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Stiehl, D P; Wirthner, R; Köditz, J; Spielmann, P; Camenisch, G; Wenger, R H. Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J. Biol. Chem. 2006, 281(33):23482-91.

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Originally published at:
J. Biol. Chem. 2006, 281(33):23482-91
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Abstract

Prolyl 4-hydroxylase domain (PHD) proteins are oxygen-dependent enzymes that hydroxylate hypoxia-inducible transcription factor (HIF) alpha-subunits, leading to their subsequent ubiquitination and degradation. Paradoxically, the expression of two family members (PHD2 and PHD3) is induced in hypoxic cell culture despite the reduced availability of the oxygen co-substrate, and it has been suggested that they become functionally relevant following re-oxygenation to rapidly terminate the HIF response. Here we show that PHDs are also induced in hypoxic mice in vivo, albeit in a tissue-specific manner. As demonstrated under chronically hypoxic conditions in vitro, PHD2 and PHD3 show a transient maximum but remain up-regulated over more than 10 days, suggesting a feedback down-regulation of HIF-1alpha which then levels off at a novel set point. Indeed, hypoxic induction of PHD2 and PHD3 is paralleled by the attenuation of endogenous HIF-1alpha. Using an engineered oxygen-sensitive reporter gene in a cellular background lacking endogenous HIF-1alpha and hence inducible PHD expression, we could show that increased exogenous PHD levels can compensate for a wide range of hypoxic conditions. Similar data were obtained in a reconstituted cell-free system in vitro. In summary, these results suggest that due to their high O2 Km values, PHDs have optimal oxygen-sensing properties under all physiologically relevant oxygen concentrations; increased PHDs play a functional role even under oxygen-deprived conditions, allowing the HIF system to adapt to a novel oxygen threshold and to respond to another hypoxic insult. Furthermore, such an autoregulatory oxygen-sensing system would explain how a single mechanism works in a wide variety of differently oxygenated tissues.
Increased Prolyl 4-Hydroxylase Domain Proteins Compensate for Decreased Oxygen Levels

EVIDENCE FOR AN AUTOREGULATORY OXYGEN-SENSING SYSTEM

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Prolyl 4-hydroxylase domain (PHD) proteins are oxygen-dependent enzymes that hydroxylate hypoxia-inducible transcription factor (HIF) α-subunits, leading to their subsequent ubiquitination and degradation. Paradoxically, the expression of two family members (PHD2 and PHD3) is induced in hypoxic cell culture despite the reduced availability of the oxygen co-substrate, and it has been suggested that they become functionally relevant following re-oxygenation to rapidly terminate the HIF response. Here we show that PHDs are also induced in hypoxic mice in vivo, albeit in a tissue-specific manner. As demonstrated under chronically hypoxic conditions in vitro, PHD2 and PHD3 show a transient maximum but remain upregulated over more than 10 days, suggesting a feedback down-regulation of HIF-1α which then levels off at a novel set point. Indeed, hypoxic induction of PHD2 and PHD3 is paralleled by the attenuation of endogenous HIF-1α. Using an engineered oxygen-sensitive reporter gene in a cellular background lacking endogenous HIF-1α and hence inducible PHD expression, we could show that increased exogenous PHD levels can compensate for a wide range of hypoxic conditions. Similar data were obtained in a reconstituted cell-free system in vitro. In summary, these results suggest that due to their high O2 Km values, PHDs have optimal oxygen-sensing properties under all physiologically relevant oxygen concentrations; increased PHDs play a functional role even under oxygen-deprived conditions, allowing the HIF system to adapt to a novel oxygen threshold and to respond to another hypoxic insult. Furthermore, such an autoregulatory oxygen-sensing system would explain how a single mechanism works in a wide variety of differently oxygenated tissues.

Biological systems tightly monitor acute changes in environmental conditions, initiate regulatory responses, and use negative feedback loops to limit the extent of these responses. To adapt to chronic changes, many environmental sensors are capable of adjusting their threshold values, allowing to respond again to acute deviations of a now different set point.

Cells sense changes in environmental oxygen availability by a group of enzymes that directly control the cellular response to lowered oxygen by destabilizing hypoxia-inducible factor (HIF) α-subunits, the master transcriptional regulators of the hypoxic response. These oxygen-sensing enzymes have alternatively been termed prolyl 4-hydroxylase domain (PHD), HIF prolyl hydroxylase (PHH), or egg laying defective nine homolog (EGLN). The following three family members are known up to date: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1, and PHD3/HPH1/EGLN3 (1–3). PHDs hydroxylate HIF-1α and HIF-2α at two distinct proline residues within the HIFα oxygen-dependent degradation (ODD) domain. Under normoxic conditions, prolyl 4-hydroxylase allows binding of the von Hippel-Lindau tumor suppressor protein (pVHL), leading to polyubiquitination and proteasomal destruction (4). Under hypoxic conditions, prolyl 4-hydroxylation is reduced, and HIF-1α and HIF-2α become stabilized, heterodimerize with the constitutively expressed HIF-1α subunit aryl hydrocarbon receptor nuclear translocator (ARNT), and regulate the expression of a large number of effector genes involved in adaptation to low oxygen (5). In addition, factor inhibiting HIF hydroxylates a C-terminal asparagine residue, thereby regulating the transcriptional activity of HIFs (6–8).

Upon re-oxygenation, the PHD oxygen-sensing system must be rapidly reversed. Interestingly, PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced at both the mRNA and protein levels (2). Accordingly, elevated PHD2 and PHD3 levels have been demonstrated in a broad panel of established cancer cell lines (9). Functional hypoxia-response elements are located in the promoter region of the human PHD2 gene as well as in the first intron of the human PHD3 gene, suggesting that PHD2 and PHD3 are HIF target genes themselves (10, 11). Because the essential co-factor oxygen is basi-

*This work was supported by grants from Edoardo R. Giovanni and Giuseppe and Chiara Sassella-Stiftung (to D. P. S.), the Krebsliga des Kantons Zürich (to R. H. W.), the 6th Framework Programme of the European Commission/SBF EUROXY LSCH-CT-2003-502932/SBF 03.0647-2 (to R. H. W.) and SNF 3100AO-104219 (to R. H. W. and G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table 1 and supplemental Fig. 1.

1 The abbreviations used are: HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; CA, carbonic anhydrase; F, inspiratory oxygen fraction; GLUT, glucose transporter; mAb, mouse monoclonal antibody; MEF, mouse embryonic fibroblast; ODD, oxygen-dependent degradation; PHD, prolyl 4-hydroxylase domain; pVHL, von Hippel-Lindau tumor suppressor protein; VBC, pVHL/elongin B/elongin C; siRNA, short interfering RNA; RT, reverse transcription; GST, glutathione S-transferase; HPH, HIF prolyl hydroxylase; EGLN, egg laying defective nine homolog; DMOG, dimethylglyoxal/glycine.

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cally lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance has been suggested to play a role in accelerating the termination of the HIF response following re-oxygenation (2, 9, 12, 13). Indeed, biochemical in vitro studies revealed $K_m$ values of purified PHDs for oxygen close to the oxygen partial pressure ($pO_2$) in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (14). However, tissues in situ have to deal with a great variability of generally very low $pO_2$ values, even when the inspiratory $pO_2$ is considered to be “normoxic.” We therefore raised the question whether HIF-dependent regulation of PHD levels might lead to the adaptation of the PHD-HIF oxygen-sensing system to a given tissue $pO_2$, rather than simply accelerating HIFα destruction following re-oxygenation. Such a self-regulatory loop might define a tissue-specific threshold for HIFα activation as a function of local $pO_2$. 

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All cells were maintained in Dulbecco’s modified Eagle’s medium-high glucose, containing 4.5 mg/ml glucose (Sigma) to maintain cellular energy metabolism during prolonged hypoxic culturing. Culture media were supplemented with 10% heat-inactivated fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Mouse embryonic fibroblast (MEF)-Hif1a+/+ and MEF-Hif1a−/− and mouse hepatoma Hepa1 and Hepa1c4 were cultured as described before (15, 16). For long term hypoxia, MEFs were grown under 2% O2 for up to 256 h in a gas-controlled glove box to allow for handling of the cells under constant $pO_2$ (InviVo 2 400, Ruskinn Technologies, Leeds, UK). Cells were grown on 145-mm culture dishes and split every 48 h. Reagents used for splitting and permanent culture of MEFs were subjected to immunoblot analysis. Antibodies were obtained from the following sources: anti-human HIF-1α/H9251 (Novus Biologicals, Littleton, CO); anti-V5 tag mAb, Invitrogen; anti-human PHDs rabbit polyclonal antibodies, provided by W. Kaelin, Jr., Boston, and I. Flamme, Wuppertal (Stockholm, Sweden). A PCR product spanning the HIF-1α ODD region (amino acids 359–685) was cloned into the EcoRI site of pm3-VP16 (Clontech) to obtain one-hybrid constructs harboring an N-terminal GAL4 DNA-binding domain and a C-terminal VP16 transactivation domain. Resulting plasmids were termed G4.mHIF(359–685).VP16_wt, G4.mHIF(359–685).VP16_P402A, G4.mHIF(359–685).VP16_P563A, and G4.mHIF(359–685).VP16_PP/AA. For overexpression in mammalian cells, full-length human PHD2 and PHD3 (kindly provided by W. Kaelin, Jr., Boston, and I. Flamme, Wuppertal, Germany, respectively) were subcloned into pENTR4 and recombined into pcDNA3.1/nV5-DEST using Gateway technology (Invitrogen). Similarly, vectors for expression of GST-tagged PHD isoforms in Sf9 insect cells were constructed by recombining coding sequences for PHD2 and PHD3 into pDEST20 (Invitrogen). All primary cDNA inserts were sequenced (Microsynth, Balgach, Switzerland).

**Plasmid Constructions**—Plasmids encoding full-length mouse HIF-1α, both wild-type and P402A and/or P563A mutants (note that all amino acid numbering corresponds to mouse HIF-1α sequences), were a kind gift of L. Poellinger (Stockholm, Sweden). A PCR product spanning the HIF-1α ODD region (amino acids 359–685) was cloned into the EcoRI site of pm3-VP16 (Clontech) to obtain one-hybrid constructs harboring an N-terminal GAL4 DNA-binding domain and a C-terminal VP16 transactivation domain. Resulting plasmids were termed G4.mHIF(359–685).VP16_wt, G4.mHIF(359–685).VP16_P402A, G4.mHIF(359–685).VP16_P563A, and G4.mHIF(359–685).VP16_PP/AA. For overexpression in mammalian cells, full-length human PHD2 and PHD3 (kindly provided by W. Kaelin, Jr., Boston, and I. Flamme, Wuppertal, Germany, respectively) were subcloned into pENTR4 and recombined into pcDNA3.1/nV5-DEST using Gateway technology (Invitrogen). Similarly, vectors for expression of GST-tagged PHD isoforms in Sf9 insect cells were constructed by recombining coding sequences for PHD2 and PHD3 into pDEST20 (Invitrogen). All primary cDNA inserts were sequenced (Microsynth, Balgach, Switzerland).

**Transient Transfections**—Cells were co-transfected with the indicated amounts of DNA using polyethyleneimine (Polysciences, Warrington, PA). Therefore, cells were grown on 100-mm dishes to subconfluence, and 200 μl of 150 mM NaCl containing a DNA/polyethyleneimine mixture (1:5, w/w) was added. For co-transfections, total DNA was kept constant by adding empty vector. Following overnight incubation, the cells were trypsinized and divided onto 12-well plates for luciferase assays and 100-mm dishes for immunoblotting.

**Luciferase Assays**—MEF-Hif1a−/− cells were transiently co-transfected with 2 μg of the indicated G4.mHIF(359–685).VP16 fusion constructs and up to 8 μg of the respective V5.PHD isoform, along with 1 μg of the GAL4-responsive reporter plasmid pGREG5xElb, containing the firefly luciferase gene under control of Elb promoter and five GAL4 response elements (kind gift of D. Peet, Adelaide, Australia).
Increased PHD Oxygen Sensors Compensate for Low pO2

Identical amounts of total DNA were transfected in each experimental setting. Following transfection, cultures were grown for an additional 24 h at the indicated oxygen concentration and lysed in 100 μl of passive lysis buffer (Promega, Madison, WI). Luciferase reporter gene activity was determined in a microplate luminometer (Berthold, Regensdorf, Switzerland) using luciferase firefly substrate (Promega). Protein concentration in the lysates was determined by a Bradford assay, and relative luciferase activity was calculated from the ratio between relative light units and micrograms of protein.

RNA Interference—HeLa cells were plated at a density of 2 × 10^5 cells per single well of a 6-well plate. The day after, cells were transfected in fresh media with 80 nm siRNA duplexes targeting either human PHD2 (5′-ggagcaagcaggggugcu-uguua-3′, sense strand) or PHD3 (5′-gcuaucggggaauggacag-gua-3′, sense strand) using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After 4 h of culturing at 20% oxygen, cells were subjected to the indicated oxygen concentrations and grown for an additional period of 24 h prior to lysis.

Expression and Purification of PHDs—GST-PHD2 and GST-PHD3 were expressed in baculovirus-infected Sf9 insect cells according to the manufacturer’s instructions (Invitrogen). After 80 h of infection, Sf9 cells were lysed in ice-cold 0.1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 mM glycine, and 10 μM dithiothreitol. Crude lysate was cleared by centrifugation at 20,000 × g for 20 min, and supernatants were incubated with equilibrated glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4°C with gentle agitation. Beads were washed three times with phosphate-buffered saline, and bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 5% glycerol, and 2 μM FeSO4. Purity of recombinant proteins was estimated by SDS-PAGE and Coo-massie Blue staining.

In Vitro Prolyl 4-Hydroxylation Assays—Enzymatic activity of recombinant PHD2 and PHD3 was determined in an in vitro hydroxylation assay performed essentially as described before (17, 21). Briefly, biotinylated mouse HIF-1α-derived peptides (amino acids 555–573, either wild-type or P563A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce). Hydroxylase reactions using purified recombinant GST-PHD2 or GST-PHD3 enzyme were carried out for 1 h at room temperature. A polyclonostic expression vector for His6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania State University, University Park, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography followed by ion exchange chromatography (Amersham Biosciences), and allowed to bind to the hydroxylated peptides. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) using the 3,3′,5,5′-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H2SO4, and absorbance was determined at 450 nm in a microplate reader. For oxygen titration experiments, the assay was performed in the hypoxic glove box. All reagents and solutions were allowed to equilibrate to the indicated oxygen concentration. Inter-assay comparability was guaranteed by calibration of each experiment to an internal standard curve using hydroxyproline-containing peptides.

RESULTS

mRNA Levels of Inducible PHD Isoforms Show a Transient Maximum and Remain Up-regulated during Prolonged Hypoxia in Cell Culture—To study the kinetics of PHD expression during prolonged hypoxia (2% O2 for up to 256 h), mRNA levels of all three PHDs were determined in MEF-Hif1α+/+ and MEF-Hif1α−/− cells. PHD2 and PHD3 but not PHD1 mRNA levels were efficiently up-regulated under hypoxic conditions, peaked after 64–112 h, and remained elevated over the entire time course (Fig. 1A). As positive controls, mRNA levels of GLUT1 and CAIX, two well established HIF-1 target genes, were measured in the same time course. Interestingly, hypoxic GLUT1 up-regulation followed a similar kinetics as PHD2 mRNA expression, whereas CAIX levels were maximally induced 48 h later, as observed for PHD3 (Fig. 1A). Because both PHDs were readily induced after 4 h, we investigated the early onset of induction in a shorter time course. Induction of PHD2 and PHD3 mRNA was detectable as early as 60–120 min after hypoxic stimulation (Fig. 1B). None of the PHD isoforms was induced in hypoxic MEF-Hif1α−/− cells, suggesting a non-redundant role of HIF-1α for hypoxic up-regulation of PHD mRNA in these cell lines. In addition, a lack of PHD2 and PHD3 mRNA induction was also observed in functionally HIF-1α/ ARNT-deficient mouse Hepa1c4 but not wild-type Hepa1 hepatoma cells after 38 h of hypoxic stimulation (Fig. 1C). Thus, these genetically altered cellular models confirm HIF-1 dependent PHD2 and PHD3 gene expression in both acute and chronic hypoxia, which has been demonstrated previously mainly in siRNA experiments (12, 13, 22).

Tissue-dependent Hypoxic Induction of PHD2 and PHD3 mRNA in Mice—To date, no systematic investigation on hypoxic PHD mRNA induction in vivo was available. Thus, we assessed the grade of hypoxic PHD2 and PHD3 mRNA induction in acutely and chronically hypoxic mice.

To examine the effects of acute hypoxia, mice were treated for 4 h with an inspiratory gas mixture containing 0.1% carbon monoxide (CO), which induced a rapid onset of severe hypoxia by blocking ~50% of the oxygen-binding sites in hemoglobin (data not shown). Strong hypoxic induction of erythropoietin mRNA content in kidney as well as erythropoietin and GLUT1 mRNA in liver and brain confirmed the activation of the HIF system in these mice (Fig. 2). The various PHD mRNA isoforms showed a broad disparity of hypoxic activation; PHD1 was not markedly regulated by hypoxia in most tissues; PHD2 was widely induced by hypoxia, albeit at rather low levels; and PHD3 was strongly induced already following 4 h of tissue hypoxia in the lung (15.6-fold), liver (5.2-fold), and kidney (3.8-fold), but only moderate induction factors similar to PHD2 were observed in other organs. Of note, striated muscle tissue (heart and to some extent tongue) showed the highest normoxic expression values for the inducible PHD2 and PHD3 isoforms.

To investigate the kinetics of PHD mRNA induction under chronically hypoxic conditions, mice were exposed to
inspiratory hypoxia (7.5% O2) from 24 to 72 h, and mRNA levels were determined in liver, kidney, and brain. Although erythropoietin mRNA expression was induced to a similar extent in kidney and brain already after 24 h of hypoxia, erythropoietin mRNA in liver did not increase until 72 h of hypoxic exposure (Fig. 3A). Consistent with previous findings, serum levels of erythropoietin protein in these animals highly correlated with mRNA levels in kidney (Fig. 3B).

Unexpectedly, the hypoxic inducibility of the known HIF target genes GLUT1 and CAIX was rather small. Although strong hypoxic induction of both proteins in tumors and tumor-derived cell lines is well established, they were not induced in liver; only one of them (CAIX) was induced after prolonged hypoxia in kidney, and both of them showed a transient induction in brain (Fig. 3C). Nevertheless, these results confirmed the hypoxic status of the animals. Comparable results were obtained with the inducible PHD2 and PHD3 isoforms; only PHD2 showed a small, transient increase in the liver; only PHD3 was induced after prolonged hypoxia in the kidney; and both of them were transiently induced in the brain. Thus, under physiological inspiratory hypoxia, regulation of these genes appears to be more subtle than under in vitro conditions, in tumor hypoxia, or following severe tissue hypoxia in CO-treated mice.

**Hypoxic Induction of PHD2 and PHD3 Proteins Is Accompanied by Decreased HIF-1α Protein Levels**—Hypoxic up-regulation of PHD2 and PHD3 has the potential to affect protein abundance of HIF-1α during prolonged hypoxia. To further elaborate this hypothesis, we quantified protein levels of HIF-1α as well as PHD2 and PHD3 in different cell lines. As exemplarily shown for HEK293 cells, endogenous HIF-1α protein levels decreased concomitantly with the hypoxic increase of endogenous PHD2 and PHD3 levels when cells were cultured for up to 72 h at 1% O2 (Fig. 4A). Similar results were obtained from lysates that were prepared under strictly hypoxic conditions, ruling out the possibility that increased levels of induced PHD enzymes might have influenced the protein levels of HIF-1α during hypoxia. Further elaboration of this hypothesis, we quantified protein levels of HIF-1α as well as PHD2 and PHD3 in different cell lines. As exemplarily shown for HEK293 cells, endogenous HIF-1α protein levels decreased concomitantly with the hypoxic increase of endogenous PHD2 and PHD3 levels when cells were cultured for up to 72 h at 1% O2 (Fig. 4A). Similar results were obtained from lysates that were prepared under strictly hypoxic conditions, ruling out the possibility that increased levels of induced PHD enzymes might have influenced the protein levels of HIF-1α during hypoxia. Further elaboration of this hypothesis, we quantified protein levels of HIF-1α as well as PHD2 and PHD3 in different cell lines. As exemplarily shown for HEK293 cells, endogenous HIF-1α protein levels decreased concomitantly with the hypoxic increase of endogenous PHD2 and PHD3 levels when cells were cultured for up to 72 h at 1% O2 (Fig. 4A). Similar results were obtained from lysates that were prepared under strictly hypoxic conditions, ruling out the possibility that increased levels of induced PHD enzymes might have influenced the protein levels of HIF-1α during hypoxia.

**FIGURE 1. HIF-1 target genes remain elevated during prolonged hypoxia.** MEF-Hif1a+/+ and MEF-Hif1a−/− were cultured either at 20 or 2% oxygen for 4–256 h (A) or 15–240 min (B); mRNA levels for PHD1, PHD2, PHD3, GLUT1, and CAIX were quantified by real-time RT-PCR and normalized to ribosomal protein S12 mRNA content. C, PHD1, PHD2, and PHD3 mRNA levels in Hepa1 Arnt wild-type (wt) and Hepa1c4 Arnt mutant (mt) cells cultured at 20, 2, or 0.2% oxygen for 38 h. Data are given as mean ± S.E. of n = 3 independent experiments.
Increased PHD Oxygen Sensors Compensate for Low pO_2

A Second Hypoxic Insult Activates Another HIF-1α Response in Cells Adapted to Chronic Hypoxia—If the novel HIF-1α protein base line following adaptation to chronic hypoxia indeed resulted from an altered steady state of the HIF-PHD oxygen-sensing circuit, the adapted cells should react to a second, more severe hypoxic insult by acutely inducing HIF-1α to a similar extent as at the first hypoxic insult. To test this hypothesis, HEK293 cells were allowed to adapt to 1% oxygen for 72 h before they were exposed to 0.2% oxygen for up to 4 h. Although HIF-1α levels markedly decreased during chronic hypoxia, a rapid re-accumulation of HIF-1α protein was already observed 1 h after exposing the cells to 0.2% oxygen (Fig. 4C). A similar result was obtained when cells adapted to chronic hypoxia were treated with the PHD inhibitor dimethylfumarate (DMOG), suggesting that PHDs are responsible for HIF-1α regulation even in hypoxically adapted cells (Fig. 4D).

PHD2 and PHD3 Silencing Increases Hypoxic HIF-1α Accumulation—As in HEK293 cells, simultaneous treatment of HeLa cells with hypoxia together with the PHD inhibitor DMOG led to additional accumulation of HIF-1α at oxygen concentrations as low as 0.2% (Fig. 5A). To provide further evidence that endogenous PHDs control HIF-1α protein levels even under hypoxic conditions, we applied siRNA to knock down PHD2 and PHD3 in HeLa cells. Interestingly, silencing of either PHD2 or PHD3 equally increased HIF-1α levels, irrespective of whether the cells were cultured at 20 or 1% oxygen (Fig. 5B). Although apparently both isoforms are involved in regulating HIF-α stability over a broad range of oxygen concentrations, combined silencing of PHD2 together with PHD3 most efficiently up-regulated HIF-1α at 20% as well as 1% O_2 (Fig. 5B).

PHDs Retain Functional HIF-1α Degradation Activity Even under Severely Hypoxic Conditions in Cell Culture—Current knowledge about oxygen substrate requirements of the PHD-dependent hydroxylation reaction is exclusively based on data derived from biochemical in vitro studies using purified enzymes and short peptides containing one single HIF-1α hydroxylation site as substrates. To further examine the activity of PHD enzymes under lowered oxygen availability in a cellular context, a luciferase-based mammalian one-hybrid approach to quantify HIF-1α stability in a feedback-uncoupled cellular system was established. A series of mammalian expression vectors coding for the mouse HIF-1α ODD domain, including mutant forms of the hydroxylation sites (P402A and/or P563A), were N-terminally fused to the yeast GAL4 DNA-binding domain and C-terminally fused to the herpes simplex virus VP16 transactivation domain (Fig. 6A). The expressed fusion proteins cannot transactivate endogenous HIF target genes, including PHD2 and PHD3, but activate a co-transfected GAL4-responsive reporter gene. The use of HIF-1α-deficient MEF-Hif1α−/− cells ensures very low basal levels of endogenous PHDs (Fig. 1A) and avoids confusion with hypoxic up-regulation of endogenous PHD2 and PHD3 by endogenous HIF-1, allowing us to experimentally define the PHD levels by transfection of

not only under normoxic conditions but also under severely hypoxic conditions.

4 h of hypoxia (Fig. 4B). Interestingly, hypoxic PHD3 mRNA induction was steadily induced during the entire hypoxic incubation, whereas PHD2 mRNA levels reached maximal induction already before 4 h of hypoxia and remained constant for up to 72 h of hypoxia.

A similar pattern of HIF-1α and PHD protein levels was observed in HeLa and Hep3B cells at oxygen concentrations as low as 0.2%, supporting the idea of a widespread regulative mechanism (see supplemental Fig. 1). These observations suggest that even under very limited oxygen supply, HIF-1α hydroxylation by PHDs functionally persists, implicating that HIF-1α turnover might be controlled by the PHD sensor system.

FIGURE 2. Effects of tissue hypoxia on the inducibility of PHD isoforms in vivo. Tissues were derived from mice kept either under normal air (F,CO 0%) or in a gas mixture containing 0.1% CO (F,CO 0.1%) for 4 h. PHD1, PHD2, PHD3, erythropoietin, and GLUT1 mRNA levels were determined by real time RT-PCR and normalized to S12 mRNA. Organ-specific hypoxic induction factors are indicated below the bars. Note the different scales (n.d., not detectable).
expression vectors. Because the HIF-1α-ODD confers oxygen-dependent instability to the fusion protein, luciferase reporter gene activity directly reflects protein stability.

First, we tested the relative importance of the two PHD target proline residues within the mouse HIF-1α-ODD residues 359–685. Although reporter gene activation of the wild-type and single proline mutant fusion proteins was similarly reduced by PHD2 overexpression, mutation of both prolines was necessary to render the HIF-1α-ODD insensitive to PHD2 overexpression (Fig. 6B). Although similar effects of PHD overexpression on ODD stability have been reported previously only for normoxic conditions (23), our data show functional relevance of both hydroxylation sites also for hypoxic (2% O2) regulation of HIF-1α protein levels (Fig. 6B).

In the next step, both oxygen concentration and PHD protein abundance were titrated simultaneously in co-transfection experiments. Interestingly, co-expression of low amounts of PHD2, which were not yet detectable by immunoblotting, already reduced HIF-1α-ODD stability to a minimum, which was not further lowered by increased amounts of PHD2 (Fig. 6C). Reducing the oxygen concentration to 4% or below revealed a successive decrease of hydroxylase activity at a given amount of PHD2 enzyme. However, increasing the abundance of PHD2 by stepwise duplication of the amount of transfected PHD2 expression vector again demonstrated that induced PHD levels can compensate for reduced oxygen availability. Indeed, increased PHD2 compensated for oxygen concentrations as low as 0.2% (Fig. 6C).

Similar results were obtained by PHD3 overexpression, which also resulted in decreased HIF-1α-ODD stability under normoxic and moderately hypoxic conditions (Fig. 6D). However, forced expression of PHD3 failed to further decrease HIF-1α-ODD stability at 0.2% oxygen, suggesting a principal differ-
Increased PHD Oxygen Sensors Compensate for Low pO₂

Purified PHDs Retain Functional Hydroxylation Activity Even under Severe Hypoxic Conditions in a Cell-free System in Vitro—The differences in hydroxylase activity of PHD2 and PHD3 under severe hypoxia described above prompted us to investigate the activity of PHDs using a cell-free in vitro approach. Therefore, GST-tagged PHD2 and PHD3 were purified from baculovirus-infected Sf9 insect cells (Fig. 7A). These enzymes were used to hydroxylate a mouse HIF-1α-ODD-derived peptide coupled to 96-well plates, and binding of a purified VBC complex to the hydroxylated peptide was measured by enzyme-linked immunosorbent assay. Each PHD preparation was diluted to obtain equal VBC binding after 1 h of hydroxylation (arbitrarily defined as “1-fold input”). These enzyme concentrations were subsequently increased to mimic the hypoxic induction of PHDs in vivo. Under normoxic conditions, both PHDs showed a similar increase in activity with increasing protein amounts (Fig. 7A). Peptide hydroxylation was within the linear range of the assay over the time frame of experiments, even at highest enzyme concentrations (data not shown).

When the assay was performed at an oxygen concentration of 2%, PHD2 hydroxylation activity was only about half of the normoxic activity after 1 h of reaction (hydroxyproline contents of 10.1 ± 0.2% at 20% O₂ and 5.7 ± 0.5% at 2% O₂; n = 3, mean ± S.D.; 8-fold enzyme input). However, HIF-1α-ODD peptide hydroxylation by PHD3 was only slightly decreased under these conditions (10.3 ± 0.2% at 20% O₂ and 8.2 ± 1.1% at 2% O₂; n = 3, mean ± S.D.; 8-fold enzyme input). Further reduction in oxygen availability decreased proline hydroxylation, but significant hydroxylation could still be observed at oxygen concentrations as low as 0.2%. Indeed, an 8-fold protein input of PHD2 or PHD3 resulted in similar peptide hydroxylation at 0.2% O₂ as a 4-fold input at 0.5% O₂ or a 2-fold input at 1% O₂, suggesting that increased PHDs can compen-

ence in oxygen-dependent hydroxylase activities between PHD2 and PHD3 under various degrees of hypoxia in vivo (Fig. 6D).
sate for decreased oxygen also in vitro (Fig. 7A). Moreover, both enzymes showed a strikingly linear increase of activity for the range of 0.2 to 2% oxygen, thus fulfilling one of the most important criterions for a cellular oxygen sensor at physiologically relevant tissue O2 concentrations (Fig. 7B).

DISCUSSION

The existence of a functional feedback loop limiting the hypoxic response has been suspected already when HIF was cloned, because nuclear HIF-1α/HIF-1β declined despite ongoing hypoxia (24). Similar observations were then reported by a multitude of other groups. However, the underlying mechanism remained unknown. We previously reported that forced expression of HIF-1α followed a similar feedback kinetics, but in this case under normally oxygenated conditions (25). This observation suggested that HIF itself, rather than the hypoxic stimulus, is required for the feedback regulation. One such mechanism might be HIF-dependent induction of pVHL by “late hypoxia” (26). However, other studies could not find any
Increased PHD Oxygen Sensors Compensate for Low \( pO_2 \)

induction of pVHL under hypoxic conditions (27, 28), and we did not observe any oxygen- or HIF-1α-dependent difference in pVHL mRNA levels during the 256-h time course described in this study (data not shown).

The identification of the PHD oxygen sensors shed new light on the process of HIF feedback control, as it became clear that PHD2 and PHD3 are hypoxia-inducible HIF target genes (2, 9–13, 29). It has been postulated that this PHD up-regulation confers accelerated degradation of HIFα upon re-oxygenation (9, 12, 22). Indeed, re-oxygenation from 1 to 20.9% (air) oxygen after 18 h decreased the half-life of HIF-1α when compared with similarly treated cells that were exposed to hypoxia for only 1 h (12).

However, physiological tissue \( pO_2 \) corresponds to ~2–5% oxygen concentrations in air rather than the widely used 20.9% normoxic oxygen concentration. Even though the pericellular \( pO_2 \) is lower at the bottom of unstirred tissue culture dishes (30), standard cell culture conditions are to be considered “hyperoxic,” which reflects an unphysiological tissue condition to which PHDs are not normally exposed. Rather, PHDs \( \text{in vivo} \) are constantly functioning under \( pO_2 \) levels far below their \( \text{in vitro} K_m \) values (14). These considerations suggested a PHD-HIF feedback loop that might be active even under chronically low tissue oxygenation.

In line with this hypothesis, we observed undulating HIF-1α-dependent mRNA levels of CAIX, GLUT1, PHD2, and PHD3 in MEF cells, and we detected tissue-specific variations in hypoxically induced PHD2 and PHD3 mRNA levels in mice \( \text{in vivo} \). So far, PHD expression levels were only known from established tumor cell lines or normoxic tissues (9, 10, 22, 31–33). We found an impressive up-regulation of PHD3 mRNA levels in hypoxic lung tissue, confirming previous reports for lung cancer-derived A549 cells (9, 28). Similarly, the observed induction factors in liver were roughly the same as determined in Hepa1 hepatoma cells used in this study.

We then concentrated on three major organ systems that are particularly sensitive to hypoxia and hence involved in the pathophysiology of shock. Interestingly, although the examined HIF target genes were all induced in the brain, different kinetics for GLUT1, CAIX, PHD2, and PHD3 were observed in kidney and liver. Expression levels of CAIX were highly increased in the kidney after 72 h of hypoxia, whereas a reverse effect was observed in liver. Because the kidney, besides the lung, is the major organ involved in systemic pH regulation, these results suggest a physiological role for CAIX in pH maintenance during hypoxia, as has been proposed for solid tumors (34). Regarding PHD mRNA induction, temporal up-regulation of at least one isoform was observed in each of the three tissues in hypoxic animals, confirming that PHD up-regulation indeed occurs in a hypoxic organism, although the extent of induction is tissue-specific. Not surprisingly, decreasing the oxygen transport capacity in mice by 0.1% CO inspiration was much more efficient in up-regulation of HIF target genes than inspiratory hypoxia itself, suggesting that adaptational mechanisms, such as increased heart rate, depth of breath, and reduced oxygen consumption compensated for decreased inspiratory \( O_2 \) concentration when oxygen transport is intact. We have shown previously that temporal and spatial accumulation of HIF-1α in \( \text{in vivo} \) greatly differs, even in neighboring cells, suggesting that factors other than oxygen availability affect the PHD-HIF system (35). In fact, we recently found that the FK506-binding protein FKBP38 regulates specifically the abundance of PHD2.3

Whatever regulates cellular PHD levels, functional PHD induction requires that these enzymes must be active under a broad range of physiologically relevant conditions. In order to experimentally support this hypothesis, we used a HIF-1α-ODD reporter construct in a HIF-1α negative cell line to breach the suggested gene activation-protein degradation loop. The HIF-1α-ODD confers normoxic degradation to the resulting hybrid fusion protein (36), and similar constructs have been used lately as bioluminescent reporter systems monitoring the state of tissue oxygenation \( \text{in vivo} \) (37). Our experiments revealed that PHD-dependent fusion-protein destabilization was effective at oxygen concentrations as low as 0.2% \( O_2 \) and that increasing the amount of enzyme partially compensated for reduced oxygen availability. These data are consistent with a recent work (38) employing hydroxylation-specific antibodies that showed persistent HIF-1α hydroxylation at equally low oxygen concentrations.

Although we observed a comparable oxygen dependence between PHD2 and PHD3 purified from S9 insect cells \( \text{in vitro} \), the ability of PHD3 to compensate for low oxygen concentrations in the cellular model was consistently lower than that of PHD2 (compare Fig. 6, C and D). In line with the former observation, \( \text{in vitro} \) transcribed and translated PHD2 and PHD3 enzymes have been shown to be equally active in another study (39). Thus, isoform-specific regulation of distinct PHDs \( \text{in vivo} \) is likely to depend on critical co-factors that might be depleted in purified enzyme preparations or become limited when PHDs are overexpressed. Supporting this notion, PHD3 has recently been described to serve as a substrate of the eukaryotic chaperonin complex TRiC that might be required for appropriate folding and full enzymatic activity (40).

Another recent work demonstrated increased binding of PHD3 to HIF-1α in the presence of OS-9 (41). As suggested by these authors, PHDs form functional multiprotein complexes that could affect binding affinity and target sequence selectivity in a cellular context. However, binding of OS-9 to human HIF-1α necessarily requires amino acids 692–785 that were neither present in the peptide used for the hydroxylation assays nor in the one-hybrid construct. Thus, PHD3 activity rather than binding might be attenuated in our cellular model system. Of note, protein degradation of PHD3 (and PHD1) has been shown to be enhanced under hypoxia by a mechanism involving the ubiquitin ligase Siah2, adding yet another layer of regulation on the HIF-PHD system (42).

Also, binding of PHD2 to HIF-1α has been suggested to repress the \( N \)-transactivation domain activity (43). Interestingly, we observed residual binding of recombinant PHD2 to immobilized HIF-1α ODD peptides \( \text{in vitro} \), suggesting a repressive function of PHD2 particularly on truncated HIF-1α (data not shown). Although these effects were rather small compared with the HIF-1α-desaturating function of enzymatic

3 S. Barth, R. Wirthner, R. H. Wenger, and G. Camenisch, manuscript in preparation.
Increased PHD Oxygen Sensors Compensate for Low pO2

PHD activity, they still could have partially influenced the transcriptional activity of the fusion constructs used in our study.

In conclusion, the present work demonstrates that an essential pre-requiement for a PHD abundance-dependent modulation of the oxygen-sensing system is fulfilled; PHDs are operative under a wide variety of even severely hypoxic oxygen concentrations. These findings led to the intriguing suggestion of a flexible oxygen threshold of the PHD-HIF system that steadily adapts to altered tissue oxygenation.


REFERENCES