Peroxisomal Localization of Hypoxia-Inducible Factors and Hypoxia-Inducible Factor Regulatory Hydroxylases in Primary Rat Hepatocytes Exposed to Hypoxia-Reoxygenation

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Many signals involved in pathophysiology are controlled by hypoxia-inducible factors (HIFs), transcription factors that induce expression of hypoxia-responsive genes. HIFs are post-translationally regulated by a family of O₂-dependent HIF hydroxylases: four prolyl 4-hydroxylases and an asparaginyl hydroxylase. Most of these enzymes are abundant in resting liver, which is itself unique because of its physiological O₂ gradient, and they can exist in both nuclear and cytoplasmic pools. In this study, we analyzed the cellular localization of endogenous HIFs and their regulatory hydroxylases in primary rat hepatocytes cultured under hypoxia-reoxygenation conditions. In hepatocytes, hypoxia targeted HIF-1 α to the peroxisome, rather than the nucleus, where it co-localized with von Hippel-Lindau tumor suppressor protein and the HIF hydroxylases. Confocal immunofluorescence microscopy demonstrated that the HIF hydroxylases translocated from the nucleus to the cytoplasm in response to hypoxia, with increased accumulation in peroxisomes on reoxygenation. These results were confirmed via immunotransmission electron microscopy and Western blotting. Surprisingly, in resting liver tissue, perivenous localization of the HIF hydroxylases was observed, consistent with areas of low pO₂. In conclusion, these studies establish the peroxisome as a highly relevant site of subcellular localization and function for the endogenous HIF pathway in hepatocytes. (*Am J Pathol* 2006, 169:1251–1269; DOI: 10.2353/ajpath.2006.060360)

Oxygen homeostasis relies on highly conserved mechanisms required for the survival of nearly all organisms. In most settings, O₂ delivery and consumption increase with metabolic demand; however, extreme shifts in tissue oxygenation can be detrimental. Mammalian cells use an O₂-responsive pathway to sense and to adapt to fluctuations in their microenvironment. This ubiquitous system involves hypoxia-inducible factors (HIFs), transcription factors that up-regulate the expression of hypoxia-responsive genes. HIFs are members of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors, which includes three HIF- α subunits (HIF-1 α , HIF-2 α /EPAS1, HIF-3 α /IPAS) and three HIF- β subunits (HIF-1 β /ARNT1, HIF-2 β /ARNT2, and HIF-3 β /ARNT3). HIF- α subunits dimerize exclusively with HIF- β subunits. In contrast, HIF- β subunits can also dimerize with aryl hydrocarbon receptors, providing cross-talk with xenobiotic metabolism.¹

HIF-1 α was first identified by Wang and Semenza,² and it serves as the prototype for studying cellular mechanisms of O₂ sensing. Under normoxic conditions, cytosolic HIF-1 α proteins are constitutively expressed but rapidly degraded because of posttranslational hydroxylations. HIF-1 α consists of both an asparagine-containing transactivation (CTAD) domain³ and a proline-rich oxygen-dependent degradation domain,⁴ both of which are essential for HIF function. These domains are enzymatically modified by recently identified nonheme HIF asparaginyl and prolyl hydroxylases, members of a dioxygenase superfamily whose activity requires 2-oxoglutarate (2-OG), Fe(II), ascorbate, and, most importantly, molecular O₂. When O₂ is abundant, hydroxylation of key prolines in the oxygen-dependent degradation domain by

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Table 1. Antibodies and Conditions Used in This Study

Protein	Primary antibody dilution (source and catalogue number)	Fluorescence secondary antibody dilution (source)
HIF-1 α	1:500 WB NB100-105; 1:1000 IHC B100-131; 1:100 IF NB100-123; all Ms mAb (NO)	Gt anti-Ms Cy3 1:1000 (JA)
HIF-2 α	NB100-132 Ms mAb; 1:100 IF (NO)	Gt anti-Ms Cy3 1:1000 (JA)
HIF-3 α	601-401-435 Rbt pAb; 1:100 IF (RO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
HIF-1 β	NB100-110 Rbt pAb; 1:100 IF (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
PHD1	NB100-310 Rbt pAb; 1:1000 WB, IHC; 1:100 IF; 1:50 TEM (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
PHD2	NB100-138 Rbt pAb; 1:1000 WB, IHC; 1:200 IF; 1:50 TEM (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
PHD3	NB100-139 Rbt pAb; 1:1000 WB, IHC; 1:100 IF; 1:50 TEM (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
PHD4	NB100-295 Rbt pAb; 1:100 WB, IF; 1:1000 IHC; 1:50 TEM (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
FIH-1	NB100-428 Rbt pAb; 1:100 WB; 1:1000 IHC; 1:200 IF; 1:50 TEM (NO)	Gt anti-Rbt IgG Alexa 488 1:500 (MO)
VHL	SC1535-R20 Gt pAb; 1:100 IF (SC)	Dky anti-Gt IgG Alexa 488 1:500 (MO)
Catalase (peroxisomes)	Ms mAb; 1:50 TEM (SI)	N/A
PMP70 (peroxisomes)	W90080C Shp pAb; 1:1000 WB; 1:200 IF (BI)	Dky anti-Shp IgG Cy3 1:200 (JA)
Pan-actin (loading control)	ALX210205C100 Rbt pAb; 1:500 WB (AL) mAb1501 Ms mAb; 1:2000 WB (CH)	N/A N/A

Dky, donkey; Gt, goat; IF, immunofluorescence; IHC, immunohistochemistry; mAb, monoclonal antibody; Ms, mouse; pAb, polyclonal antibody; Rbt, rabbit; Shp, sheep; TEM, immunotransmission electron microscopy; WB, Western blotting; AL, Alexis Corp., San Diego, CA; AM, Amersham, Uppsala, Sweden; BI, Biodesign International, Saco, ME; CH, Chemicon, Temecula, CA; NO, Novus Biologicals, Littleton, CO; JA, Jackson ImmunoResearch, West Grove, PA; MO, Molecular Probes, Eugene, OR; RO, Rockland Immunochemicals, Gilbertsville, PA; VE, Vector Laboratories, Burlingame, CA; SA, Santa Cruz Biotechnologies, Santa Cruz, CA; SI, Sigma, St. Louis, MO.

(table continues)

HIF prolyl 4-hydroxylases (PHDs) (The HIF prolyl 4-hydroxylases were termed PHDs, EGLNs, or HPHs by various groups. In this study, we refer to the PHD nomenclature. PHD1/PHD2/PHD3 are equivalent to HPH3/HPH2/HPH1 or EGLN2/EGLN1/EGLN3, respectively.) allow the von Hippel-Lindau tumor suppressor protein (pVHL) to tag HIF-1 α for polyubiquitination and subsequent proteasomal degradation.^{5,6} This continuous turnover results in the very short half-life of HIF-1 α in normoxic conditions.⁷ Furthermore, normoxia curtails HIF-1 α transcriptional activity via the asparaginyl hydroxylase, factor-inhibiting HIF-1 (FIH-1), which acts on an asparagine residue in the CTAD,⁸ providing yet another brake in the system.

Because of their requirement for molecular O₂, the HIF hydroxylases can be considered principal O₂ sensors within the cell, preventing aberrant HIF-dependent transcription in the presence of O₂. Accordingly, when cells undergo hypoxic stress, the hydroxylation and degradation of HIF-1 α is inhibited.^{9,10} As a result, HIF-1 α stabilizes, accumulates in the cytoplasm, and translocates to the nucleus, where it forms a heterodimer with its constitutively expressed nuclear binding partner HIF-1 β .¹¹ After transactivation,^{12,13} HIF-1 heterodimer binds to hypoxia-response elements, consensus sequences in the promoter or enhancer regions of target genes.¹⁴ To date, more than 70 HIF-induced genes have been identified, encoding such adaptive proteins as EPO, VEGF-A, iNOS, PAI-1, c-MET, IGFBP-1, and all glycolytic enzymes.^{15,16}

Because of its physiological O₂ gradient, the liver is a unique organ where maintenance of O₂ homeostasis is critical for its specialized function. Despite much of the pioneering work on HIF originating in hepatoma cell lines,¹⁴ little is known about its regulation in the liver itself.

Unlike other organs, liver receives most of its blood supply from the portal vein, which carries venous blood with lower O₂ tension. This gradient can further be disrupted by chronic liver disease or acute insults such as ischemia-reperfusion injury. O₂ zonation is therefore an additional consideration when studying the HIF pathway in liver, and perivenous mRNA expression of all three HIF- α subunits has been described.¹⁷ Although PHD1 to PHD3 are highly expressed in mouse liver,¹⁸ the significance of HIF hydroxylases in hepatic physiology and pathology is primarily unexplored. To better understand how the endogenous HIF pathway is affected by hypoxia/reoxygenation, we investigated the subcellular distribution of HIFs and their regulatory hydroxylases in primary rat hepatocytes. We show that in hepatocytes, HIF-1 α targets to the peroxisome rather than the nucleus, where it co-localizes with vHL and the HIF hydroxylases. Peroxisomal sequestration may provide an additional point of regulation for HIF signaling in the liver.

Materials and Methods

Materials

All chemicals were from Sigma (St. Louis, MO) unless otherwise noted. Antibody sources and conditions are outlined in Table 1.

Hepatocyte Cell Culture

Animals were treated according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Rat hepatocytes were isolated from male Fisher 344 rats (Harlan, Indianapolis, IN)

Table 1. *Continued*

Gold-conjugated secondary antibody for immuno-TEM dilution (source)	HRP-conjugated secondary antibody for Western blotting dilution (source)	Biotinylated secondary antibody for immunohistochemistry dilution (source)
N/A	Dky anti-Ms IgG 1:75,000 (JA)	Anti-Ms ImmPRESS reagent (VE)
N/A	N/A	N/A
N/A	N/A	N/A
N/A	N/A	N/A
Gt anti-Rbt IgG 10-nm gold 1:25 (AM)	Dky anti-Rbt IgG 1:75,000 (JA)	Gt anti-Rbt IgG 1:500 (CH)
Gt anti-Rbt IgG 10-nm gold 1:25 (AM)	Dky anti-Rbt IgG 1:75,000 (JA)	Gt anti-Rbt IgG 1:500 (CH)
Gt anti-Rbt IgG 10-nm gold 1:25 (AM)	Dky anti-Rbt IgG 1:75,000 (JA)	Gt anti-Rbt IgG 1:500 (CH)
Gt anti-Rbt IgG 10-nm gold 1:25 (AM)	Dky anti-Rbt IgG 1:75,000 (JA)	Gt anti-Rbt IgG 1:500 (CH)
Gt anti-Rbt IgG 10-nm gold 1:25 (AM)	Dky anti-Rbt IgG 1:75,000 (JA)	Gt anti-Rbt IgG 1:500 (CH)
N/A	N/A	N/A
Gt anti-Ms 5-nm gold 1:25 (AM)	N/A	N/A
N/A	Dky anti-Shp IgG 1:20,000 (JA)	N/A
N/A	Dky anti-Rbt IgG 1:75,000 (JA)	N/A
N/A	Dky anti-Ms IgG 1:75,000 (JA)	N/A

using the modified two-step collagenase perfusion technique.^{19,20} Freshly isolated hepatocytes of >90% viability, as assessed by trypan blue exclusion, were added to plating medium (minimal essential medium containing 50 μ g/ml bovine insulin and 0.1% gentamicin). Hepatocytes were plated on rat-tail collagen I-coated cultureware at a density of 3 to 4 $\times$ 10⁶ cells/100-mm plastic dish or 1 to 2 $\times$ 10⁵ cells/22-mm collagen-coated glass coverslip (BD Biocoat, Bedford, MA), incubated at 37°C (5% CO₂), and checked for adherence of monolayers after 2 to 4 hours. Once adhered, the medium was changed to serum-free basal hepatocyte growth media (without ITS, dexamethasone, or growth factors).²¹ The next day, normoxic cells were cultured for 6 hours at 37°C in a standard 5% CO₂ humidified incubator (Heraeus, Asheville, NC), while parallel sets of hypoxic cells were cultured for 6 hours at 37°C in a 1% O₂ ProOx system balanced with 5% CO₂/95% N₂ (Biospherix, Redfield, NY). This degree and timing of hypoxia was chosen based on established protocols for HIF-induced transcription.²² Duplicate hypoxic cultures were also returned for reoxygenation overnight (18 hours) in the normoxic incubator. Hypoxic culture was evaluated by incubating cells with 200 μ M of Hypoxyprobe-1 Plus (Chemicon, Temecula, CA) during hypoxic incubation and subsequent detection of pimanidazole adducts.²³ Cell survival was confirmed by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability and lactate dehydrogenase (LDH) cytotoxicity assays (Biovision, Mountain View, CA).^{24,25} At the indicated time points, hepatocytes were harvested immediately on ice for protein or RNA, or fixed in 2% paraformaldehyde for imaging (described below).

Hepatoma Cell Line Culture, Transplantation, and in Vivo Tumor Formation

Previously frozen stocks of JM1 Fisher 344 rat hepatoma cells²⁶ were grown in T-75 flasks containing Dulbecco's modified Eagle's medium supplemented with 2 mmol/L glucose, 2 mmol/L L-glutamine, 10% fetal bovine serum, and 0.1% gentamicin. Once confluent, JM1 cells were trypsinized and washed in Hanks' balanced salt solution. Three million cells were transplanted into each liver of 8- to 10-week-old, 180 to 200 g, male Fisher 344 rats via surgical injection with a 26-gauge needle into the superior mesenteric vein. Negative control rats were injected with cell-free Hanks' balanced salt solution. After 2 and 4 weeks of syngeneic engraftment, rats were injected intraperitoneally with a 100 mg/kg dose, dissolved in phosphate-buffered saline (PBS), of Hypoxyprobe (pimonidazole hydrochloride) hypoxia marker 1 hour before sacrifice. Livers were then harvested, snap-frozen, and/or processed for histology. Formalin-fixed tissues were embedded in paraffin blocks. A total of two series were performed, each consisting of three rats: control (vehicle alone), 2-week hepatoma, and 4-week hepatoma.

Immunotransmission Electron Microscopy (Immuno-TEM)

Rat livers were perfused-fixed and cultured primary rat hepatocytes were fixed with 2% paraformaldehyde in PBS (1 hour for cells, overnight for tissues), processed, and analyzed as described.²⁷ Sections were observed on a JEM 1210 electron microscope (JEOL, Peabody,

Table 2. RT-PCR Primer Pairs Used in This Study

Gene target	Accession number (all are rat)	Forward primer (FP)/reverse primer (RP)	Size (bp)
HIF-1 α	AF057308	5'-TGCTTGGTGCTGATTGTGA-3' 5'-GGTCAGATGATCAGAGTCCA-3'	209
HIF-2 α	RNO277828	5'-TGACTTCACTCATCTTGGACCA-3' 5'-ATTCATAGGCAGAGCGGCAAGTA-3'	443
HIF-3 α	RNO277827	5'-AAGAAGGGTATCCCAGGCAACAGT-3' 5'-TGACGAGCCAACATCTCCAAGT-3'	361
HIF-1 β	U61184	5'-TGCACCAACACCAACGTGAAGAAC-3' 5'-TGGTTGTGCTGATGTTGGCTGAAC-3'	915
PHD1	NM_001004083	5'-AGCAACAGCACTACCCATAGCAGT-3' 5'-TGTGACACGGGTACTTGAACACCT-3'	755
PHD2	NM_178344	5'-AAGATCACCTGGATCGAGGGCAAAA-3' 5'-TCGCTCGTCTGCATCGAAATACCA-3'	426
PHD3	NM_019371	5'-AGAGGCACCTTGAAACCCCTAACA-3' 5'-TTGCTTGGAAGTCTGCATGGCTG-3'	897
FIH-1	XM_219961	5'-TGCAGCAAACACTCAATGACACCG-3' 5'-TCAAGAGGCAAGGGTGAGAAACCT-3'	821
VEGF-A all isoforms	NM_031836	5'-CTCACCAAAGCCAGCACATA-3' 5'-AAATGCTTTCTCCGCTCTGA-3'	160
PAI-1	NM_012620	5'-GACAATGGAAGAGCAACATG-3' 5'-ACCTCGATCTTGACCTTTTG-3'	205
ADM	NM_012715	5'-GGCAGCATTAACAGTCG-3' 5'-AAGGCAGTGGCTCAGACC-3'	223
GAPDH	NM_017008	5'-CTCACTGGCATGGCCTTCCG-3' 5'-ACCACCCTGTTGCTGTAGCC-3'	200
β -Actin	BC063166	5'-GAGCTATGAGCTGCCTGACG-3' 5'-AGCACTTGCGGTCCACGATG-3'	361

MA), and digital images obtained using a bottom-mount 2k AMT digital camera (Advanced Microscopy Technologies, Danvers, MA).

Scanning Laser Confocal Immunofluorescence Microscopy

Primary rat hepatocytes cultured on collagen I-coated coverslips were placed on ice and rapidly washed in cold PBS containing 1:200 dilution of protease and phosphatase inhibitor cocktails (Sigma), fixed in 2% paraformaldehyde in PBS for 15 minutes, and processed as described.²⁷ Primary-deleted negative controls for background were treated with the antibody diluent alone. HIF-1 α and HIF-2 α immunofluorescence staining was performed using a modification of the method recommended by the manufacturer (Novus Biologicals, Littleton, CO). In brief, cells were permeabilized and blocked overnight at 4°C with 2% bovine serum albumin/0.1% Triton X-100 in PBS. Subsequent labeling was performed in 0.5% bovine serum albumin in PBS. All fluorescence labeling was imaged on a Fluoview 1000 confocal scanning microscope (Olympus, Melville, NY). Imaging conditions were maintained at identical settings within each antibody-labeling experiment with original gating performed using the negative control.

Immunohistochemistry

Serial sections of formalin-fixed or zinc-fixed liver tissue were cut at 5- μ m thickness onto Superfrost Plus glass

slides (Fisher Scientific, Pittsburgh, PA) and heat-fixed for 1 hour at 65°C. With the exception of Hypoxyprobe and HIF-1 α , all immunohistochemistry was performed using the Vectastain Elite ABC kit according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA). In brief, after deparaffinization and rehydration of sections, endogenous peroxidase activity was quenched 20 minutes in methanol containing 3% H₂O₂, and 10 minutes of antigen retrieval was performed in boiling 10 mmol/L citrate buffer (pH 6.0) with slow cooling. Sections were blocked 30 minutes at room temperature with Blueblock (Thermo Electron, Pittsburgh, PA) and then incubated with primary antibodies overnight at 4°C. Primary-deleted negative controls for background were treated with the antibody diluent alone. After incubation for 30 minutes at room temperature with affinity-purified biotinylated secondary antibodies, sections were treated with ABC reagent followed by diaminobenzidine chromagen (Vector Laboratories). Mouse ImmPRESS reagent (Vector Laboratories) was used for HIF-1 α staining. Hypoxyprobe staining followed the manufacturer's protocol (Chemicon). All sections were counterstained in hematoxylin, dehydrated, and coverslipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were lysed in RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN), and total RNA was purified using the RNeasy kit with DNase treatment (Qiagen, Valencia, CA). Total RNA (500 ng) was

Figure 1. Establishment of hypoxic cultures. Primary rat hepatocytes were cultured under normoxic (control) or hypoxic (1% O₂) conditions for 6 hours. Parallel hypoxic cultures were returned to the normoxic incubator for 18-hour reoxygenation. **A:** Confocal immunofluorescence detection of pimanidazole adducts (green, FITC-conjugated primary antibody) in hepatocytes after 6 hours of hypoxia confirms hypoxic incubation. **B:** Representative data from MTT and LDH viability assessments are shown as percentage of normoxic control. **C:** RT-PCR demonstrates up-regulation of known HIF target genes, *PAI-1*, *adrenomedullin*, and *VEGF-A* isoforms, indicating a canonical hypoxia response in hepatocytes. Scale bar = 5 μ m.

reverse-transcribed, and the resulting cDNA template was amplified with hot-start PCR using Jumpstart ReadyMix *Taq* polymerase according to the manufacturer's recommendations (Sigma). Primer pairs are outlined in Table 2. PCR products were visualized on 1.2% agarose-TBE gels, stained with ethidium bromide, and imaged with AlphaImager software (Alpha Innotech, San Leandro, CA).

Preparation of Nuclear Extracts

For HIF Western analysis, nuclear proteins from both snap-frozen rat liver tissue and primary hepatocytes cultured on 5 $\times$ 100-mm collagen-coated plates were extracted as described previously²⁸; however, no milk was included in the hypotonic buffer.

Preparation of Membrane-Enriched Fractions

Hepatocytes cultured on 5 × 100-mm plates were washed and scraped into 10 ml of ice-cold isotonic isolation buffer [0.1 mmol/L ethylenediaminetetraacetic acid, 250 mmol/L sucrose, 4% PEG-6000, 5 μmol/L MES (pH 7.4), plus fresh 1:100 protease/phosphatase inhibitor cocktails (Sigma)], resuspended in 2 ml of isolation buffer, and lysed by nitrogen cavitation (600 psi/15 minutes; Parr Bomb, Moline, IL) on ice.²⁷ The resulting cell lysate was centrifuged for 10 minutes at 10,000 × *g* to separate a nuclear fraction (pellet) and a cytosolic/membrane fraction (supernatant). After ultracentrifugation (10 minutes at 100,000 × *g*, Beckman Airfuge, A-100 rotor; Beckman Coulter, Fullerton, CA) of the supernatant, intact peroxisomes were obtained in the organelle-enriched membrane fraction and cytoplasmic proteins remained in the cytosolic fraction. The membrane fraction was solubilized in 1% sodium dodecyl sulfate. All fractions were stored at −80°C until use. Isolation of intact peroxisomes in the membrane was confirmed via Western blotting for PMP70, a peroxisomal membrane protein.

Western Blotting

Protein concentrations were determined by BCA assay (Pierce, Rockford, IL), and 20 to 50 μg of protein were heated to 65°C in 2× Laemmli buffer and separated on 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.²⁹ After electrotransfer onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA), protein bands were reversibly stained with Ponceau S to confirm complete transfer. Membranes were blocked for 1 hour in Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat dry milk, then incubated overnight at 4°C with primary antibodies diluted in 1% or 5% nonfat dry milk/TBST. Membranes were washed and incubated 1.5 hours at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted in 1% nonfat dry milk/TBST. After several TBST washes, membranes were incubated with Supersignal West Pico enhanced chemiluminescence and exposed to CL-Xposure film (Pierce). For Western blots designed for maximum sensitivity, three mouse anti-HIF-1α monoclonal antibodies (NB100-131, NB100-123, NB100-105; Novus) were combined and used at 1:500 on Western blots containing 50 to 100 μg of nuclear extracts.

HIF-1α Enzyme-Linked Immunosorbent Assay (ELISA)

To ensure that the samples' salt and detergent concentrations were compatible with this ELISA-based transcription factor assay, nuclear extracts were prepared using buffers accompanying the kit, and the *Trans*-AM ELISA for HIF-1α DNA binding was used according to the manufacturer's protocol (Active Motif, Carlsbad, CA).³⁰

Results

Establishment of Hypoxic Cultures of Primary Rat Hepatocytes

To investigate the effects of hypoxia-reoxygenation on endogenous HIF proteins in hepatocytes, we first characterized our *in vitro* culture system. We isolated and plated primary rat hepatocytes, allowing them to adhere and equilibrate overnight (18 hours). The following day, we cultured them under conditions of 6 hours normoxia (control), 6 hours hypoxia (1% O₂), or 6 hours hypoxia followed by overnight (18 hours) reoxygenation. To confirm initially that our hepatocytes were indeed hypoxic, we used the hypoxia marker pimanidazole (Figure 1A), a nitroheterocyclic drug whose hypoxia-dependent activation by cellular nitroreductases leads to the formation of covalent intracellular adducts between cellular macromolecules and the drug itself.²³ As seen in Figure 1A, hypoxic cells did indeed stain for the adduct, and subsequent images showing staining of nuclei (Hoechst) and F-actin (phalloidin) confirmed that the cells were viable after hypoxic incubation. We also routinely assessed hepatocyte viability and found that hypoxic cultures had ~73.7% viability (MTT assay) and ~20.6% cytotoxicity (LDH assay) compared with normoxic controls (Figure 1B). Analysis of gene expression by RT-PCR next confirmed the hypoxic induction of HIF target genes *PAI-1*, *adrenomedullin*, and *VEGF-A*, but not the housekeeping gene *GAPDH* (Figure 1C).

Hypoxia Does Not Induce Nuclear Localization of HIF-1α in Primary Rat Hepatocytes

After our assessment of hypoxic culture conditions, we next sought to investigate the expression of endoge-

Figure 2. Absence of nuclear HIF-1α induction in primary rat hepatocytes. Primary rat hepatocytes were cultured under normoxic (control) or hypoxic (1% O₂) conditions for 6 hours. Parallel hypoxic cultures were returned to the normoxic incubator for 18 hours of reoxygenation. **A:** RT-PCR shows expression of HIF-1α, HIF-2α, HIF-3α, and HIF-1β in hepatocyte cultures. **B:** Maximum sensitivity Western blot of nuclear extracts reveals lack of HIF-1α induction in primary hepatocytes compared with JM1 tumor cells. A similar pattern is observed in normal rat liver (NRL) tissue versus JM1 tumor tissue nuclear extracts. **C:** Scanning confocal immunofluorescence images demonstrate nuclear localization of HIF-1α in syngeneic JM1 rat hepatoma cells during hypoxia, but not in primary rat hepatocytes. Scale bar = 10 μm. **D:** HIF-1α DNA binding, as assessed by transcription factor ELISA, further confirms a lack of HIF-1α activation in normal hepatocyte nuclear extracts. JM1 hepatoma cells, in contrast, do show HIF-1α activation under identical hypoxic conditions. Positive control was CoCl₂-treated COS-7, and further internal controls were competitive inhibition of HIF-1α binding by the addition of excess wild-type (WT) oligonucleotide and no effect by the addition of excess mutant (MUT) oligonucleotide. These results, in conjunction with Figures 1 and 2, demonstrate that although hepatocytes do respond to hypoxia, the contribution of HIF-1α to this response may be minor or very transient.

Figure 4. Subcellular localization of endogenous HIFs in primary rat hepatocytes. Primary rat hepatocytes were cultured under normoxic (control) or hypoxic (1% O₂) conditions for 6 hours. Parallel hypoxic cultures were returned to the normoxic incubator for 18 hours of reoxygenation. **A:** Confocal laser-scanning immunofluorescence microscopy demonstrates HIF-1α (green) co-localization (yellow) with the peroxisomal membrane marker PMP-70 (red) after reoxygenation. Note the donut-like red outline of the peroxisomes. Hepatocyte subcortical membrane cytoskeleton is labeled with Alexa Fluor 647 phalloidin (blue), and HIF-1α (green) is often also co-localized at these sites. **B:** Unlike HIF-1α, hypoxia-reoxygenation leads to nuclear induction of HIF-2α (green), which co-localizes (yellow) with the constitutive HIF-1β (red). **C:** Nuclear HIF-3α (green) is observed in normoxic hepatocytes, and hypoxia-reoxygenation leads to co-localization with catalase (red) in peroxisomes. N, nucleus. Scale bars = 5 μm. Insets shown at ×2 large panel.

nous HIFs in primary rat hepatocytes, beginning with HIF-1α. Interestingly, although we observed expression of *HIF-1α* via RT-PCR (Figure 2A), we were unable to detect nuclear induction of HIF-1α protein in hypoxic

primary rat hepatocytes by either imaging or Western blotting (Figure 2, B and C). We repeated our experiments using parallel cultures of primary rat hepatocytes and JM1 cells, a syngeneic rat hepatoma cell line

Figure 3. Comparison of hypoxic regions in syngeneic rat hepatomas. JM1 Fisher 344 rat hepatoma cells were transplanted into livers of same-strain rats. After 4 weeks of tumor formation, rats were injected intraperitoneally with Hypoxyprobe marker and immunostained for pimanidazole adducts (**A–C**). **A:** Low-magnification micrograph of immunohistochemical localization of hypoxic regions contained in tumor and compressed normal adjacent (host) liver. Normal adjacent liver shows gradient of Hypoxyprobe emanating from the perivenous areas, whereas the periportal shows no labeling. **B:** Hypoxyprobe-positive tumor cells outline the necrotic region (N) of tumor. **C:** Hypoxyprobe-positive host liver (between arrows) is compressed by tumors extending bilaterally. **D:** HIF-1α-positive nuclei (arrows) of tumor cells surround necrotic center (N), corresponding to hypoxic regions identified in **B**. **E:** Absence of HIF-1α nuclear labeling in tumor-adjacent hepatocytes (between arrows), despite being compressed in hypoxic regions (**C**). All labeling is observed as cytoplasmic in these hepatocytes. Original magnifications, ×100.

Figure 5. Subcellular localization of endogenous HIF hydroxylases in resting liver. Shown here are representative images of localization patterns observed. **A–C:** Perivenous (**heavy arrows**) gradient distribution of PHD4 in rat liver, with sparing of periportal regions (**light arrow**). Punctate labeling (**arrows**) is observed in perivenous hepatocytes for PHD3 in rat liver (**B**) and PHD2 in human liver (**C**) (CV, central vein). **D:** Immuno-TEM showing PHD3 (10-nm gold) in peroxisomes (P) and in mitochondria (M) of rat liver. **E:** PHD2 in rat liver is labeled with 10-nm gold particles, and peroxisomes are identified by catalase (5-nm gold particles) and the urate oxidase crystalline core. Scale bars: 500 μm (**A**); 100 μm (**B**, **C**); 100 nm (**D**, **E**).

derived from Fisher 344 rat hepatocytes.²⁶ Even when analyzed using identical experimental conditions, we could only detect nuclear HIF-1α in the JM1 cells and not the hepatocytes. As seen in Figure 2, B and C, the absence of nuclear HIF-1α in hepatocytes was observed by both confocal immunofluorescence and maximum sensitivity Western blots. An ELISA-based DNA-binding assay also confirmed this lack of HIF-1α responsiveness in hepatocytes (Figure 2D). Furthermore, when JM1 cells were reintroduced into normal Fisher 344 rat livers, the resulting tumors contained

numerous hypoxic regions (Figure 3, A–C). The tumor cells highly expressed nuclear HIF-1α in these regions; however, the normal adjacent compressed liver contained only cytoplasmic HIF-1α staining (Figure 3, D and E), indicating that such an excessive and chronic hypoxic insult to the liver was inadequate to induce nuclear HIF-1α in hepatocytes. Taken together, the results in Figures 1 to 3 provide evidence that although hepatocytes do respond to hypoxia, the contribution of HIF-1α to this adaptation may be minor or transient at best.

HIF-1 α Localizes to Peroxisomes in Primary Rat Hepatocytes

As shown in Figure 2C, although HIF-1 α does not translocate to the hepatocyte nucleus in hypoxia, there appears to be an increase HIF-1 α in the cytoplasm. Given our unexpected findings, we decided to further dissect the punctate cytoplasmic labeling we observed for HIF-1 α in hypoxic hepatocytes. Confocal immunofluorescence (Figure 4A) revealed that endogenous HIF-1 α (green) co-localized (yellow) with the peroxisomal membrane protein PMP70 (red) in hepatocytes subjected to reoxygenation after hypoxia. Because HIF-1 α was recently shown to play the least active role in primary rat hepatocytes co-transfected with HIF- α expression vectors and IGFBP-1 reporter gene constructs,³¹ we next decided to compare the distribution patterns of other endogenous HIF transcription factors in response to hypoxia. In contrast to HIF-1 α , we did observe a nuclear induction of HIF-2 α in hepatocytes, but this was only after reoxygenation experiments (Figure 4B). HIF-2 α (green) co-localized (yellow) with nuclear HIF-1 β (red), the constitutive nuclear binding partner of HIF- α . There was also an increase in cytoplasmic HIF-2 α , but this was not co-localized to peroxisomes (data not shown). Interestingly, basal levels of HIF-3 α (green) were observed in the nuclei of normoxic (control) hepatocytes, and this HIF-3 α shifted out of the nucleus in hypoxia (Figure 4C). Unlike HIF-2 α , HIF-3 α did co-localize (yellow) with the peroxisomal enzyme catalase (red), suggesting a similar targeting mechanism as HIF-1 α .

HIF Hydroxylases Localize to Peroxisomes in Resting Liver

Because much less is known about the HIF regulatory hydroxylases in comparison to the HIF transcription factors, we decided to expand our study to include the subcellular distribution of HIF hydroxylases in intact rat and human liver. As seen in Figure 5A, there is a zonal distribution of PHD4 around the central veins, and this zonal pattern is similar for the other HIF hydroxylases examined. This was surprising because the perivenous hepatocytes are exposed to the lowest pO₂ along the liver's physiological O₂ gradient, making this region less suited for HIF hydroxylase activity. At higher magnification, we found that in perivenous areas, HIF hydroxylases localized to some hepatocyte nuclei; however, there was also an intense punctate labeling in the cytoplasm (Figure 5, B and C). These findings were intriguing given the paucity of previous reports on endogenous HIF hydroxylases in normal tissue and liver in particular. We next performed additional high-resolution studies to identify the subcellular localization of endogenous HIF hydroxylases in resting rat liver. The particulate pool of HIF hydroxylases in the hepatocyte cytoplasm was contained in specific organelles. PHD2 and PHD3 localized to mitochondria as well as peroxisomes (Figure 5D). As demonstrated by immuno-TEM

Figure 6. Expression of HIF hydroxylase transcripts in hepatocyte cultures. Primary rat hepatocytes were cultured under normoxic (control) or hypoxic (1% O₂) conditions for 6 hours. Parallel hypoxic cultures were returned to the normoxic incubator for 18 hours of reoxygenation. RT-PCR analysis of total RNA is shown for HIF hydroxylases indicating constitutive expression of transcripts in hepatocytes. *PHD3* expression is up-regulated by hypoxia, consistent with published reports. Repeated analyses using human *PHD4* primers were unsatisfactory, and rat sequence for *PHD4* was not available at time of experimentation.

in Figure 5E, PHD2 co-localizes in peroxisomes, which are identified by both catalase and the recognizable urate oxidase crystalline core. Although localization of transiently transfected PHD3 (SM20) has been described previously in rat sympathetic neurons,³² peroxisomal localization has never been reported for any of the known HIF hydroxylases. Interestingly, unlike the other HIF hydroxylases, PHD1 also localized to the bile canalicular membrane of hepatocytes (see Figure 7).

Expression of HIF Hydroxylases in Primary Rat Hepatocytes

To investigate the effects of hypoxia-reoxygenation on HIF hydroxylases in hepatocytes, we once again used our *in vitro* culture system. We analyzed gene expression by RT-PCR of the HIF hydroxylases with available rat sequences, *PHD1–3* and *FIH-1*, in our hepatocyte cultures and found an up-regulation of *PHD3* but not *PHD2* by 6 hours of hypoxia (Figure 6). This is in contrast to previous reports by others that *PHD2* and *PHD3* are both hypoxia-induced genes; however, those findings were based on longer hypoxic incubation times (18 hours).³³

Figure 7. Subcellular localization of endogenous PHD1 in hepatocytes. **A:** PHD1 is found in a punctate staining pattern within hepatocyte cytoplasm, as well as concentrated around the bile canaliculi (arrow) of resting human liver. **B:** Immuno-TEM showing PHD1 localized (arrows) in close proximity to the membrane of the bile canaliculus (BC) of rat liver (M, mitochondrion). **C:** PHD1 signal is also observed on the canalicular membranes (arrows) of cultured normoxic primary rat hepatocytes. **D:** Confocal immunofluorescence microscopy visualizing cells labeled with catalase (red) and PHD1 (green). As with the other HIF hydroxylases, hypoxia-reoxygenation results in a reversible nuclear-to-cytoplasmic translocation for PHD1. Increased peroxisomal co-localization (yellow, see insets) is also observed in response to these treatment conditions; however, unlike the other HIF hydroxylases, PHD1 also localizes to the hepatocyte membrane (arrows), N, nucleus. Insets represent $\times 2$ magnification of large panels. Scale bars: 10 μm (A); 500 nm (B); 5 μm (C, D).

Table 3. Summary of Confocal Immunolocalization for Endogenous Members of the HIF Pathway in Primary Rat Hepatocytes

Protein	Treatment condition			Comments on localization
	Normoxia	Hypoxia	Reoxygenation	
HIF-1 α	Membrane	Membrane	Cytoplasm	<ul style="list-style-type: none"> ● Peroxisomal in reoxygenation ● Not in nucleus
HIF-2 α	Cytoplasm	Cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Not in peroxisomes
HIF-3 α	Nucleus cytoplasm	Cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Peroxisomal ● Leaves nucleus in hypoxia
HIF-1 β	Nucleus	Nucleus	Nucleus	<ul style="list-style-type: none"> ● Constitutive
PHD1	Nucleus	Cytoplasm membrane	Nucleus cytoplasm Membrane	<ul style="list-style-type: none"> ● Peroxisomal
PHD2	Nucleus cytoplasm	Cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Bile canaliculi ● Peroxisomal in hypoxia
PHD3	Nucleus cytoplasm	Nucleus cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Some mitochondrial ● Peroxisomal
PHD4	Nucleus cytoplasm	Nucleus cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Some mitochondrial ● Peroxisomal ● Lowest abundance ● Very novel enzyme
FIH-1	Nucleus cytoplasm	Nucleus cytoplasm	Nucleus cytoplasm	<ul style="list-style-type: none"> ● Peroxisomal
VHL	Cytoplasm	Cytoplasm	Cytoplasm	<ul style="list-style-type: none"> ● Peroxisomal ● Not in nucleus

Hypoxia Induces Nuclear-to-Cytoplasmic Translocation and Peroxisomal Sequestration of HIF Hydroxylases in Cultured Hepatocytes

We next used our *in vitro* hypoxia-reoxygenation model to further characterize the dynamic relationship of subcellular localization and HIF hydroxylases in hepatocytes in culture. As seen in Figure 7, scanning laser confocal immunofluorescence microscopic analysis revealed that endogenous PHD1 (green) shifts from the nucleus to the cytoplasm in hypoxia, and this shuttling is reversed on reoxygenation. As nuclear PHD1 decreases, more PHD1 is associated with the hepatocyte membrane, and there is an increase in PHD1 co-localization (yellow) with catalase (red) in peroxisomes. Although nuclear PHD1 is restored with reoxygenation, peroxisomes still appear to sequester a sizable fraction of PHD1. Figure 8, A–D, shows analogous findings for PHD2 to PHD4 and FIH-1; however, only PHD1 localizes to the bile canaliculi (Figure 7). The peroxisomal pool exists in normoxia, but increases with hypoxia-reoxygenation. This finding is observed even with PHD4 (Figure 8C), which is the least expressed of the HIF hydroxylases in these hepatocyte cultures, suggesting a common sequestration event among these family members.

Immuno-TEM and Western Analysis Confirm Peroxisomal Localization of HIF Hydroxylases in Cultured Hepatocytes

To verify our microscopic observations, we commenced additional studies on primary rat hepatocytes. Immuno-TEM of hypoxic hepatocytes in culture confirmed the presence of PHD2 in peroxisomes, identified by catalase and the urate oxidase crystalline core (Figure 9A). This

was also noted for the other HIF hydroxylases, with PHD4 showing the least amount of labeling. As shown for PHD3 (Figure 9B), there is a striking lack of this protein in the nucleus compared with the peroxisome in these cells. We next performed subcellular fractionation of hepatocytes in an isotonic buffer to obtain a heavy pellet containing an organelle-enriched membrane fraction. Immunoblotting of the membrane fraction isolated from cultured rat hepatocytes further confirmed the presence of PHD1 to PHD3 and FIH-1 in the peroxisome-containing fractions (Figure 9C). Hypoxia-reoxygenation also altered the size of some HIF hydroxylases, suggesting either posttranslational modifications or the existence of other isoforms. For instance, we observed a decrease in the faster-migrating species of the nuclear PHD1 doublet, which has also been described by others.^{34,35}

Hypoxia-Reoxygenation Leads to Peroxisomal Localization of vHL

Having identified the presence of HIF-1 α and PHDs in peroxisomes, we next investigated whether vHL, known to be associated with the transport of HIF-1 α , also resided here. As the substrate recognition unit of the E3 ubiquitin ligase multiprotein complex (elongins B and C, cullin2, Ring-box 1), pVHL tags hydroxylated HIF-1 α for polyubiquitination and degradation by the 26S proteasome.^{5,6} As seen in Figure 10, vHL does indeed co-localize in peroxisomes with HIF-1 α and catalase. These findings may suggest a potential link between HIF-1 α shuttling to the peroxisomes and HIF hydroxylation.

Discussion

The subcellular localization patterns of endogenous HIFs and HIF regulatory hydroxylases in our primary rat hepato-

Figure 9. Peroxisomal localization of endogenous HIF hydroxylases in hepatocytes. **A** and **B**: Immuno-TEM of 6-hour hypoxic primary rat hepatocytes. PHD2 (**A**) and PHD3 (**B**) are labeled by 10-nm gold particles, whereas peroxisomes are identified by catalase (5-nm gold particles) and the urate oxidase crystalline core (asterisk). N, nucleus. **C**: Hepatocyte subcellular fractionation in an isotonic buffer was used to obtain both a nuclear fraction and intact peroxisomes in the organelle-enriched membrane fraction. Western blots showing localization of HIF hydroxylases in both the nuclear and membrane fractions of primary rat hepatocytes. PHD proteins display obvious isoforms under different conditions and fractions, although their significance is not known. PHD4 was undetectable in either fraction via Western blot. COS-7 cell nuclear extract was used as positive control for each protein. PMP-70 Western blot confirms isolation of intact peroxisomes in the membrane fraction and absence of contaminating intracellular membrane in the nuclear fraction. Scale bar = 50 nm.

cyte cultures are summarized in Table 3. Interestingly, although we observed both expression of *HIF-1 α* via RT-PCR (Figure 2A) and an up-regulation of HIF target genes (Figure 1C), we were unable to detect by imaging, high-sensitivity Western blot, or ELISA a nuclear induction of HIF-1 α protein in hypoxic primary rat hepatocytes (Figure 2). This may be because of the predominance of other HIF- α species in hepatocytes, because HIF-2 α did translocate to the nucleus after hypoxia-reoxygenation. In addition, the role of nuclear HIF-3 α in normoxic hepatocytes is less clear, because several splice variants are known to exist, including those that may act as decoys (eg, IPAS), negatively regulating the other HIF- α subunits.³⁶ It was recently demonstrated that HIF-2 α and HIF-3 α were the predominant activators of hypoxia-induced IGFBP-1 transcription in transfected primary

rat hepatocytes.³¹ The minor role of HIF-1 α in hepatocytes may be because of its translocation to peroxisomes rather than to the nucleus in hypoxia. This peroxisomal import is accompanied (or perhaps facilitated) by the HIF regulatory hydroxylases and vHL in hypoxia-reoxygenation.

Our observations of vHL in hepatocytes are intriguing given that Groulx and Lee³⁷ found in HeLa cells that vHL engages in a constitutive nuclear-cytoplasmic shuttle unaffected by pO₂ or levels of nuclear HIF- α substrate. The peroxisomal targeting sequences PTS1 and PTS2 are consensus sequences that are involved in peroxisomal import, and they are recognized by the peroxisomal import receptors Pex5 and Pex7, respectively.³⁸ On closer examination of the primary sequence of vHL, we identified a potential 100% canonical PTS2 import sequence in the N-terminal end of the middle of the molecule, making this PTS site the most potentially active among the protein sequences we compared. Furthermore, even if a protein's sequence is not 100% identical to the canonical PTS sites, other enzymes have been described that target to the peroxisome with similar, but nonconventional PTS sequences; these include acetoacetyl-CoA thiolase,³⁹ alanine:glyoxylate aminotransferase,⁴⁰ isopentenyl diphosphate dimethylallyl diphosphate isomerase,⁴¹ and iNOS.^{27,42} In fact, very large protein oligomers lacking PTS motifs have been shown to piggy-back onto other conventional peroxisomal proteins and gain entry into the peroxisomal matrix in their native three-dimensional configuration.⁴³ Specifically, the primarily hydrophobic β -domain of vHL contains two critical hydrophilic residues (His-115 and Ser-111), which must hydrogen bond with either hydroxyproline or H₂O molecules.^{44,45} Theoretically, vHL could gain entry into the peroxisome while complexed with HIF-1 α and its associated HIF hydroxylase(s), and hydroxylated HIF-1 α may serve as an intermediate between them. Because achieving highly efficient transfection of primary hepatocyte cultures can be a challenge, additional experiments in liver-specific vHL-null cells⁴⁶ would provide further clues about the peroxisomal import of these proteins.

The significance of our findings can be further extrapolated to the liver's microenvironment, where hepatocytes and neighboring cells are exposed to a physiological gradient of O₂ and other nutrients from the portal circulation. Not only does the portal vein contribute significantly to the liver's low-oxygen-containing blood supply, there is an ~50% drop in pO₂ along the course of the sinusoid toward the central vein, from 60 to 65 mm Hg (afferent) to 30 to 35 mm Hg (efferent).⁴⁷ Because basal HIF-1 α is constitutively expressed but rapidly degraded, a mechanism must exist to poise the cell for swift responses to hypoxic insult, while at the same time keeping nuclear levels of HIF-1 α in check when not needed. Nuclear HIF hydroxylases are thought to participate in the regulation of HIF-1 α turnover and activity, because the

Figure 8. Subcellular localization of endogenous HIF hydroxylases in cultured rat hepatocytes. Primary rat hepatocytes were cultured under normoxic (control) or 6-hour hypoxic (1% O₂) conditions. Parallel hypoxic cultures were returned to the normoxic incubator for 18-hour reoxygenation. Confocal immunofluorescence microscopy was used to visualize cells labeled with catalase (red) and each of the HIF hydroxylases indicated (green). In general, hypoxia-reoxygenation results in increased peroxisomal co-localization (yellow, see **inserts**); however, each hydroxylase has distinct levels of expression. **A**: PHD2; **B**: PHD3; **C**: PHD4; **D**: FIH-1 (N, nucleus). Insets represent a $\times 2$ magnification from the large panels. Scale bar = 5 μ m.

Figure 10. Peroxisomal co-localization of vHL with HIF-1α. Primary rat hepatocytes were cultured under normoxic (control) or hypoxic (1% O₂) conditions for 6 hours. Parallel hypoxic cultures were returned to the normoxic incubator for overnight reoxygenation. Confocal scanning laser immunofluorescence microscopy demonstrates the peroxisomal co-localization (light yellow, **arrows** indicate several examples) of HIF-1α (blue), catalase (red), and vHL (green) in hepatocytes subjected to hypoxia-reoxygenation. N, nucleus. Scale bar = 5 μm.

degradation of HIF-1 α can occur with the same half-life in both the nucleus and the cytoplasm.⁴⁸ This indicates that unlike p53, which must exit the nucleus before its degradation, both nuclear and cytoplasmic proteasomes are fully competent to degrade HIF-1 α in an O₂-dependent manner, thereby preventing even ongoing HIF transcriptional activity. Moreover, *in vitro* enzyme kinetic assays have found that PHD2 and PHD3 possess the highest relative prolyl hydroxylation activities,⁴⁹ suggesting that the robust expression of these enzymes in resting liver would counteract much of the HIF-1 α activity one would have expected to find in perivenous hepatocytes. This lack of HIF-1 α activity is in contrast to organs such as the adult kidney, which acts as an important physiological O₂ sensor and rapidly adapts to systemic hypoxia by increasing EPO production.⁵⁰

In general, the PHDs can shuttle from the hepatocyte nucleus to the cytoplasm in response to hypoxia. On reoxygenation, there exists a substantial pool of endogenous HIF hydroxylases sequestered in peroxisomes, which is a novel finding. In contrast to the HIFs, subcellular localization of HIF hydroxylases has only been published for cell lines transfected to overexpress each respective enzyme. Mitochondrial localization has been shown for the PHD3 homolog SM20,³² and we observed some mitochondrial localization in hepatocytes as well (Figure 5D). In U2OS cells expressing transiently transfected human HIF hydroxylase-GFP fusion proteins, the localization of PHD1 was completely nuclear, PHD2 and FIH-1 were mostly cytoplasmic, and PHD3 was homogeneously distributed between the nucleus and the cytoplasm.⁵¹ In contrast, transfection of COS-1 cells revealed both nuclear and cytoplasmic distribution for PHD1 to PHD3 and FIH-1.⁵² When FLAG-tagged PHD4 was transiently transfected in COS-7 cells, it excluded the nucleus and localized to the endoplasmic reticulum, although no consensus ER retention signal was identified in its peptide sequence.⁵³ These pioneering experiments may be useful in predicting the diverse functions of HIF hydroxylases in different cell types; however, they only add to the complexity of how and where these O₂-dependent hydroxylases are shuttled in response to hypoxia. To date, a detailed analysis of the subcellular localization of endogenous HIF hydroxylases in primary cells has not been published.

In this study, we identify dynamic changes in subcellular localization of HIF-1 α , vHL, and the HIF regulatory hydroxylases, all of which co-localize to peroxisomes in hepatocytes. The question remains as to whether these enzymes are actively hydroxylating key proline residues while sequestered in the peroxisome. Before the discovery of HIF regulation, collagen was the only known hydroxyproline-containing protein. Unlike the collagen prolyl 4-hydroxylase, which acts on the tripeptide X-pro-gly, the HIF PHDs require a much longer (~19mer) minimal HIF-derived peptide for optimal activity.⁵⁴ Furthermore, each PHD may preferentially hydroxylate one or both of the two proline residues in the HIF-1 α oxygen-dependent degradation domain, suggesting specialized roles for acute and chronic adaptation.⁵⁴ Interestingly, a short but growing list is emerging for O₂-dependent hy-

droxylation of non-HIF proteins by PHDs and FIH-1, such as the large subunit (Rbp1) of RNA polymerase II.⁵⁵ Even though the PHDs are highly conserved and ubiquitously expressed, there is also evidence of alternative splicing, with some variants no longer capable of hydroxylating HIF- α .⁵⁴ These isoforms may represent the various sizes of PHDs observed in the Western blots (Figure 9C). In general, numerous hydroxylases exist in peroxisomes as well as in the bile canaliculi, where PHD1 was observed around the canalicular membrane. Although we observed a peroxisomal pool of HIF-1 α in hepatocytes, we are uncertain whether any of the HIF hydroxylases sequestered in peroxisomes still retain their activity, and if so, what their potential substrates may be. In the case of iNOS, a nonconventional peroxisomal enzyme in hepatocytes, the fraction of iNOS sequestered in peroxisomes is an inactive monomer, perhaps protecting the cell from incompetent enzyme.⁴² On the other hand, phytanoyl Co-A hydroxylase is a classic PTS2-containing enzyme that leads to Refsum's disease if defective.⁵⁶ Not only is phytanoyl Co-A hydroxylase active in peroxisomes, it is actually a member of the same O₂-, Fe²⁺-, and 2-oxoglutarate-dependent oxygenase family as the HIF hydroxylases; therefore, within the peroxisomes there may exist a medium containing the co-factors necessary for PHD and FIH-1 activity.⁵⁷ Modification of existing biochemical techniques for measuring hydroxylated HIF peptides *in vitro* could provide an alternative method for testing enzymatic activity of HIF hydroxylases in intact peroxisomes.

The *in vitro* hypoxia-reoxygenation model alters the O₂ availability necessary for HIF hydroxylase function. A potential mechanism for targeting HIF hydroxylases to other organelles may involve the oxygen-redirection hypothesis,⁵⁸ which states that inhibition of mitochondrial respiration may lead to subcellular redirection of O₂ from mitochondria to nonrespiratory O₂-dependent compartments. In hepatocytes, the peroxisome represents one such compartment, constituting ~1% of total cell volume, yet consuming up to 30% of the O₂ in resting liver.⁵⁹ Inhibitors of mitochondrial respiration such as NO have been shown to increase PHD activity and decrease nuclear HIF-1 α in hypoxia because of subcellular O₂ redirection from mitochondria to PHDs.^{60,61} A very recent study⁶² has shown that HIF-1 α can actively down-regulate mitochondrial O₂ consumption by repressing the TCA cycle in cell lines. This results in adaptation to hypoxia, because mitochondrial O₂ redistribution leads to a relative increase in intracellular O₂ concentration and availability. The effects of HIF-1 α on mitochondria were found to be functional, not structural, and decreased cell death was observed in chronic hypoxia. Other groups have also linked the TCA cycle as a metabolic switch for cellular adaptation to hypoxia because of its production of intermediates such as the PHD co-factor 2-OG.^{63,64}

In summary, we have identified an unexpected subcellular distribution pathway in hepatocytes in response to hypoxia and reoxygenation, where both HIF-1 α and the O₂-sensing hydroxylases by which they are regulated and are all shuttled to the peroxisome, such that nuclear induction of HIF-1 α is undetectable. It would be interesting to elucidate the molecular mechanisms of this se-

questration in more detail and to determine precisely how vHL or other carrier proteins might be functioning in hepatocytes under these conditions. Further consideration should also be given to the effects of required HIF hydroxylase co-factors (Fe^{2+} , 2-OG, ascorbate), as well to glucose itself, because like O_2 , the latter is also distributed across a gradient in the liver. Finally, *in vivo* correlations with ischemia-reperfusion models may provide new insights on how HIF regulatory hydroxylases are altered in liver injury. In conclusion, our results suggest a novel site for the regulation of the O_2 -dependent HIF pathway in hepatocytes, and they expand on the role of peroxisomes as an O_2 sink in the redox microenvironment of the liver.

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