cardiac Protection in Hif1a<sup>−/−</sup> Mice
To establish a role for HIF-1 in delayed cardiac protection induced by IH, we analyzed mice heterozygous (HET) for a knockout allele at the Hif1a locus. Hif1a<sup>−/−</sup> cells manifest reduced expression of HIF-1α protein and HIF-1 target genes under hypoxic conditions. Previous studies have demonstrated impaired physiological responses to hypoxia in Hif1a<sup>−/−</sup> mice. Untreated HET mice demonstrated poor recovery of cardiac function (LVDP<sub>60 minutes</sub>/LVDP<sub>0 minutes</sub>=12.5%; Figure 1B) and large infarcts (49.6±8.8%; Figure 1C), similar to WT littermates (control WT mice analyzed above). Exposure of HET mice to hypoxia 24 hours before ischemia (HET-IHD) had no protective effect on functional recovery (LVDP<sub>60 minutes</sub>/LVDP<sub>0 minutes</sub>=20.3%; Figure 1B) or infarct size (42.2±9.9%; Figure 1C). Thus, partial loss of HIF-1α expression results in a complete loss of protection induced by IH.

Analysis of Gene Expression in Hypoxic Mice
To analyze the effects of hypoxia on the expression of HIF-1 target genes, total RNA was isolated from hearts of WT mice that were untreated, exposed to continuous hypoxia, and immediately killed or exposed to IH and killed 0, 1, 2, 4, or 8 hours later. Reverse transcriptase (RT)-PCR analyses of mRNAs encoded by HIF-1 target genes, including GLUT1 (Figure 2F), VEGF (Figure 2E), and NOS2 (data not shown)
mRNA, failed to show increased expression in response to intermittent or continuous hypoxia.

HIF-1 was originally identified as a factor that activated transcription of the EPO gene, and, among HIF-1 target genes, EPO shows the greatest induction in response to hypoxia. Analysis of RNA isolated from mouse kidneys (the primary source of plasma EPO) revealed a 5-fold increase in EPO mRNA expression after continuous or intermittent hypoxia (Figure 2A). In contrast, the expression of VEGF mRNA was not significantly increased in the kidneys of mice subjected to hypoxia (Figure 2D). EPO mRNA expression in the heart was at the limits of detection and could not be reliably quantified. Thus, among the genes analyzed, exposure of mice to 1 hour of IH selectively induced EPO mRNA expression in the kidney.

Increased Plasma EPO Levels After Intermittent Hypoxia

Plasma EPO levels were significantly increased after IH (Figure 2B). EPO mRNA levels in the kidney peaked at 0 to 1 hour after IH (Figure 2A), thus preceding the peak plasma EPO levels at 1 to 2 hours (Figure 2B). The lag between mRNA synthesis and protein secretion may explain the less robust increase in plasma EPO levels after continuous hypoxia, because plasma EPO levels were only measured immediately after the hypoxic exposure.

Loss of Hypoxia-Induced EPO Expression in Hif1a+/− Mice

To demonstrate that HIF-1 is required for the induction of EPO mRNA, WT and HET mice were exposed to IH and killed immediately after the fifth cycle of hypoxia-reoxygenation (IH-0 time point). RT-PCR analysis of kidney RNA demonstrated that in WT mice, EPO mRNA expression was induced 6.4-fold (Figure 2C). In contrast, HET mice showed no significant increase in EPO mRNA expression.

Cardiac Expression of EPO Receptor mRNA

The biological activity of EPO is mediated by binding to EPOR, its cognate receptor. EPOR mRNA was detected in the hearts of WT and HET mice by RT-PCR (Figure 3). In contrast to the expression of EPO mRNA in the kidney (Figure 2A), expression of EPOR mRNA in the heart was not induced immediately after IH (IH-0 time point; Figure 3).

Protective Effect of EPO Administration

The results described above did not prove that increased EPO expression was necessary for the protection induced by IH. However, because EPO is a drug with an extensive history of clinical use, we next tested whether EPO administration was sufficient to induce protection against I-R injury. Rats received an intraperitoneal injection of saline or recombinant human EPO at a dose of 5000 U/kg. Hearts were isolated for Langendorff perfusion 24 hours later and subjected to I-R. Compared with the poor recovery in controls (LVDF at minute 60).
LVDP_{60 \text{ minutes}} = 18.4\% \text{; Figure 4A), hearts from EPO-treated rats showed significantly increased recovery of function (LVDP_{60 \text{ minutes}} / LVDP_{0 \text{ minutes}} = 54.1\%). EPO had no significant effect on HR (Figure 4B) or CFR (Figure 4C).

Reduction of Ischemia-Induced Apoptosis in the Hearts of EPO-Treated Rats

EPO inhibits apoptosis in erythroid progenitors, neurons, and endothelial cells.\textsuperscript{17–20} To determine whether EPO protected against myocardial apoptosis, rats were treated with saline or 5000 U/kg of EPO, and 24 hours later isolated hearts were subjected to ischemia. As before, EPO treatment was associated with increased recovery of LVDP. After 45 minutes of reperfusion, the hearts were processed for TUNEL assay (Figure 5). In the LV free wall of both hearts, most apoptotic cells were localized to the subendocardial region, whereas in the interventricular septum, the distribution of apoptotic cells was transmural. There was no TUNEL staining in areas of necrosis in either heart. EPO treatment was associated with a dramatic reduction in the total number of apoptotic cells (Figure 5).

As an independent measure of apoptosis, hearts from EPO- and saline-treated rats were subjected to I-R, lysates were prepared, and levels of activated caspase 3 were determined. Activation of caspase 3 is a critical event linking signals from the endoplasmic reticulum, mitochondria, or plasma membrane with downstream effectors of apoptosis. Compared with the control, the heart from the EPO-treated rat showed improved recovery of function (Figure 6A) and decreased caspase 3 activity as measured by incubation of lysates with a fluorogenic caspase 3 substrate (Figure 6B). Immunoblot analysis of lysates revealed the presence of activated caspase 3 in the control heart but not in the heart from the EPO-treated rat (Figure 6C). Thus, data from 3 independent assays demonstrate that systemic EPO administration reduces apoptosis in the postischemic heart.

Discussion

In this study we report 4 related but distinct findings. First, we have demonstrated for the first time that exposure of mice to brief episodes of IH induces protection against myocardial ischemia 24 hours later. While this work was in progress, another group reported delayed protection after continuous exposure of mice to 10\% \text{O}_2 for 4 hours.\textsuperscript{21} In that study, NOS2 protein expression was increased in the heart, and inhibition of NOS2 activity blocked the protective effect of hypoxia. We found no evidence of increased NOS2 mRNA in the hearts of mice after IH but did not analyze NOS2 protein expression. Thus, the role of NOS2 in protection against myocardial ischemia induced by IH remains to be determined. We have found that at lower oxygen concentrations (7\% \text{O}_2), exposure of rats to continuous hypoxia for 1 hour is sufficient to induce protection against I-R injury 24 hours later (Z. Cai and G.L. Semenza, unpublished data, 2003). Our studies suggest that the systemic stimulus of hypoxia-reoxygenation may induce protection via a systemic response that would represent a mechanism that is distinct from the local protective response induced by brief coronary occlusion-reperfusion.\textsuperscript{1–4} The absence of acute protection in hearts of mice subjected to hypoxia-reoxygenation is consistent with this hypothesis.

Second, we demonstrated that the protective effect of intermittent hypoxia was lost in Hif1a\textsuperscript{-/-} mice. Previous studies demonstrated dramatic defects in these mice, including a loss of hypoxic ventilatory responses mediated by the carotid body.\textsuperscript{13} The complete loss of hypoxia-induced cardiac protection in mice that are only partially HIF-1\alpha deficient indicates that one functional Hif1a allele is not sufficient to mediate the adaptive responses induced by IH.

Third, we demonstrated that EPO mRNA expression is induced in the kidneys of WT but not Hif1a\textsuperscript{-/-} mice. The selective induction of EPO expression (relative to the other HIF-1 target genes analyzed) in response to brief episodes of hypoxia-reoxygenation may reflect the fact that compared

Astrocytes produce EPO in response to hypoxia and neurons express EPOR, suggesting a paracrine mechanism for cerebral protection induced by hypoxic preconditioning. Although we demonstrated EPOR mRNA expression in the mouse heart, cardiac EPO mRNA expression was at the limits of detection, arguing against a paracrine mechanism. Renal EPO mRNA expression and plasma EPO levels were significantly induced by IH, suggesting an endocrine mechanism of cardiac protection. However, additional studies are required to confirm this model. In addition to its expression by endothelial cells, EPO protein expression has been demonstrated in the myocardium and endocardium of 12.5-day mouse and 18-week human embryos. Additional studies are required to determine whether EPOR expression in the adult heart is localized to myocytes or endothelial cells.

Our data provide the first evidence that EPO administration is protective against myocardial injury after I-R. Although the Langendorff-perfused heart preparation is a well-established model for studying I-R injury, it differs from coronary artery occlusion in situ in several important respects, including the presence of asystole during the ischemic episode and the absence of an inflammatory response. Thus, additional preclinical studies involving coronary artery occlusion in situ are necessary to determine whether these observations can be translated to the treatment of patients with myocardial ischemia.

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References


Hearts From Rodents Exposed to Intermittent Hypoxia or Erythropoietin Are Protected Against Ischemia-Reperfusion Injury

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