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Loss or Silencing of the PHD1 Prolyl Hydroxylase Protects Livers of Mice Against Ischemia/Reperfusion Injury

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BACKGROUND & AIMS: Liver ischemia/reperfusion (I/R) injury is a frequent cause of organ dysfunction. Loss of the oxygen sensor prolyl hydroxylase domain enzyme 1 (PHD1) causes tolerance of skeletal muscle to hypoxia. We assessed whether loss or short-term silencing of PHD1 could likewise induce hypoxia tolerance in hepatocytes and protect them against hepatic I/R damage. **METHODS:** Hepatic ischemia was induced in mice by clamping of the portal vessels of the left lateral liver lobe; 90 minutes later livers were reperfused for 8 hours for I/R experiments. Hepatocyte damage following ischemia or I/R was investigated in PHD1-deficient ($PHD1^{-/-}$) and wild-type mice or following short hairpin RNA-mediated short-term inhibition of PHD1 in vivo. **RESULTS:** $PHD1^{-/-}$ livers were largely protected against acute ischemia or I/R injury. Among mice subjected to hepatic I/R followed by surgical resection of all nonischemic liver lobes, more than half of wild-type mice succumbed, whereas all $PHD1^{-/-}$ mice survived. Also, short-term inhibition of PHD1 through RNA interference–mediated silencing provided protection against I/R. Knockdown of PHD1 also induced hypoxia tolerance of hepatocytes in vitro. Mechanistically, loss of PHD1 decreased production of oxidative stress, which likely relates to a decrease in oxygen consumption as a result of a reprogramming of hepatocellular metabolism. **CONCLUSIONS: Loss of PHD1 provided tolerance of hepatocytes to acute hypoxia and protected them against I/R-damage. Short-term inhibition of PHD1 is a novel therapeutic approach to reducing or preventing I/R-induced liver injury.**

Keywords: PHD1; Prolyl Hydroxylase; Ischemia/Reperfusion.

Liver dysfunction due to ischemia/reperfusion (I/R) damage is a frequent clinical complication of interrupted hepatic blood flow that occurs in low-flow states and in patients undergoing liver surgery. I/R injury is the main cause of failure of liver allografts and an important problem influencing outcomes of transplantations in clinical practice.[1] Liver I/R injury arises as a result of various consecutive pathophysiologic processes.[2] Initially, oxygen shortage causes metabolic pertubation with mitochondrial dysfunction and energy deficiency in ischemic hepatocytes.[3] The resultant hepatocyte swelling contributes to narrowing of sinusoidal blood vessels and accumulation of leukocytes in sinusoids.[4] A substantial inflammatory response follows, causing parenchymal injury, in part through excess production of toxic reactive oxygen species (ROS)[5] and degradative enzymes.[6] Thus, mitochondrial dysfunction and energy deficiency early in the ischemic phase trigger a chain of deleterious pathophysiologic responses, ultimately causing hepatocyte death and liver dysfunction.[2]

The prolyl hydroxylase domain (PHD) enzymes PHD1, PHD2, and PHD3 are oxygen sensors that regulate the stability of hypoxia inducible transcription factors (HIFs) in an oxygen-dependent manner, thus mediating cellular adaptive responses to changes in oxygen supply.[7,8] We recently demonstrated that loss of PHD1 protects ischemic skeletal muscle against mitochondrial dysfunction and toxic ROS formation.[9] Hypoxia tolerance of $PHD1^{-/-}$ skeletal muscle cells relied on metabolic reprogramming that attenuated mitochondrial oxidative metabolism and oxygen consumption. These changes already occurred in baseline conditions and were mediated via HIF-2α (and HIF-1α), as well as by enhanced expression of the metabolic adaptors, pyruvate dehydrogenase kinase (PDK) isoenzymes PDK1 and -4.[9]

Abbreviations used in this paper: **ALT, alanine aminotransferase; HIF, hypoxia inducible factor; I/R, ischemia/reperfusion; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PDC, pyruvate dehydrogenase enzyme complex; PDK, pyruvate dehydrogenase kinase; PHD, prolyl hydroxylase domain; ROS, reactive oxygen species; WT, wild-type.**

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Here, we determined whether loss or silencing of PHD1 likewise protected hepatocytes against hypoxic damage and, if so, whether this hypoxic protection was sufficient to attenuate hepatic I/R injury. We report that PHD1-deficiency reduces liver cell damage and hepatic dysfunction in a model of murine liver I/R. Notably, this beneficial effect was also achieved by short-term silencing of PHD1 in the liver. These findings highlight the therapeutic potential of short-term inhibition of PHD1 to prevent ischemic liver injury.

Materials and Methods

Mouse Models

Inactivation of PHD1 has been described elsewhere (Supplementary Note 1).[9] Male WT and PHD1$^{-/-}$ mice aged 10 to 12 weeks were used for experiments. To induce hepatic ischemia, portal vessels of the left lateral liver lobe were clamped. Sham-operated animals underwent median laparotomy and liver manipulation without clamping. Animals were sacrificed after 90 minutes of ischemia or after 90 minutes of ischemia followed by 8 hours of reperfusion (I/R). In survival experiments, the lateral liver lobe was clamped for 90 minutes and non-ischemic liver lobes were ligated and resected immediately after removal of the clamp. Thirty percent of WT and 40% of PHD1$^{-/-}$ mice died within 2 hours of hepatectomy due to surgical trauma and were excluded from further analysis. Sinusoidal perfusion was assessed by intravital microscopy. The lateral liver lobe was exteriorized on a mechanical stage, its upper surface placed under a fluorescent microscope for epi-illumination, and continuously superfused with prewarmed Ringer's solution. A 1:1 solution of fluorescein isothiocyanate–labeled erythrocytes was administered via a jugular catheter. Mean erythrocyte velocity was analyzed offline applying a computer-assisted software (Cap Image, Zeintl, Heidelberg, Germany) based on intravital imaging movies of perfused liver acini, and sinusoidal diameter was measured perpendicularly to the vessel path. The ratio of perfused sinusoids (representing the percentage of perfused sinusoids in relation to all visible sinusoids) was around 95% in WT and PHD1$^{-/-}$ mice, and was not significantly impaired when erythrocyte velocity was recorded immediately after ischemia. Real-time reverse transcription polymerase chain reaction and immunoblotting were performed as described previously (Supplementary Note 2).

Histology and Immunostaining

For histological assessment of liver cell damage, paraffin sections were stained with hematoxylin and eosin, and the area of hepatocytes with clearly definable borders (at least 120 cells per specimen) was determined morphometrically. For immunostaining, sections were digested with 0.2% trypsin (Sigma, Hamburg, Germany), blocked, and incubated overnight with primary antibodies: rat anti-CD45 (BD Pharmingen, San Diego, CA; 1/100), rat anti-CD31 (BD Pharmingen; 1/100), or mouse anti–8-hydroxy-2′-deoxyguanosine (8-OHdG) (Oxis International, Beverly Hills, CA; dilution 1/20). Sections were incubated with appropriate secondary antibodies and developed with 3,3-diaminobenzidine (Sigma). For detection of hypoxic cells, mice were treated with pimonidazole (Chemicon, Temecula, CA) 1 hour before collecting the liver; and liver sections were stained with the Hydroxyprobe1 antibody (Chemicon). Apoptosis was evaluated by TUNEL staining (Roche Diagnostics, Mannheim, Germany). All microscopic quantification analyses were performed in 12 standardized optical fields per specimen by 2 individual investigators using Zeiss KS300 morphometry software (Zeiss, Jena, Germany).

Metabolite Concentrations and PDC Activity

Liver metabolites and levels of energy-rich substrates were measured by enzymatic methods, and determination of pyruvate dehydrogenase enzyme complex (PDC) activity was performed on pulverized and homogenized liver tissue as described.[10] The cellular energy charge was calculated by applying the formula ([ATP] + 0.5[ADP] / [ATP] + [ADP] + [AMP]).[11] Relative ratio of glutathione-disulfide to total glutathione was assessed applying a glutathione colorimetric assay kit (Oxis GSH/GSSG-412) according to manufacturer's instructions.

Cell Culture Experiments

Hepatocytes were isolated using a 2-step collagenase perfusion technique as described.[12] Oxygen consumption was assessed in a high-resolution respirometer (OROBOROS, Innsbruck, Austria) at 37°C as described.[13] To simulate I/R in vitro, hepatocytes were incubated in Krebs-Ringer-HEPES buffer at acidic pH in a hypoxic chamber (0.5% O_2) for 4 hours.[14] Subsequently, hypoxic Krebs-Ringer-HEPES was replaced by aerobic growth medium containing WST-1 cell proliferation reagent (Roche) to simulate reperfusion. The quantity of formazan dye resulting from mitochondrial cleavage of the tetrazolium salt WST-1, which is indicative of the number of viable cells, was quantified after 2 hours by measuring the absorbance at 450 nm.

Short-Term Inhibition of PHD1 in Mice

An *shPHD1^{KD}* construct (5′–CACCGCTGCATCACCTGTATCTATTTCTCTTGAAA ATAGATACAGGTGATGCAGC) was designed as described (www.invitrogen.com/mai) to produce short hairpin interference RNA against PHD1 in vivo.[9] As control, we used an *shPHD1^{CTR}* construct (5′–CACCGCTTAACCCGTATTGCCTATTTCTCTTGAAAATA GGCAATACG GGTTAAGC). *shPHD1^{CTR}* and *shPHD1^{KD}* were cloned in an adenoviral expression system (pAd/BLOCK-iT-DEST; Invitrogen, Carlsbad, CA) applying the Invitrogen Gateway Technology (Ad*shPHD1^{CTR}*

and Ad*shPHD1*^{KD}, respectively; Supplementary Note 3). Ad*shPHD1*^{KD} and Ad*shPHD1*^{CTR} (5×10^9 plaque-forming units) in 100 μL saline were administered via tail vein injection.

Statistics

All values are represented as mean ± standard error of mean. The difference between means was analyzed with Student's *t* test. *P* values <.05 were considered significant. Log-rank test was applied to assess survival of mice subjected to liver ischemia and resection.

Results

Hypoxia Tolerance in Ischemic PHD1$^{-/-}$ Livers

We first determined the expression of the PHDs in the liver. Each PHD was detectable in the liver of WT mice; in PHD1$^{-/-}$ livers, PHD1 was undetectable, while PHD2 and PHD3 were not compensatorily up-regulated (Supplementary Note 4; Supplementary Figure S1*A*). Livers of PHD1$^{-/-}$ mice appeared normal (Figure 1*A*; Supplementary Note 1). To assess whether loss of PHD1 protects hepatocytes against ischemic damage, portal vessels were occluded for 90 or 180 minutes. Loss of PHD1 did not protect hepatocytes against 3 hours of ischemia (Supplementary Note 5; Supplementary Figure 1*B*). This finding is not surprising, given that mammalian cells ultimately need oxygen to survive. However, PHD1$^{-/-}$ hepatocytes exhibited a remarkable protection against ischemic stress of 90 minutes. Indeed, histology of ischemic WT livers after 90 minutes ischemia revealed hepatocyte swelling, vacuolization, and karyolysis, while signs of hypoxic cell damage were markedly attenuated in ischemic PHD1$^{-/-}$ livers (Figure 1*B*). We determined hepatocyte size as an index of cellular injury, as it was a reliable quantifiable parameter. Analysis revealed that hepatocyte size in WT livers was increased in ischemic conditions (consistent with previous observations[15]); in contrast, no significant swelling of ischemic hepatocytes was observed in PHD1$^{-/-}$ livers (Figure 1*C*).

Because hepatic sinusoidal vessels are compressed by swelling of ischemic hepatocytes,[15] we measured their diameter. As shown in Figure 1*D*, lumen size of these vessels was decreased in WT livers after 90 minutes of ischemia, but maintained in ischemic PHD1$^{-/-}$ livers. We also determined sinusoidal blood flow by intravital fluorescence microscopy after releasing of 90 minutes vascular occlusion. In baseline conditions, sinusoidal erythrocyte velocity was comparable in both genotypes (Figure 1*E*). After ischemia, sinusoidal flow in postischemic lobes was increased in both genotypes, reflecting reactive hyperemia.[16] However, sinusoidal microcirculation was higher in postischemic PHD1$^{-/-}$ livers (Figure 1*E*), consistent with reduced damage of PHD1$^{-/-}$ hepatocytes. This protection against ischemic injury was not due to a genotypic difference in the supply of oxygen in baseline conditions, as PHD1$^{-/-}$ mice had normal blood oxygen and hemoglobin levels,[9] and formed a normal liver microvasculature (Supplementary Note 6; Supplementary Figure S2), Thus, loss of PHD1 attenuated hypoxic swelling of hepatocytes and resultant compression of sinusoidal vessels in ischemic livers.

The observation that PHD1$^{-/-}$ livers were hypoxia-tolerant suggested that PHD1$^{-/-}$ hepatocytes were still capable of producing sufficient energy to survive, even despite the reduced oxygen supply. We therefore measured the cellular energy charge. In baseline conditions, this parameter was comparable in WT and PHD1$^{-/-}$ livers (Figure 1*F*). After 20 minutes of ischemia, the energy charge steeply decreased to 40% of baseline levels in WT hepatocytes and continued to decline progressively during the 60 minutes thereafter (Figure 1*F*). In PHD1$^{-/-}$ livers, the energy charge similarly dropped acutely during the initial 20 minutes of ischemia but thereafter did not decrease as rapidly and progressively as in WT hepatocytes (Figure 1*F*). Liver cells are sensitive to changes in cellular energy charge, and even small changes below a critical threshold can have a substantial impact on cellular function and survival.[17,18] Nonetheless, while these data indicate that PHD1$^{-/-}$ hepatocytes were capable of preserving vital amounts of adenosine triphosphate in ischemic conditions, sufficient to prevent swelling, the absence of more dramatic genotypic differences in cellular energy charge suggested that additional mechanisms (reduced oxidative stress) might also contribute to protection of PHD1$^{-/-}$ hepatocytes against ischemia. In particular, it is conceivable that HIF-induced target genes, such as endothelial nitric oxide synthase, may enhance postischemic microcirculation in PHD1$^{-/-}$ livers (Supplementary Note 7).[16]

Loss of PHD1 Protects Hepatocytes Against I/R Injury

Hepatocyte swelling and narrowing of sinusoidal vessels are key events in the induction of hepatic I/R damage.[2,3] Given that these parameters were reduced in PHD1$^{-/-}$ livers, we also analyzed whether PHD1$^{-/-}$ livers were more tolerant against I/R injury. Therefore, animals were subjected to hepatic ischemia (90 minutes) and reperfusion (8 hours) when all analyses were performed. Hepatocellular injury, as measured by serum levels of the liver enzyme alanine aminotransferase (ALT), was substantially attenuated in PHD1$^{-/-}$ mice after I/R (serum ALT: 2960 ± 543 IU/L in WT vs 1210 ± 351 IU/L in PHD1$^{-/-}$; n = 6; P = .02). The genotypic difference in liver damage was specific for the I/R, as serum ALT levels were similar in sham-operated mice (serum ALT: 271 ± 30 IU/L in WT vs 238 ± 73 IU/L in PHD1$^{-/-}$; n = 6; P = not significant).

Histological evaluation of I/R-exposed WT livers revealed extensive necrosis. Consistent with the oxygen-gradient in ischemic liver,[3] necrosis occurred mostly in

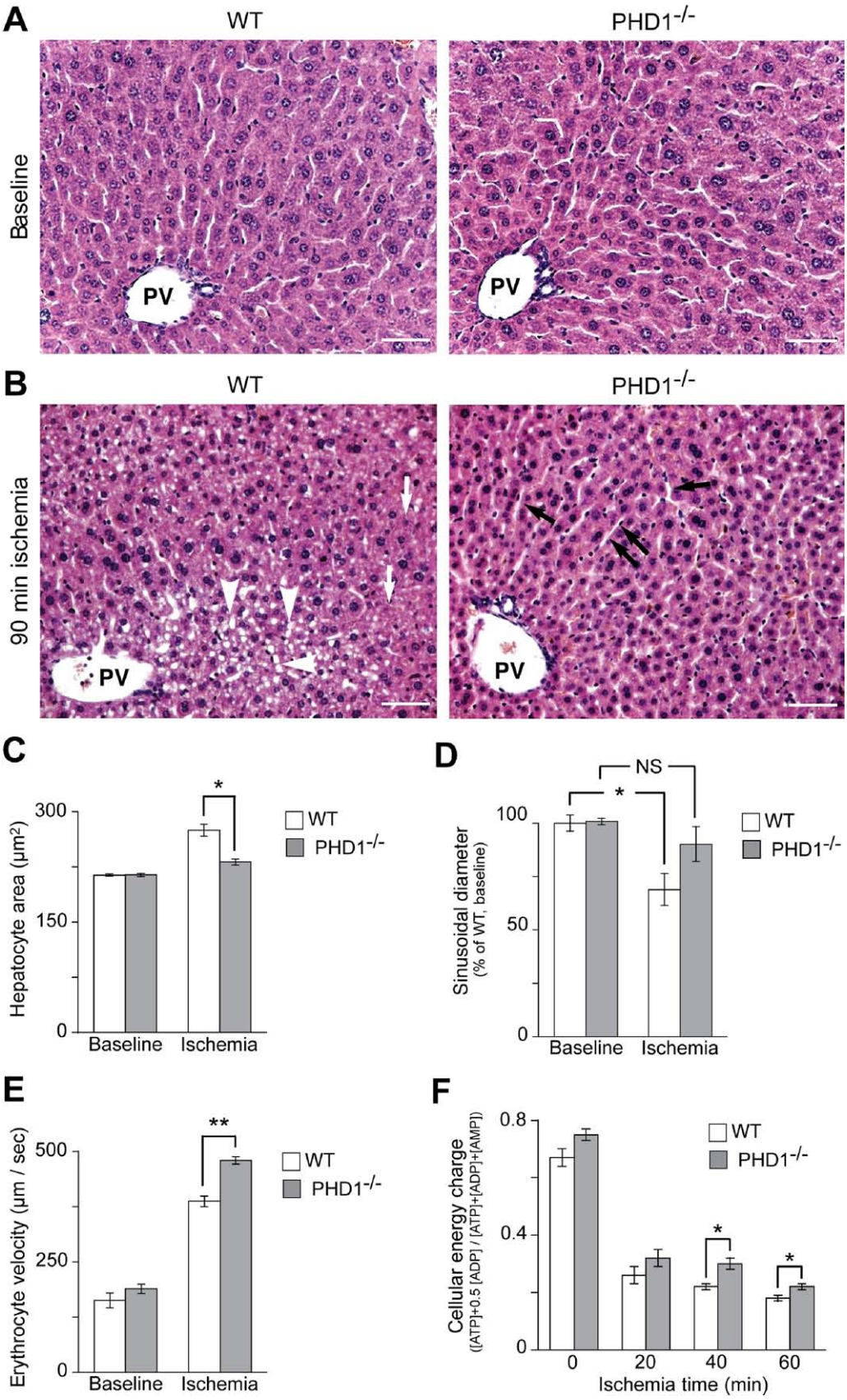

Figure 1. Reduced hypoxic hepatocyte damage in ischemic PHD1−deficient (PHD1$^{-/-}$) livers. (*A*) Hematoxylin and eosin staining revealing normal appearance of healthy PHD1$^{-/-}$ (*right*) and wild-type (WT) livers (*left*). (*B*) Hematoxylin and eosin-staining reveals hepatocyte vacuolization (*arrowheads*) and swelling (*white arrows*) in WT (*left*), but not PHD1$^{-/-}$ livers (*right*) in ischemic conditions. Note preserved sinusoidal spaces in PHD1$^{-/-}$ livers (*black arrows*). (*C*−*E*) Quantification of hepatocyte size (*C*), sinusoidal diameter (*D*), and erythrocyte velocity (*E*) revealing reduced ischemic hepatocyte swelling and preserved sinusoidal lumen in ischemic PHD1$^{-/-}$ livers. $^{*}P < .01$, $^{**}P < .001$, n = 4. (*F*) Cellular energy charge, revealing improved preservation in ischemic PHD1$^{-/-}$ livers. $^{*}P < .02$, n = 4. Bar graphs: mean ± standard error of mean; *scale bars* in (*A,B*) = 50 µm. PV, branch of portal vein; NS, not significant.

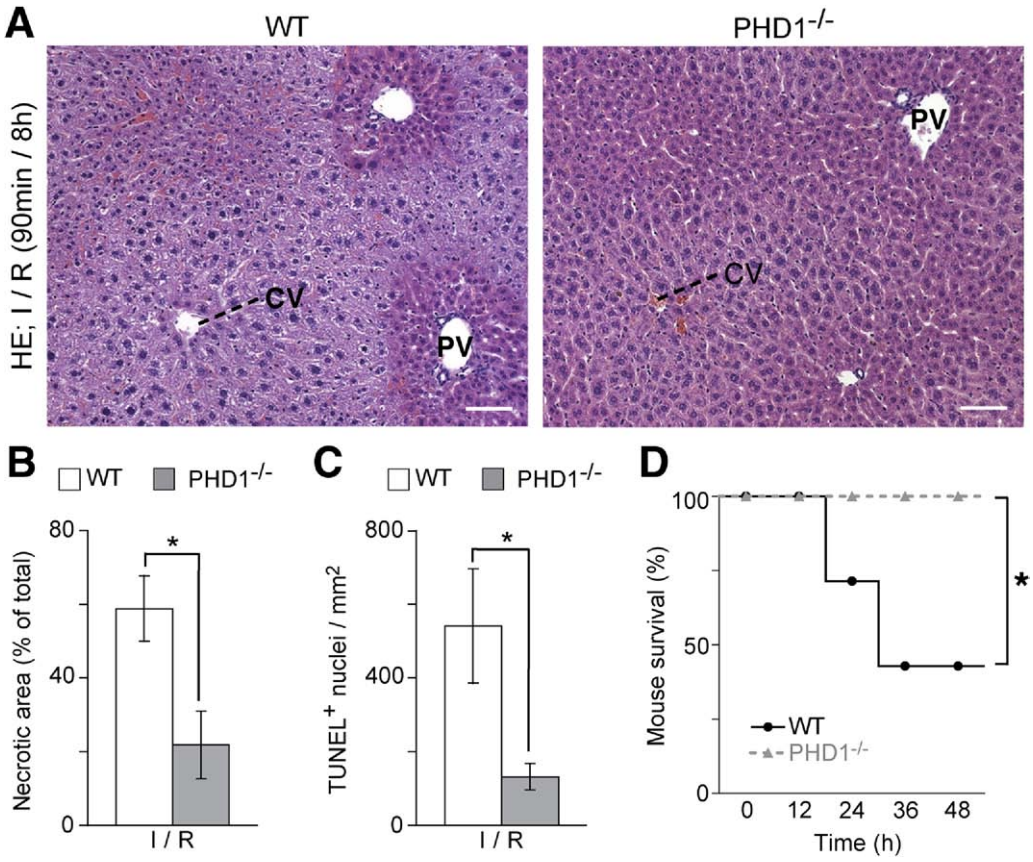

Figure 2. Loss of PHD1 protects against hepatic I/R damage. (*A*) Hematoxylin and eosin (HE) staining revealing extensive hepatocyte damage in wild-type (WT) (*left*) but less in PHD1-deficient (PHD1$^{-/-}$) livers (*right*) after CV, central vein; PV, portal vein. Scale bars = 100 μm. (*B, C*) Quantification of necrotic areas (*B*) and TUNEL-positive nuclei (*C*), revealing decreased I/R-induced hepatocyte death in PHD1$^{-/-}$ livers. Bars represent mean ± standard error of mean *$P < .02$, n = 5. (*D*) Survival curves, revealing improved survival of PHD1$^{-/-}$ mice after liver I/R and resection of nonischemic liver lobes. *$P = .03$, n = 6 by log rank test.

perivenular zones with sparing of periportal fields (Figure 2*A*). Decreased cytoplasmic staining, loss of nuclei, and red cell congestion, all characteristic features of liver necrosis, were attenuated in post-I/R livers of PHD1$^{-/-}$ mice (Figure 2*A*). Morphometry revealed necrosis in up to 60% of I/R-subjected liver lobes in WT mice, while only 30% of ischemic liver regions showed more subtle signs of cellular dysfunction and demise in PHD1$^{-/-}$ mice (Figure 2*B*). TUNEL-staining also revealed more severe hepatocyte death in I/R-challenged WT than PHD1$^{-/-}$ livers (Figure 2*C*). To confirm the protective effect of PHD1-deficiency against liver I/R damage, we assessed survival of mice subjected to hepatic I/R, followed by surgical resection of all nonischemic liver lobes. Up to 57% of all WT mice subjected to liver I/R and resection died within 48 hours of surgery, whereas 100% of PHD1$^{-/-}$ mice undergoing the same procedure survived (Figure 2*D*). Thus, loss of PHD1 increased the resistance of hepatocytes against lethal I/R damage.

Short-Term Silencing of PHD1 Protects Against Hepatic I/R Damage In Vivo

We next determined whether short-term silencing of PHD1 in vivo might suffice to protect mice against hepatic I/R damage. This would allow us to address whether permanent inactivation of PHD1 was required to establish the protective state, or whether short-term inhibition might already suffice—a question of medical relevance when considering PHD1 inhibition as a possible therapeutic option. Because selective PHD1 inhibitors are not available, we silenced PHD1 by using a construct designed to produce a short hairpin interference RNA against PHD1 (*shPHD1^{KD}*). As control, we applied a construct that differed by a mismatch of 10 nucleotides (*shPHD1^{CTR}*). For in vivo use, we cloned adenoviral vectors Ad*shPHD1^{CTR}* and Ad*shPHD1^{KD}*, expressing these short hairpin interference RNAs. The efficiency and specificity of Ad*shPHD1^{KD}* to silence PHD1 were first tested in AML12 hepatocytes (Supplementary Note 3). We took advantage of the fact that intravenous injection of adenoviral vectors nearly selectively transduces the majority of hepatocytes.[19] Because pilot experiments revealed that intravenous injection of Ad*shPHD1^{KD}* in WT mice reduced hepatic expression of PHD1 only incompletely (not shown), PHD1$^{+/-}$ mice were used, which express reduced PHD1 levels (PHD1 messenger RNA levels, percent of WT: 65% ± 8%; n = 10; $P < .05$). Intravenous injection of Ad*shPHD1^{KD}* in PHD1$^{+/-}$ mice (nearly) completely eliminated hepatic expression of PHD1, at both messenger RNA and protein levels, while expression of PHD2 or PHD3 was not altered; in contrast, Ad*shPHD1^{CTR}* treatment did not affect hepatic PHD1 expression (Figure 3*A* and *B*).

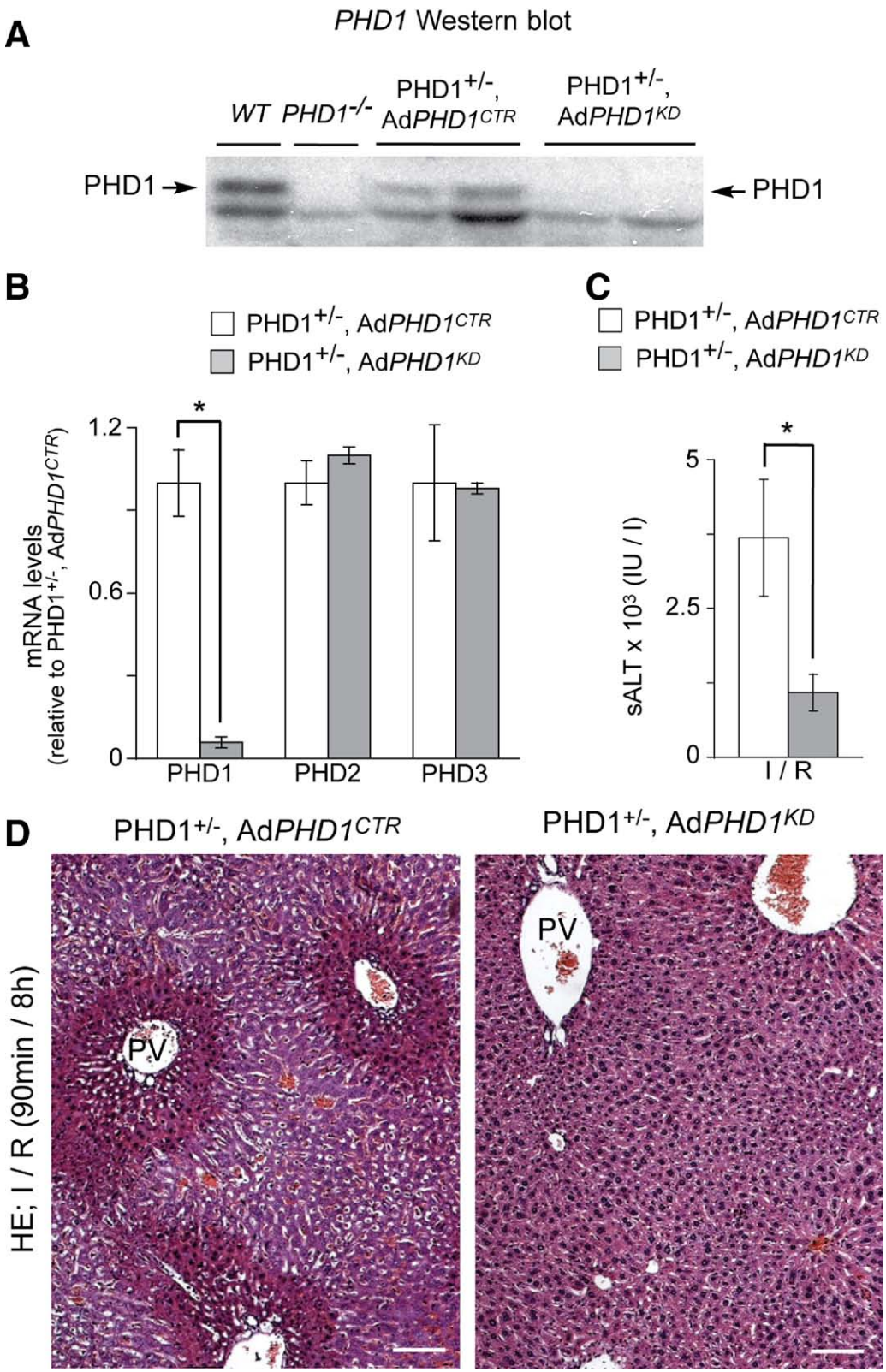

Figure 3. Short-term inhibition of PHD1 protects against hepatic I/R damage. (*A*) Immunoblot of liver lysates, revealing effective knock-down of PHD1 protein in PHD1$^{+/-}$ mice treated with Ads*hPHD1*^{KD}, but not Ads*hPHD1*^{CTR}. PHD1 protein expression in livers from healthy wild-type (WT) and PHD1$^{-/-}$ mice was assessed as control. Equal amounts of protein were loaded. (*B*) Real-time reverse transcription polymerase chain reaction analysis revealing residual expression of PHD1, PHD2, and PHD3 transcripts in livers from PHD1$^{+/-}$ mice treated with Ads*hPHD1*^{KD} or Ads*hPHD1*^{CTR}. *$P < .0001$, n = 7. (*C*) Attenuated serum alanine aminotransferase levels in I/R-treated PHD1$^{+/-}$ mice, following short-term inhibition of PHD1 via Ads*hPHD1*^{KD}. *$P = .03$, n = 10. (*D*) Hematoxylin and eosin (HE) staining, revealing extensive I/R damage in Ads*hPHD1*^{CTR}–treated- (*left*), but not in Ads*hPHD1*^{KD}–treated animals (*right*). PV, branch of portal vein. *Bars* represent mean ± standard error of mean. *Scale bars* in (*D*) = 100 μm.

Knockdown of PHD1, performed 5 days prior to hepatic ischemia (to allow sufficient adenoviral transgene expression and turnover of preexisting PHD1 protein), induced resistance of PHD1$^{+/-}$ livers against I/R damage. Indeed, serum ALT levels were reduced in Ads*hPHD1*^{KD}-treated PHD1$^{+/-}$ mice as compared to littermates injected with Ads*hPHD1*^{CTR} (Figure 3*C*). Notably, liver I/R damage in Ads*hPHD1*^{CTR} injected PHD1$^{+/-}$ and untreated

WT mice was comparable (serum ALT: 2965 ± 543 IU/L in untreated WT vs 3690 ± 982 IU/L in Ad*shPHD1*^{CTR} treated PHD1$^{+/-}$; n = 7; *P* = not significant). Consistently, silencing of PHD1 attenuated I/R-induced hepatocyte death (percent necrotic liver area: 53 ± 11 in Ad*shPHD1*^{CTR}-treated PHD1$^{+/-}$ vs 15 ± 5 in Ad*shPHD1*^{KD}-treated PHD1$^{+/-}$; n = 4; *P* = .027; Figure 3*D*). Thus, short-term inhibition of PHD1 protects hepatocytes against I/R.

Loss of PHD1 Reduces Oxidative Stress in I/R-Treated Livers

After having established that permanent loss or short-term silencing of PHD1 provided protection of hepatocytes against I/R injury, we performed experiments in an effort to start unraveling the underlying mechanisms of this protection. We first investigated whether the protection of PHD1$^{-/-}$ livers against I/R-damage might be related to genotypic differences in the production of toxic ROS, known to contribute to hepatic I/R-damage.[2] Therefore, livers were immunostained for 8-OHdG, a biomarker of oxidative stress.[9] Livers of healthy or sham-operated animals did not exhibit any signs of 8-OHdG staining (not shown). In I/R-exposed WT livers, a substantial fraction of hepatocyte nuclei stained positively (Figure 4*A*). By contrast, many fewer nuclei stained for 8-OHdG in I/R-exposed PHD1$^{-/-}$ livers (Figure 4*A* and *B*). As an alternative readout for ROS-production in I/R-treated livers, we determined the oxidation of the antioxidant glutathione to glutathione-

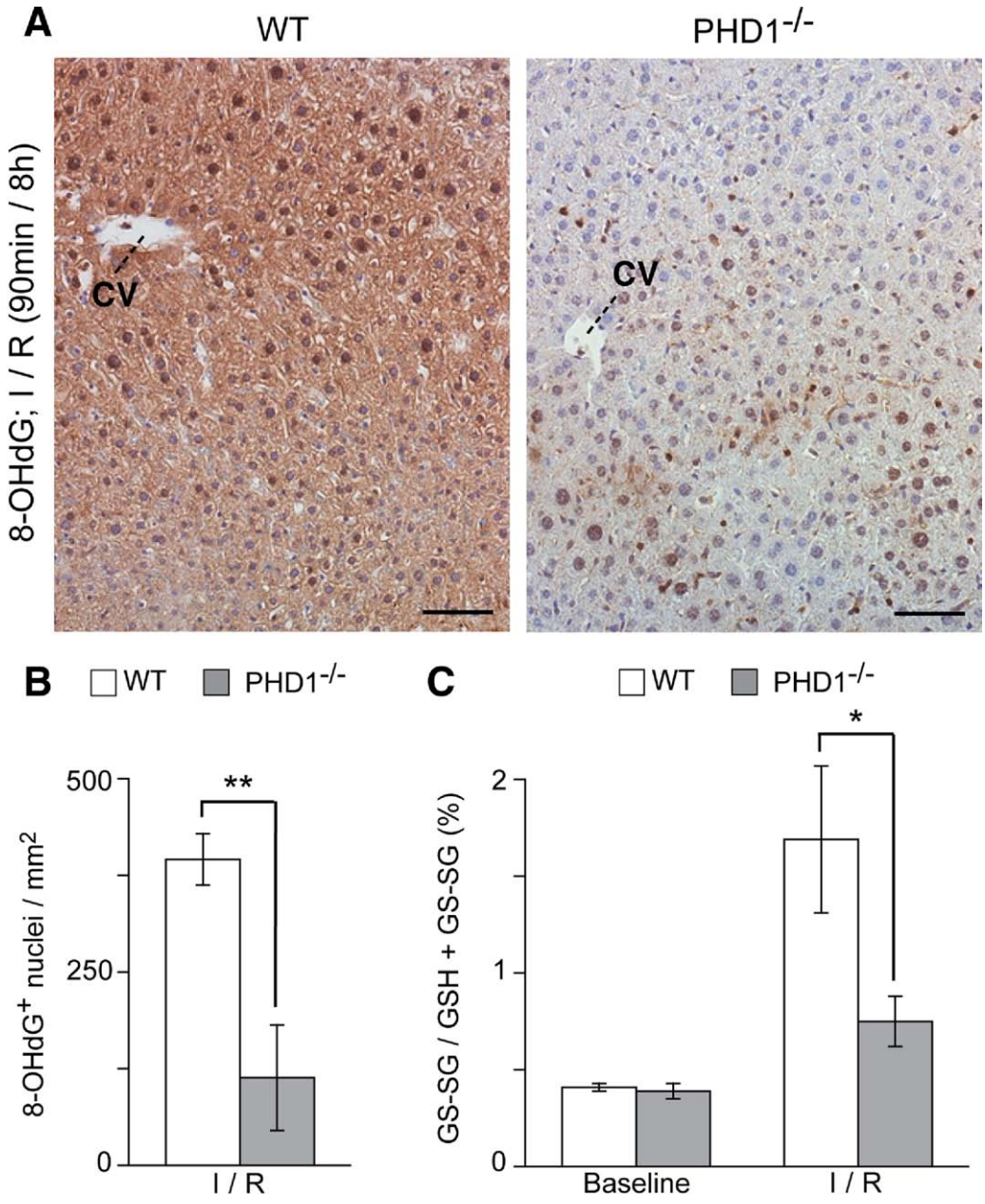

Figure 4. Oxidative stress in reperfused PHD1−deficient (PHD1$^{-/-}$) livers. (*A*) 8-OHdG staining, revealing substantial oxidative damage in hepatic I/R-exposed wild-type (*left*), but only minimal in PHD1$^{-/-}$ livers (*right*). CV, central vein. *Scale bars* = 50 μm. (*B*) Histomorphometric quantification of 8-OHdG-stained hepatocyte nuclei in I/R-challenged livers. (*C*) Relative ratio of glutathione-disulfide to total glutathione in baseline and I/R-challenged livers. Bars represent mean ± standard error of mean; *$P < .05$, n = 5; **$P < .01$, n = 4.

disulfide. The relative ratio of glutathione-disulfide to total glutathione was markedly increased in WT livers after I/R-exposure, indicating oxidative stress (Figure 4*C*). In comparison, this ratio was significantly attenuated in I/R-treated PHD1$^{-/-}$ livers (Figure 4*C*). Thus, PHD1-deficiency attenuated oxidant stress after I/R. Additional analysis revealed that only minor genotypic differences in leukocyte infiltration occurred upon I/R challenge (Supplementary Figure S3*A* and *B*).

Loss of PHD1 Lowers Oxygen Consumption

We then sought to delineate some of the initial molecular mechanisms through which loss of PHD1 reduces oxidative stress in ischemic hepatocytes. We recently demonstrated that loss of PHD1 protected against skeletal muscle cell death induced by oxidative stress; this protection relied on a decrease in oxygen consumption, as respiration generates ROS in hypoxic conditions.[20] Oxidative metabolism and oxygen consumption were reduced in PHD1$^{-/-}$ muscle, because PHD1 deficiency up-regulated PDK1, which inhibits PDC and also, indirectly, entry of glycolytic intermediates into the TCA cycle and oxidative metabolism.[9] To assess whether this mechanism was also operational in PHD1$^{-/-}$ hepatocytes, we determined the expression of PDK isoenzymes in the liver. By real-time reverse transcription polymerase chain reaction and immunoblotting, levels of PDK1 were increased in PHD1$^{-/-}$ livers (PDK1 protein, percent of WT: 154% ± 16%; n = 6; $P = .03$, Figure 5*A* and *B*). Accordingly, PDC activity was slightly but significantly attenuated in livers from healthy PHD1$^{-/-}$ mice (Figure 5*C*). The up-regulation of PDK1 in PHD1$^{-/-}$ livers was specific, as the expression of other PDKs was not altered (Supplementary Note 8).

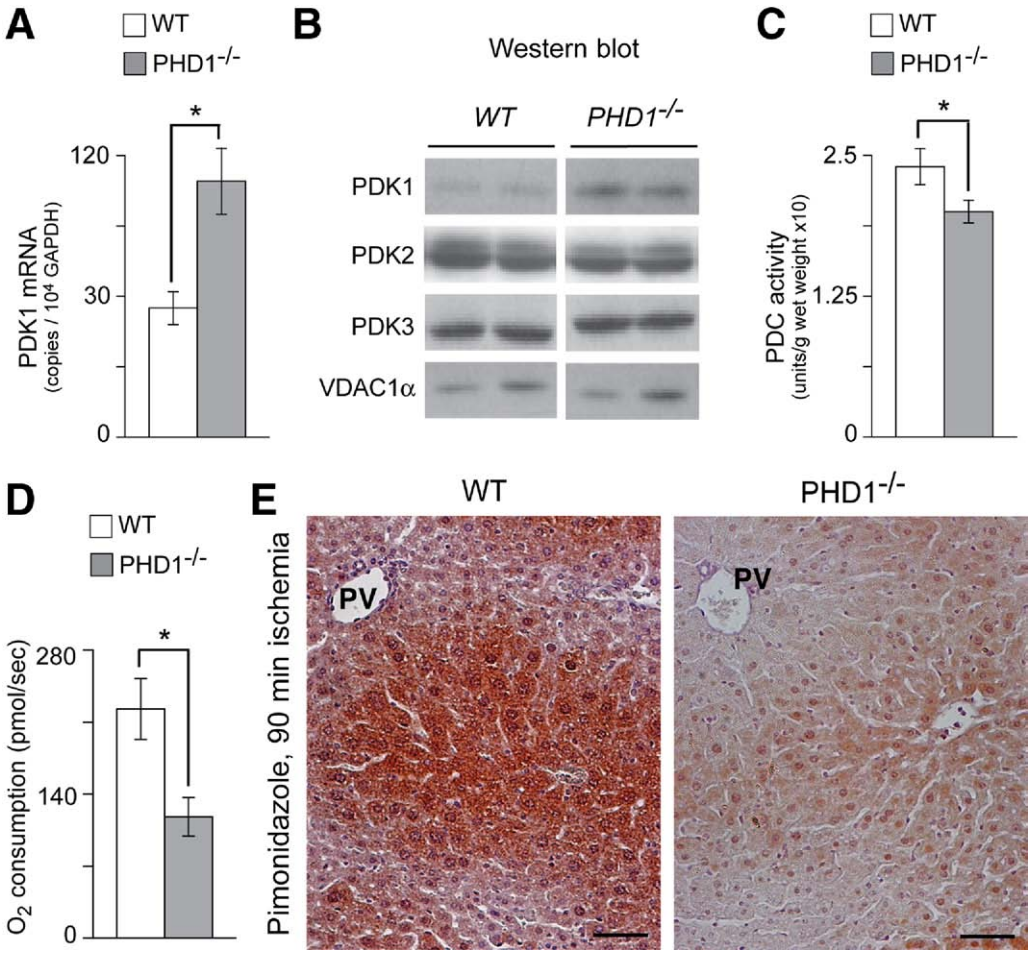

Figure 5. Decreased oxygen consumption in PHD1−deficient (PHD1$^{-/-}$) livers. (*A*) Real-time reverse transcription polymerase chain reaction revealing enhanced PDK1 expression in PHD1$^{-/-}$ livers. (*B*) Representative immunoblots, revealing enhanced expression of PDK1, but not PDK2- or PDK3 protein in liver lysates from healthy PHD1$^{-/-}$ mice. Voltage-dependent anion channel (VDAC1α) was used to assure equal loading. (*C*) Measurements of the activity of the PDC in healthy liver tissue. (*D*) Respirometry, revealing reduced oxygen consumption of PHD1$^{-/-}$ hepatocytes supplied with glucose as a metabolic substrate. Triplicate measurements, 1 of 3 representative experiments is shown. (*E*) Pimonidazole staining, revealing severe hypoxia in ischemic WT hepatocytes (*left*), and attenuated hypoxia in ischemic PHD1$^{-/-}$ hepatocytes (*right*). Bars represent mean ± standard error of mean; *$P < .05$, n = 3. *Scale bars* in (*E*) = 50 μm. PV, branch of portal vein.

To investigate whether PHD1$^{-/-}$ hepatocytes also conserved oxygen, we used respirometry to measure oxygen consumption of isolated hepatocytes when supplied with glucose, as this is the key fuel during liver ischemia. In these conditions, PHD1$^{-/-}$ hepatocytes consumed less oxygen (Figure 5*D*). Also, silencing of PHD1 reduced oxygen consumption of AML12 hepatocytes in the presence of glucose (Supplementary Note 9; Supplementary Figure S4*A*). Consistently staining for the hypoxia marker pimonidazole revealed that hepatocytes of both genotypes had comparable intracellular oxygen levels in baseline conditions (Supplementary Figure S4*B*), but after 90 minutes of ischemia, WT hepatocytes exhibited more severe signs of intracellular hypoxia than PHD1$^{-/-}$ hepatocytes (Figure 5*E*). Quantification revealed a 40% ± 6% reduction of pimonidazole-stained areas in ischemic PHD1$^{-/-}$ livers (n = 5; $P < .01$).

Initial studies of anaerobic glucose metabolism suggested that increased glycolytic production of energy, besides the reduced oxidative stress, might contribute to the improved tolerance of PHD1$^{-/-}$ livers against ischemic and I/R injury (Supplementary Note 10; Supplementary Figure S4*C*).

Role of Downstream HIFs in Hypoxia Tolerance

We finally explored whether the hypoxia tolerance was conferred by stabilization of hypoxia inducible factors (HIFs) in PHD1$^{-/-}$ livers. In baseline conditions, HIF-2α protein levels were higher in PHD1$^{-/-}$ than WT livers (Figure 6*A*). After onset of ischemia, HIF-2α levels were up-regulated in both genotypes, indicating that they perceived hypoxia; as in baseline conditions, HIF-2α levels were higher in ischemic PHD1$^{-/-}$ than WT livers (not shown). I/R-treatment also up-regulated HIF-2α levels in both genotypes, but HIF-2α levels were again higher in PHD1$^{-/-}$ livers (Figure 6*A*). Hepatic HIF-1α protein was barely detectable in baseline conditions, and also up-regulated by ischemia (not shown) and I/R-treatment (Figure 6*A*). In general, loss of PHD1 up-regulated HIF-2α levels more than HIF-1α levels (Figure 6*A*).

To assess the relevance of HIF-2α and HIF-1α, we silenced their expression in hepatocytes isolated from WT or PHD1$^{-/-}$ mice. Transfection with interference RNA resulted in incomplete silencing of HIF-1α or HIF-2α messenger RNA levels by ~50% (Supplementary Note 11). To test whether these reduced HIF levels were capable of reverting the hypoxia tolerance of PHD1$^{-/-}$ hepatocytes, we used a model of simulated I/R in vitro.[14] In accordance with our in vivo findings, loss of PHD1 increased the survival of hepatocytes exposed to simulated I/R (Figure 6*B*). Partial silencing of HIFs did not affect the viability of WT hepatocytes (Figure 6*B*). By contrast, incomplete silencing of HIF-2α partially reverted the hypoxia protection of PHD1$^{-/-}$ hepatocytes, while silencing of HIF-1α was ineffective (Figure 6*B*), suggesting that HIF-2α may have a more prominent role in regulating the hypoxia tolerance of PHD1$^{-/-}$ livers.

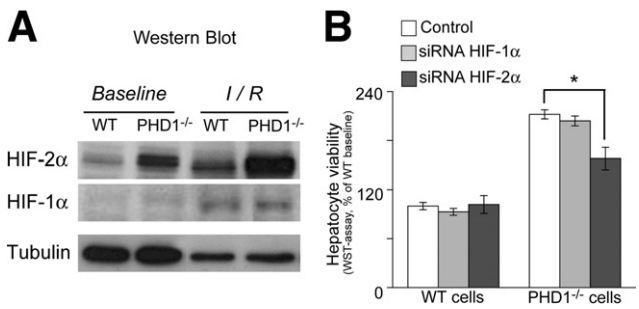

Figure 6. Role of HIF-2α. (*A*) Immunoblot of nuclear extracts, revealing elevated protein levels of HIF-2α and, less extensively, also of HIF-1α in PHD1−deficient (PHD1$^{-/-}$) liver at baseline conditions, and following I/R. Tubulin was used as a loading control. (*B*) Survival of isolated hepatocytes exposed to simulated I/R in vitro revealing that silencing of HIF-2α, but not HIF-1α, partly reverts the hypoxia tolerance of PHD1$^{-/-}$ cells. Hepatocyte viability was determined by WST-1 assay. Measurements were carried out 4 times; 1 of 3 representative experiments is shown. Bars represent mean ± standard error of mean; *$P < .01$, n = 4.

Discussion

Here, we used mice lacking PHD1, as well as interference RNA−mediated hepatocyte-specific knockdown of the PHD1 gene in vivo to demonstrate that germline loss or short-term silencing of PHD1 provides hypoxia tolerance to the liver. These results extend previous findings that PHD1-deficiency conveys hypoxia tolerance to metabolically active tissues such as skeletal muscle,[9] and underline the potential therapeutic value of targeting PHD1 in order to prevent hypoxia-induced liver damage.

Liver cells are highly dependent on oxygen and are therefore vulnerable to hypoxia or I/R-injury.[3] For example, even a drop in cellular energy charge of <10% below a critical vital threshold suffices to induce hepatocyte death.[17,18] The key finding of this study is that loss of PHD1 confers improved protection against acute ischemia and I/R injury. Furthermore, this protective state does not require permanent inactivation of PHD1, neither is its absence in all tissues required, but can be induced by short-term silencing of PHD1 in the liver alone. Not only were there clear morphological signs of enhanced cellular protection (as evidenced by the reduced hepatocyte swelling, vacuolization, and karyolysis) resulting in improved residual perfusion after releasing vascular occlusion (as evidenced by the flow measurements), but PHD1$^{-/-}$ hepatocytes also exhibited reduced signs of oxidative stress and were capable of maintaining their cellular energy charge at higher levels.

Excessive oxidative stress inactivates key mitochondrial and cellular enzymes and perturbs structural components, overall leading to cellular demise. Loss of PHD1 provided hepatocytes a remarkable protection against

oxidative stress, by lowering/preventing generation of excessive amounts of ROS, as also occurs in PHD1$^{-/-}$ skeletal muscle fibers.[9] Similar to the latter,[9] expression of PDK1 was up-regulated with an accompanying reduction in PDC activity in PHD1$^{-/-}$ hepatocytes. Because PDC acts as a gatekeeper to regulate the entry of glycolytic intermediates in the Krebs cycle, a decrease (even when incomplete) of its activity might be expected to slow down oxidative metabolism and, hence, to conserve oxygen.[9] This metabolic adaptation is crucial for cells to survive life-threatening episodes of hypoxia as it reduces/prevents mitochondrial generation of excessive amounts of ROS. Indeed, even though ROS are a natural byproduct of mitochondrial respiration, they can be generated in excess in hypoxic conditions.[21,22] When ischemic cells continue to consume oxygen in mitochondrial respiration (as occurs in ischemic WT hepatocytes or myofibers; see pimonidazole staining), production of ROS is increased and reaches toxic levels, which cause irreversible damage to mitochondrial respiratory enzymes.[9] Thus, similar to ischemic PHD1$^{-/-}$ skeletal muscle cells,[9] the reduced oxygen consumption in PHD1$^{-/-}$ hepatocytes prevents excessive formation of toxic ROS and is, thereby, likely a predominant mechanism whereby loss of PHD1 provides hepatocytes hypoxia tolerance and protection against I/R injury.

While reperfusion is well known to initiate a cascade of deleterious events,[2] part of the protection of PHD1$^{-/-}$ hepatocytes against I/R injury may result from cellular changes during the ischemic challenge, ie, prior to reperfusion. Indeed, already during the ischemic stress, PHD1$^{-/-}$ hepatocytes were less hypoxic (pimonidazole staining), indicating that they conserved oxygen despite the reduced oxygen supply, in contrast to their WT counterparts, which continued to consume oxygen. Furthermore, measurements of the cellular energy charge revealed that PHD1$^{-/-}$ hepatocytes experienced an acute drop on vascular occlusion, but then seemed to better preserve their energy charge levels above critical thresholds. Probably as a consequence, PHD1$^{-/-}$ hepatocytes exhibited reduced signs of swelling at the end of the ischemic period, thereby improving flow upon reopening of the vascular clamps and attenuating microcirculatory perfusion failure, which is a well-known major determinant of liver dysfunction following hepatic I/R.[23] All this evidence indicates that loss of PHD1 already induced critical changes during ischemia, which prepared hepatocytes better to survive the subsequent reperfusion injury. However, given that hepatocyte oxidative stress was strikingly attenuated after 8 hours of reperfusion, when mitochondria represent a major source of ROS-production in I/R,[2] it is conceivable that cellular adaptations provoked by loss of PHD1 likewise affected hepatocyte survival during the reperfusion phase as well.

An interesting question is whether HIF-1α or HIF-2α are downstream mediators of PHD1 in providing hypoxia tolerance to hepatocytes. As expected, HIF levels in WT hepatocytes were elevated after onset of ischemia due to shortage of oxygen (not shown) and even more so after onset of I/R, likely because abundant generation of ROS inhibits PHD activity. In baseline ischemic and post-I/R conditions, HIF-1α but especially HIF-2α levels were more abundant in PHD1$^{-/-}$ livers. This genotypic difference in HIF levels cells is caused, in part, by the absence of PHD1, which elevates HIF levels even when the reduced oxygen consumption in these mice counteracts this up-regulation (through redistribution of oxygen into the cytosol); similar findings were obtained previously in PHD-knockout mice.[9,24] In post-I/R conditions, PHD1$^{-/-}$ hepatocytes suffer less oxidative stress but are better perfused and more viable and therefore more competent to up-regulate HIFs, and are also capable of maintaining better cellular respiration, which leads to a redistribution of oxygen away from the cytosol, and a further up-regulation of HIF levels.

Functional evidence for the importance of HIF-2α was provided by findings that the hypoxia tolerance of hepatocytes was at least partly reverted by silencing HIF-2α expression in vitro, whereas this was not the case for HIF-1α. PDK1 is a direct target for HIF-1α,[21,22] but hepatic regulation of PDK1 by HIF-2α should not be excluded because recent reports assigned HIF-2α a predominant role in hepatic regulation of archetypical HIF-1α target genes, such as Bnip3.[25,26] Although a possible role of HIF-1α should not be discarded prematurely (perhaps at deeper levels of hypoxia), these findings suggest that HIF-2α is involved in the hypoxia tolerance pathway not only in the skeletal muscle,[9] but also in the liver.

Our previous studies in the PHD1$^{-/-}$ muscle indicate that compensatory glycolytic generation of energy contributed to the hypoxia tolerance of myofibers and their protection against ischemia.[9] This seems to be also the case in ischemic PHD1$^{-/-}$ hepatocytes. Indeed, in PHD1$^{-/-}$ liver, glycogen stores were increased in baseline conditions, while glycogen breakdown rates were higher in ischemic conditions, probably due to an increased utilization of glycogen for glycolytic generation of energy. Of note, high glycogen reserves promote hepatocyte survival in rodent models of hepatic ischemia,[21] as well as in clinical settings.[27] Thus, besides the reduced oxidative damage, an increased anaerobic glucose catabolism may have allowed PHD1$^{-/-}$ hepatocytes to survive better the ischemic and I/R injury.

This study highlights possible medical relevance of specific PHD1-inhibition to treat medical conditions caused by severe acute hypoxia. We show here, for the first time, that short-term inhibition of PHD1 reduces hypoxic liver damage in vivo. This finding is of clinical relevance, as the increased incidence of liver transplantation for patients with end-stage liver disease demands novel therapeutic strategies to protect donor organs from hypoxic damage.[1,28] Moreover, our data may also suggest

that therapeutic PHD1-targeting might be applicable to improve residual liver function in subjects undergoing liver ischemia and resection, as occurs in resection of liver metastases. The therapeutic potential of pharmacologic HIF stabilization to alleviate acute hypoxic tissue damage has been highlighted in rodents.[29–31] However, different from nonspecific inhibition of all PHDs, we observed hypoxia tolerance in mice lacking PHD1 selectively, but expressing normal levels of PHD2 and PHD3, highlighting that hepatic protection against hypoxia is a specific effect of PHD1-inhibition. Thus, further investigation may be warranted to design clinical strategies that specifically target PHD1 to protect the liver against severe hypoxia in conditions of transplantation, surgical liver resection, veno-occlusive disease, hemorrhagic shock resuscitation or perhaps even heart failure.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.09.057.

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Drs Schneider and van Geyte contributed equally to this work.

Conflicts of interest

The authors disclose the following: P.R. and P.M. are scientific co-founders of ReOx Ltd, a University spin-out company that seeks to develop HIF Hydroxylase Inhibitors. The remaining authors disclose no conflicts.

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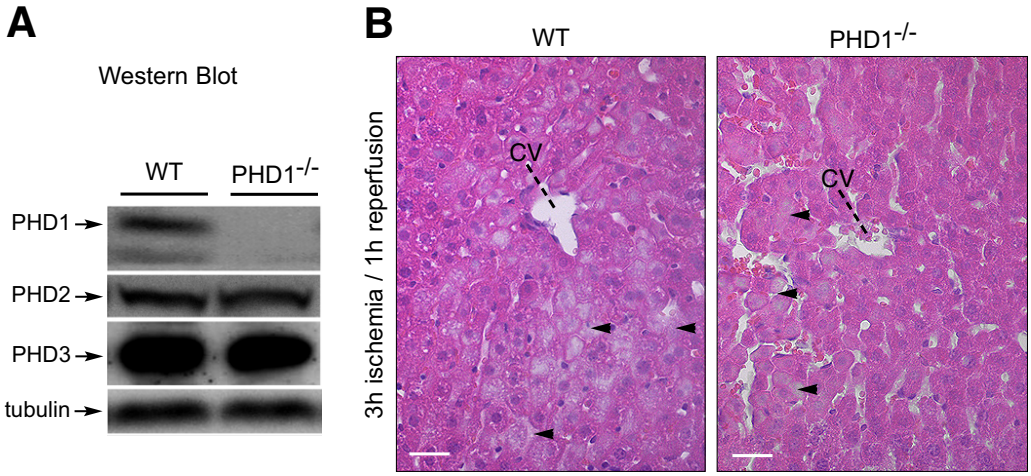
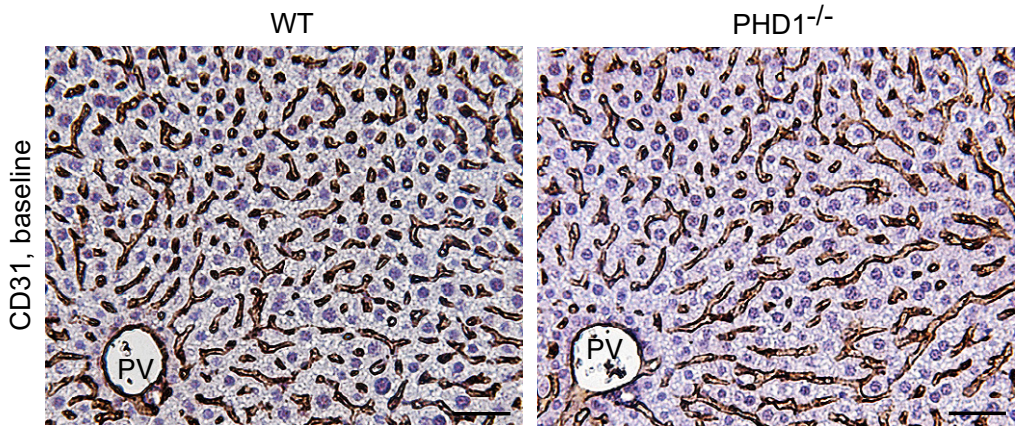
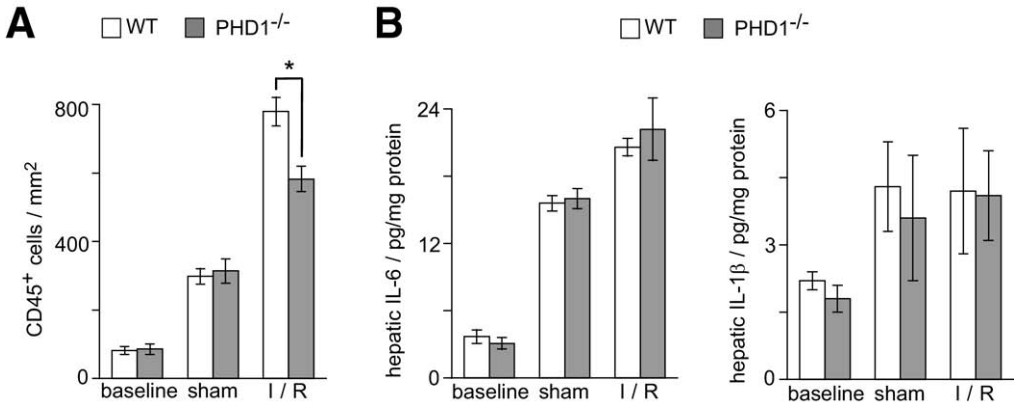


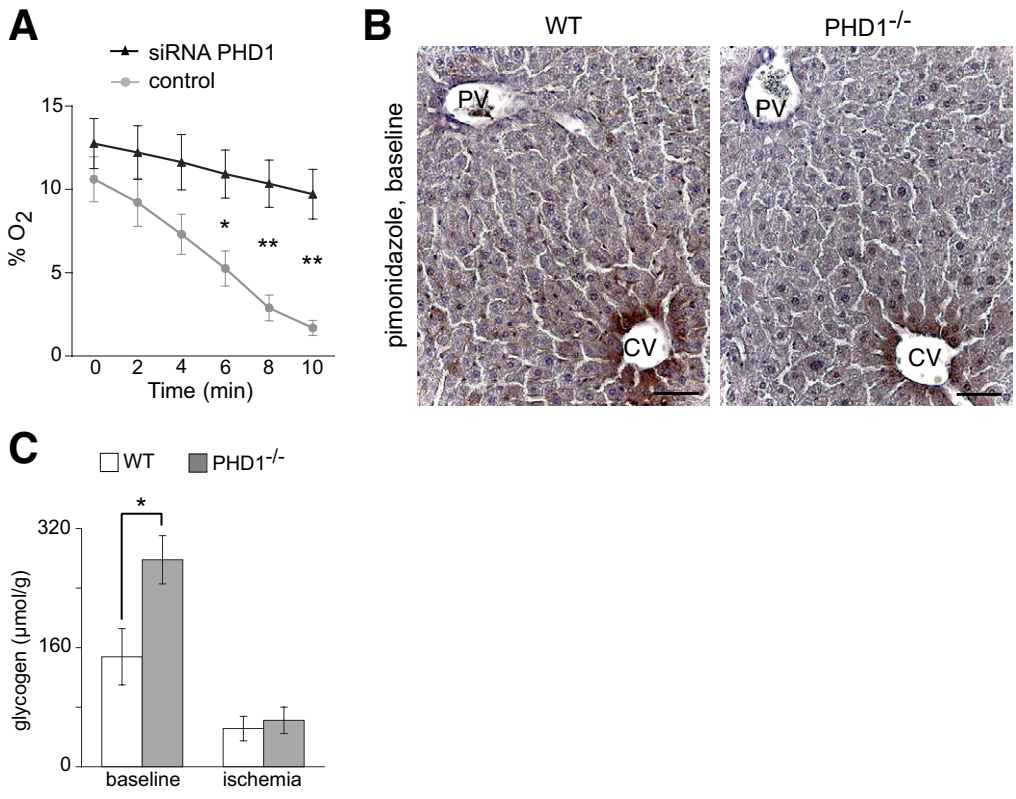
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.