Nuclear Oxygen Sensing: Induction of Endogenous Prolyl-hydroxylase 2 Activity by Hypoxia and Nitric Oxide

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Utta Berchner-Pfannschmidt†, Suzan Tug‡, Buena Trinidad‡, Felix Oehme§, Hatice Yamac‡, Christoph Woztal‡, Ingo Flamme§, and Joachim Fandrey†,‡

From the †Institut für Physiologie, Universität Duisburg-Essen, D-45122 Essen, Germany and the ‡Institute for Cardiovascular Research, Bayer HealthCare AG, D-42096 Wuppertal, Germany

The abundance of the transcription factor hypoxia-inducible factor is regulated through hydroxylation of its α-subunits by a family of prolyl-hydroxylases (PHD1–3). Enzymatic activity of these PHDs is O2-dependent, which enables PHDs to act as cellular O2 sensor enzymes. Herein we studied endogenous PHD activity that was induced in cells grown under hypoxia or in the presence of nitric oxide. Under such conditions nuclear extracts contained much higher PHD activity than the respective cytoplasmic extracts. Although PHD1–3 were abundant in both compartments, knockdown experiments for each isoenzyme revealed that nuclear PHD activity was only due to PHD2. Maximal PHD2 activity was found between 120 and 210 μM O2. PHD2 activity was strongly decreased below 100 μM O2 with a half-maximum activity at 53 ± 13 μM O2 for the cytosolic and 54 ± 10 μM O2 for nuclear PHD2 matching the physiological O2 concentration within most cells. Our data suggest a role for PHD2 as a decisive oxygen sensor of the hypoxia-inducible factor degradation pathway within the cell nucleus.

The capability of mammalian cells to sense oxygen and adapt gene expression according to O2 availability is critical for the maintenance of oxygen homeostasis within the tissue. The heterodimeric transcriptional regulator hypoxia-inducible factor (HIF) is central to the regulation of gene expression in response to decreased oxygen levels, i.e. hypoxia (1). HIFs are composed of the constitutive β-subunit and one of three O2-labile α-subunits HIF-1, -2, or -3 (2, 3). Both the stability and the activity of the HIF α-subunits are regulated by oxygen-dependent post-translational modifications.

Under normoxic conditions HIFαs are hydroxylated by a family of prolyl-4-hydroxylases (PHD1–3) at two conserved proline residues in HIF-1α and -2α (Pro502/564 or Pro505/531, respectively) or a single proline residue in HIF-3α (Pro490) (4–6). Hydroxylated HIFαs is recognized by the von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase complex and targeted for proteasomal degradation (7–9). Hypoxia reduces prolyl-hydroxylation by PHDs, resulting in stabilization of the α-subunit. This allows dimerization with the β-subunit and induces expression of about 100 genes involved in adaptation to hypoxia (10). Transcriptional activity of the HIF complex is regulated by hydroxylation of an asparaginyl residue within the C-terminal trans-activating domain of HIF-1α and -2α by an asparagyl-hydroxylase termed factor-inhibiting HIF; active factor-inhibiting HIF hydroxylates Asp803 in HIF-1α under normoxia, which impedes binding of the transcriptional coactivator p300/CBP (11, 12). By hydroxylating HIF-α in an oxygen-dependent manner, PHDs and factor-inhibiting HIF function as oxygen-sensing enzymes of HIF activation.

The three PHD isoenzymes belong to the superfamily of iron and 2-oxoglutarate-dependent dioxygenases. These enzymes need O2 as cosubstrate, which provides the molecular basis for their O2-sensing function. Oxygen dependence of PHDs is reflected by their Km(O2) values, which are in the range of 85–250 μM depending on the source of recombinant enzyme, substrate length and assay condition (13–15). In general all three PHDs preferentially hydroxylate Pro504 in HIF-1α and Pro531 in HIF-2α, whereas Pro502 and Pro505 in HIF-1α and HIF-2α are less or in the case of PHD3 are not at all hydroxylated (16–18). Although the availability of O2 serves as a general determinant of PHD activity, the cellular capacity for HIFα hydroxylation by PHDs is also affected by their abundance under the respective conditions. Importantly PHD2 and PHD3 expression, but not that of PHD1, is induced by hypoxia in an HIF-1-dependent manner (18–20). This HIF-1-dependent induction of the cellular O2-sensors generates an auto-regulatory loop controlling HIF-1α stability under hypoxia and reoxyge- 

Differential expression and regulation of PHD isoenzymes may enable fine tuning of hypoxic responses in different tissues and under different conditions. In cultured cells, all three PHDs can regulate HIFαs, but PHD2 was found to be the most active isoform in a number of cell lines. PHD2 has a preference for HIF-1α, whereas PHD1 and PHD3 prefer HIF-2α (25).
Although PHD2 plays a dominant role under normoxia and hypoxia (21, 26), all three PHDs can contribute to PHD activity under prolonged hypoxic stress (27). In addition, all three PHD enzymes exhibit different tissue distribution and distinct patterns of subcellular localization. We reported that overexpressed PHDs fused to green fluorescence protein (GFP) were differentially localized in cells with PHD1 solely found in the nucleus, PHD2 mainly in the cytoplasm, and PHD3 evenly distributed between both compartments (28). Interestingly, endogenous PHDs were mainly located in the cytoplasm (29), but increased PHD2 protein expression in tumor tissues was located in the cell nuclei (30).

Recently we reported that inhibition of PHDs by NO results in the up-regulation of PHD2 and 3 expression because of HIF-1α accumulation in addition to hypoxia (26). Subsequently, hypoxic HIFα induction was reduced by increased PHD levels, suggesting a role for NO as an inducer of PHD activity. Here we highlight the unexpected finding that the O2-dependent activity of endogenous PHD2 is enhanced in cells preincubated under hypoxia or NO. In contrast to the proposed cytosolic localization of PHD2, we observed that nuclear PHD2 activity was higher than cytoplasmic PHD2 activity. Moreover, nuclear PHD2 activity was steeply decreased at O2 concentration below 100 μM (half-maximum activity at 54 μM O2), which matches the physiological range of O2 concentration of most cells. Our data support the notion that PHD2 acts as the prominent oxygen sensor enzyme of the HIF pathway, but it is particularly active in the cell nucleus. We suggest a role for nuclear oxygen sensing via PHD2 under conditions of hypoxia or NO when HIF-1α is present in the nucleus.

**EXPERIMENTAL PROCEDURES**

**Reagents**—S-Nitrosoglutathione (GSNO) was synthesized as described previously (31).

**Cell Culture**—The human osteosarcoma cells (U-2OS), renal clear carcinoma cells (RCC4), embryonal kidney cells (HEK293), and colon carcinoma cells (DLD1) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a normoxic atmosphere of 21% O2, 74% N2, and 5% CO2 (by volume). For cell culture experimented with 10% fetal calf serum, 100 units/ml penicillin, and (HEK293), and colon carcinoma cells (DLD1) were grown in an atmosphere of 1% O2, 74% N2, and 5% CO2 (by volume). For cell culture experimented with 10% fetal calf serum, 100 units/ml penicillin, and (HEK293), and colon carcinoma cells (DLD1) were grown in an atmosphere of 1% O2, 74% N2, and 5% CO2 (by volume).

**Protein Extraction**—Whole cell lysates were prepared from 35-mm dishes of cells that were about 80% confluent. The cells were lysed in 50 μl of extraction buffer (300 mM sodium chloride, 10 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Nonidet P-40, 1× protease inhibitor mixture; Roche Applied Science) for 20 min on ice and centrifuged (3600 × g at 4 °C for 5 min). The supernatant was used as whole cell extract. Cytoplasmic and nuclear extracts were obtained from 60-mm dishes of cells that were about 80% confluent by using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The cell membranes were lysed in 100 μl of ice-cold cytoplasmic extraction reagent I (1× protease inhibitor mixture; Roche Applied Science) for 10 min on ice. 5.5 μl of ice-cold cytoplasmic extraction reagent II was added, incubated for 1 min, and centrifuged (16,000 × g at 4 °C for 10 min). The supernatant was used as cytoplasmic extract fraction. The insoluble pellet that contains nuclei was washed two times with phosphate-buffered saline to remove cytoplasmic remains. The washed pellet was resuspended in 50 μl of ice-cold nuclear extraction reagent (1× protease inhibitor mixture; Roche Applied Science), incubated for 40 min on ice, and centrifuged (16,000 × g at 4 °C for 10 min). The supernatant was used as nuclear extract. Extracts obtained by this procedure generally had less than 10% contamination between nuclear and cytoplasmic fractions. The protein concentration was determined with a commercial protein assay reagent (Bio-Rad).

**Western Blot Analysis**—70 μg of whole cell lysate or 25 μg of cytoplasmic and nuclear extracts were loaded per lane onto a 7.5% or 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes after electrophoresis. The membranes were probed for detection of HIF-1α (or HIF-2α), PHD2, α-tubulin, and Lamin A or PHD3, PHD1, and endoplasmatic reticulum marker Grp78 (glucose-regulated protein 78), and Lamin A, respectively. As primary antibodies the following monoclonal antibodies were used: anti–HIF-1α (diluted 1:750; Transduction Laboratories, San Diego, CA), anti–α-tubulin (diluted 1:750; Santa Cruz Biotechnology, Heidelberg, Germany), anti–Grp78 (diluted 1:250; Abcam, Cambridge, UK), and anti–Lamin A (diluted 1:750; Abcam). The mouse monoclonal anti-PHD3 antibody (diluted 1:20) was a kind gift from P. Ratcliffe (Oxford, UK) and has been characterized previously (25). Rabbit polyclonal antibodies used as primary antibody were anti–HIF-2α (diluted 1:1000; Abcam), anti–PHD2 (diluted 1:3000; Abcam), and anti–PHD1 (diluted 1:1000; Abcam). Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:1000,000 dilution; Sigma) were used as secondary antibodies. The ECL Western blotting system (Amersham Biosciences) was used for detection.

To obtain the relative PHD2 protein amounts, the immunoblot signals were quantified by densitometry. PHD2 protein amounts of whole cell lysates and cytosolic extracts were normalized to the respective β-tubulin signal, whereas PHD2 amounts of nuclear extracts were normalized to the nuclear envelope marker Lamin A.

**PHD Activity Assay**—The enzymatic activity of whole cell lysate or of cytoplasmic and nuclear extracts was determined by an in vitro hydroxylation assay as described before for recombinant PHDs (32). Whole cell lysates, cytosolic, and nuclear extracts were dialyzed using the Slide-A-Lyzer dialysis cassettes (Pierce) to exclude effects on enzyme activity caused by buffer components. Briefly, 10 ng of biotinylated HIF-1α-derived peptides (amino acids 556–574) either wild type (wt), the P564A mutant, or the corresponding HIF-2α-derived peptide (amino acids 523–542, including the target proline 531) were bound to NeutrAvidin–coated goat anti-mouse IgG or goat anti-rabbit IgG (1:1,000,000 dilution; Sigma) were used as secondary antibodies. The ECL Western blotting system (Amersham Biosciences) was used for detection.

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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. Each experiment was calibrated to an internal standard curve using hydroxyproline (Pro564-containing HIF-1α peptide (amino acids 556–574) and VBC complex. The assay allowed the detection of a hydroxylated HIF-1α substrate peptide in a linear concentration range between 1 and 40 nm. For oxygen titration experiments, the hydroxylation reaction was performed in a hypoxic work station (Invtigo2 400; Ruskinn, Leicester, UK). All of the reagents and solutions were allowed to equilibrate to the indicated oxygen concentration. PHD activity is given as the amount of HIFα peptide that was hydroxylated by 1 μg protein of the respective cell extract (fmol/μg). Normalized PHD2 activity was determined by dividing the PHD activity to the relative PHD2 protein amounts obtained by densitometry of immunoblots as described above.

Short Interfering RNA (siRNA) Treatment—For siRNA experiments, the cells were seeded at 30–50% confluence and transfected with double-stranded antisense oligonucleotides specific for PHD2 (sense, 5'-CAAGGUAAGUGGAUGUAUU-3'; antisense, 5'-UAUACCUCACUUACCUGGU-3'; GenBank™ accession number EGLN1 NM_022051), PHD3 (sense, 5'-GUACUUGUGACUGGAAUU-3'; antisense, 5'-UUCUUAGCAUCAAAGACU-3'; GenBank™ accession number EGLN3 NM_022073), or PHD1 (sense, 5'-ACAGAAAGGUCCAGAAU-3'; antisense, 5'-AUCCUGACCUUCCUGUUU-3'). The cells were transfected twice with siRNA directed against PHD2 (10 nm) or PHD3 (25 nm) or PHD1 (2.5 nm) at 24 and 48 h using Oligofectamine (Invitrogen) according to the manufacturer's instructions. siRNA against Luciferase was used as a nontargeting control, and mock control cells were transfected without oligonucleotides under the same conditions. After transfection the cells were grown for 24 h and then exposed to normoxic or hypoxic atmosphere with or without GSNO for the indicated time. The cells were lysed and whole cell lysates or cytosolic and nuclear extracts were obtained as described above.

Immunofluorescence and Microscopy—The cells were grown on poly-d-lysine-coated glass coverslips in 24-well dishes overnight. Subconfluent cells were subjected to hypoxia for 6 h, fixed by ice-cold methanol/acetic acid (1:1) for 10 min on ice, and blocked with 3% bovine serum albumin in phosphate-buffered saline. As primary antibody the rabbit polyclonal anti-PHD2 (diluted 1:200; Abcam) was used, and as secondary antibody an Alexa-532-conjugated goat anti-mouse IgG (1:400; Molecular Probes) antibody was used. The coverslips were mounted on the slides with Mowiol (Calbiochem, Bad Soden, Germany). Three-dimensional visualization was performed with laser scanning microscope (PCM2000; Nikon) equipped with a two photon Coherent Mira 900M laser as described before (34, 35). Yellow fluorescence of Alexa-532 was collected through a 585-nm long pass filter. The objective lens was a 63× NA 1.40 Plan-Apochromat. Fluorescence intensities were visualized in false colors as indicated.

**RESULTS**

Induction of Endogenous PHD Activity under Hypoxia or NO Is Specifically Caused by PHD2—Incubation of U-2OS cells under hypoxia or NO lead to induction of PHD2 protein levels (1.8-, 2.0-, and 2.2-fold compared with controls; Fig. 1A). We have previously shown that this induction of PHD2 protein accumulation is a result of HIF-1α-dependent gene expression of PHD2 in response to hypoxia and NO (26). In consequence, HIF-1α was reduced by increased PHD2 under hypoxia and NO compared with hypoxia alone (Fig. 1A). To test for PHD activity

![Graph](https://via.placeholder.com/150)

**FIGURE 1. Cellular PHD2 activity is induced by hypoxia or NO.** U-2OS cells were incubated in the presence (+) or absence (−) of 250 μM GSNO under NOx or under HOX conditions for 6 h to induce PHD2 protein levels. A, whole cell lysates were analyzed for the expression of HIF-1α and PHD2 by Western blot analysis. α-Tubulin (α-Tub) was used as a loading control. The numbers indicate fold induction of PHD2 protein amounts compared with normoxic cells. Immunoblots shown are representative for at least three separate experiments. B, whole cell lysates were subjected to an in vitro PHD activity assay using the wt HIF-1α(556–574) peptide or the mutated (mut) HIF-1α(556–574) peptide containing alanine instead of the target proline 564 in the presence of 210 μM O₂. The data are means of three independent experiments. The error bars represent standard deviation. The numbers above the bars indicate fold induction compared with PHD2 activity of normoxic cells. C–E, PHD2, 3, or 1 were suppressed by transfecting U-2OS cells with the respective specific siRNA duplexes or treated with transfection reagent alone (Mock). The cells were then incubated under NOX, HOX, with or without GSNO, respectively as described above and subjected to **in vitro** PHD activity assay and Western blot analysis. The data are shown as representative of two separate experiments. F, normalized PHD2 activity was determined by dividing the PHD activity to the PHD2 protein amounts quantified by densitometry of immunoblots. Normalized PHD2 activity is given as fold induction compared with PHD2 activity of normoxic cells. The data are the means of three independent experiments. The error bars represent standard deviation.
under these conditions, a PHD activity assay (32) was established for cell extracts using a HIF-1α(556–574) peptide containing the target proline 564 as substrate. As shown in Fig. 1B, PHD activity was low in normoxic cells but induced in hypoxic cells (2.6-fold induction) or cells that were treated with NO both under normoxia (3.6-fold induction) or hypoxia (5.8-fold induction). Because this PHD activity assay was not fully established for cell extracts, we tested a P564A mutated HIF-1α peptide to show that PHD activity measured in cell extracts was completely dependent on the presence of the target proline (Fig. 1B, mut).

The induction of PHD activity in response to hypoxia or NO closely correlated with the induction of the respective PHD2 protein amounts (Fig. 1A). To verify that these changes were responsible for the increase in PHD activity, PHD2 expression was suppressed by specific siRNA. siRNA against PHD2 completely abolished the PHD activity in extracts from cells incubated under normoxia, hypoxia, or NO (Fig. 1C). It has previously been shown that the HIF-1α peptide used in our PHD activity assay can be hydroxylated by all recombinant PHD isoenzymes in an O2-dependent manner (13–15). We therefore tested whether endogenous PHD3 or PHD1 contributed to the overall PHD activity of U-2OS cells by silencing PHD3 or PHD1 expression using siRNA. Although PHD3 protein levels were induced by hypoxia or NO treatment, the suppression of PHD3 had no impact on PHD activity (Fig. 1D). Furthermore PHD1 protein levels were neither affected by hypoxia nor NO, and the suppression of PHD1 had no major effect on total PHD activity (Fig. 1E). U-2OS cells were found to hardly express HIF-2α (supplemental Fig. S1). Nevertheless, we wondered whether endogenous PHDs would hydroxylate a HIF-2α peptide in our PHD activity assay. Suppression of PHD2 by siRNA completely abolishes PHD activity, whereas suppression of PHD3 or PHD1 had no impact on hydroxylation of HIF-2α peptide (supplemental Fig. S2). In summary, the data indicate that PHD2 is the key oxygen sensor enzyme for HIFα regulation in U-2OS cells.

Because only PHD2 was responsible for total PHD activity, we further analyzed PHD2 activity by normalizing PHD activity to PHD2 protein amounts. Normalized PHD2 activity was induced by hypoxia (1.5-fold) and NO (2.0-fold under normoxia, 2.6-fold under hypoxia) when compared with normoxic cells (Fig. 1F). Taken together, hypoxia and NO induce PHD activity by induction of PHD2 protein. The additional induction of PHD2 activity observed after normalization indicate that the induction of activity cannot be explained by changes in protein amounts alone and suggest the action of an additional mechanism.

PHD2 Activity Is Prominently Induced in Cell Nuclei—To test for the subcellular distribution of PHD2 in U-2OS cells, we performed immunostaining and fluorescence microscopy. We found that PHD2 was more present in the nucleus than in the cytoplasm under normoxia, hypoxia, or NO (Fig. 2A). To further analyze whether the nuclear PHD2 contributes to the cellular PHD activity, we isolated cytoplasmic and nuclear extracts. Western blot analysis of these extracts confirmed that PHD2 was more abundant in the nucleus than in the cytoplasm under normoxia as well as after induction by hypoxia or NO (1.4–2.4-fold induction in the nuclear fractions compared with 1.0–1.7-fold induction in cytosolic fractions; see Fig. 2B). Likewise PHD3 was found in both compartments with increased protein levels upon hypoxia and NO treatment. PHD1 was localized mainly in the nucleus and was affected neither by hypoxia nor by NO (Fig. 2B). HIF-1α was not detectable under normoxic conditions but strongly accumulated in cell nuclei under hypoxia; this hypoxic induction was reduced by after NO treatment (Fig. 2B), corroborating previous data (26).
O2-dependent Regulation of Nuclear PHD2 Activity

Cytosolic and nuclear extracts were of high purity, as indicated by detection of the cytoplasmic marker protein α-tubulin and the endoplasmic reticulum marker Grp78 in cytoplasmic fractions, whereas the nuclear envelope marker protein Lamin A was detectable only in nuclear fractions (Fig. 2B). Both cytoplasmic and nuclear extracts contained PHD activity, which was inducible both by hypoxia and NO (Fig. 2C). The induction of activity coincided with increased levels of PHD2 protein in the respective extracts (Fig. 2B). Remarkably, nuclear PHD activity was considerably higher than cytoplasmic PHD activity under all of the conditions tested (Fig. 2C).

To test whether PHD2 was responsible for PHD activity in subcellular compartments, PHD2 gene expression was silenced by siRNA (Fig. 2D). For maximal induction of endogenous PHD2, the cells were treated with GSNO under hypoxia for 6 h. Silencing of PHD2 completely abolished the total PHD activity in both cytoplasmic and nuclear extracts (Fig. 2D) and induced HIF-1α accumulation in cell nuclei (Fig. 2E). In addition, the specificity of proline 564 hydroxylation was verified for the cytoplasmic and nuclear extracts by using the mutated HIF-1α peptide (P564A) as a substrate (compare Fig. 1), which resulted in no measurable PHD activity (Fig. 2D). Because PHD2 was solely responsible for total PHD activity, we normalized PHD activity to PHD2 protein amounts. As shown in Fig. 2F, normalized PHD2 activity was induced by hypoxia and NO in both the cytoplasmic and the nuclear compartment; however, normalized PHD2 activity was higher in nucleus than in the cytoplasmic fractions (Fig. 2F). In summary, our data indicate that significant induction of PHD2 activity by hypoxia and NO occurs in the nucleus.

Because our data on the subcellular distribution of PHD2 activity were so far obtained with U-2OS cells, we tested whether the studied subcellular localization of PHD2 and the subcellular distribution of endogenous PHD activity can be extended to other cell types. To this end different cell lines were incubated in the presence of NO under hypoxia to maximally induce HIF-1α-dependent expression of PHDs. Nuclear extracts of all cell types tested exhibited considerably higher PHD activity than the respective cytoplasmic extracts, albeit by siRNA confirmed that PHD2 largely contributes to the nuclear PHD activity in DLD-1 cells (Fig. 3C). Collectively, although the majority of PHD2 activity is found in the nuclei of all cells tested, the ratio of nuclear/cytoplasmic PHD2 activity differs from cell to cell.

Nuclear PHD2 Activity Is Tightly Regulated below 100 μM O2 Concentration—To further analyze the oxygen-dependent regulation of PHD2 activity, cytosolic and nuclear extracts obtained under conditions of normoxia, hypoxia, or NO were subjected to a PHD activity assay carried out under different oxygen concentrations (10, 30, 50, 80, 120, and 210 μM O2).

Cytosolic and nuclear PHD activity displayed a maximum in the range between 120 and 210 μM O2. However, PHD activity decreased in O2 concentrations below 120 μM O2 (Fig. 4). Strikingly, the main reduction of PHD activity occurred between 80 and 30 μM O2 independently of growth conditions or intracellular localization (Fig. 4). However, the nuclear extracts contained higher PHD activity than the cytoplasmic fractions under all of the oxygen concentrations tested (Fig. 4). Furthermore, PHD activity was higher in extracts obtained from cells incubated under hypoxia or NO than in extracts obtained from cells incubated under normoxia alone even under reduced oxygen concentrations. Of note, nuclear and cytoplasmic extracts from cells incubated with NO under hypoxia still showed substantial PHD activity in the presence of 10 μM O2, whereas PHD activity in extracts from normoxic or hypoxic cells was below the detection limit (Fig. 4, compare D with A–C). This confirms our previous result that NO can induce PHD activity even under low O2 concentrations (26).

PHD activity was affected by intracellular localization or treatment (hypoxia (HOX) or normoxia (NOX)) or NO in the presence of a given O2 concentration in the following order: PHD activityNuc > PHD activityCytoplasm > PHD activityHOX | NO > PHD activityNOX | HOX > PHD activityHOX | NO, O2-dependent regulation of the endogenous PHD activity occurred mainly between 80 and 30 μM O2, which was reflected by the O2 concentration of half-maximum PHD activity (cO2 50%; Table 1). In tendency, nuclear extracts obtained from normoxic cells and cytosolic extracts from cells with different ratios (Fig. 3A). Levels of nuclear PHD activity varied between the cell lines; DLD-1 colon carcinoma cells and RCC4/wt renal clear carcinoma cells deficient for pVHL exhibit much higher nuclear PHD activity than RCC4/vhl (pVHL reconstituted) cells or HEK cells (Fig. 3A). Subcellular localization of endogenous PHD2 protein was determined in immunostained cells by fluorescence microscopy, which revealed different ratios of nuclear and cytoplasmic PHD2 for the cells studied (Fig. 3B). Notably, DLD-1 cells showed a strong nuclear PHD2 staining that coincided with a high nuclear and a very low cytoplasmic PHD activity. Suppression of PHD2

FIGURE 3. Subcellular distribution of PHD2 activity is cell-specific. Different cell lines (a colon carcinoma cell line (DLD-1), a renal clear carcinoma deficient for vhl (RCC4/wt) or vhl reintroduced in RCC4 cells (RCC4/vhl) and a HEK cell line) were incubated in the presence of 250 μM GSNO under hypoxia for 6 h. A, cytoplasmic and nuclear extracts were subjected to PHD activity assay using the HIF-1α(556–574) peptide in the presence of 210 μM O2. PHD activity assays shown are representative for at least two separate experiments. B, cells were immunostained and imaged by fluorescence microscopy to show subcellular localization of PHD2 in the different cell types. C, DLD-1 cells were transfected with PHD2 siRNA or treated with transfection reagent only (Mock) and incubated under hypoxia and 250 μM GSNO for 6 h. PHD activity of the respective cytoplasmic or nuclear extracts was measured in the presence of 210 μM O2, as described above. The PHD activity assay shown is representative for at least three independent experiments.
O$_2$-dependent Regulation of Nuclear PHD2 Activity

![Graphs](image)

FIGURE 4. Nuclear PHD2 activity is tightly regulated by oxygen concentration. Cytoplasmic and nuclear extracts were obtained from U-2OS cells grown under NOX (A), normoxia and GSNO (NOX + NO) (B), HOs (C), or hypoxia and GSNO (HOS + NO) (D) for 6 h and subjected to PHD activity assay using the HIF-1α (556–574) peptide as substrate. PHD activity assays were carried out under different oxygen concentrations (cO$_2$: 10, 30, 50, 80, 120, and 210 μM). The range of cO$_2$ in which strongest regulation of PHD activity occurred (30–80 μM O$_2$) is highlighted in gray. O$_2$ dependences of nuclear and cytoplasmic PHD activities shown are representative for at least two independent experiments each. Regression analysis was performed by a sigmoid function (five parameters) with SigmaPlot 2001 (SYSTAT Software Inc.). The coefficients of determination were generally $R^2 > 0.99$ except $R^2 > 0.98$ for NOX + NO, cytoplasm.

**TABLE 1**

| Compartment | cO$_2$ 50%* | Normoxia | Normoxia + NO | Hypoxia | Hypoxia + NO | Mean* |
|-------------|-------------|----------|---------------|---------|-------------|-------|
| Cytoplasm   | ND          | 72 ± 16  | 46 ± 3.9      | 41 ± 9.8| 53 ± 13     |
| Nuclei      | 71 ± 11     | 52 ± 0.0 | 45 ± 5.8      | 49 ± 10 | 54 ± 10     |

* O$_2$ concentration required for half-maximum PHD activity was determined by taking the O$_2$-dependent activities of the PHDs given in Fig. 4. The data are the means ± S.D. of at least two independent experiments.

* The values were calculated for PHD activities at all treatments because the differences were not statistically significant (analysis of variance two-tailed t test).

incubated under normoxia and NO exhibit slightly higher values for half-maximum PHD activity (71 ± 11 and 72 ± 16 μM O$_2$, respectively) when compared with PHD activity of all other extracts ranging from 41 ± 9.8 (HOS + NO) to 52 ± 0.0 (NOX + NO) μM O$_2$. Because these differences in cO$_2$ 50% values of PHD activity between the extracts were not statistically significant (tested by analysis of variance two-tailed t test), we calculated the mean half-maximum PHD activity for cytosolic extracts at 53 ± 13 μM O$_2$ and for the nuclear extracts at 54 ± 10 μM O$_2$ (Table 1). Fig. 5 illustrates that the cytosolic and the nuclear PHD2 activity obtained under all growth conditions displayed strong regulation below 100 μM O$_2$ which correlates with the frequency distribution of O$_2$ within normoxic tissues (36). In conclusion, nuclear PHD2 activity just like cytosolic PHD2 activity is tightly regulated by the O$_2$ concentration within the physiological range corroborating its role as the important oxygen sensor in the HIF-1α degradation pathway.

**DISCUSSION**

We recently reported that NO is a strong stimulus for HIF-1-dependent PHD2 and 3 expression. PHD2 induced by NO leads to reduced accumulation of HIF-1α in response to hypoxia (26). During the course of that study we noticed that the induction of endogenous PHD2 protein was most prominent in the nucleus. However, we were not able to specifically determine PHD activity with respect to subcellular distribution, taking into consideration that PHDs showed a specific distribution pattern within the cell (28). So far, most data on PHD activity were obtained with purified recombinant enzymes (13–15, 37). This is the first study where endogenous PHD activity of cells grown under normoxia, hypoxia, or after induction by NO was studied, and the contribution of each isoenzyme was determined. Furthermore, we analyzed subcellular distribution of PHD activity and its O$_2$-dependent regulation.

**Hypoxia or NO Is an Inducer of Cellular PHD2 Activity**—Previous studies have shown that protein levels of PHD2 can be induced by hypoxia and/or NO in a HIF-1-dependent manner (22, 26). Whereas induction of PHDs by hypoxia may form a functionally important negative feedback loop (22, 24), our previous results indicated that increased PHD activity in cells incubated with NO affected cellular O$_2$ sensing. Although PHD activity of normoxic cells was low, the induction of PHD activity by hypoxia or NO correlated well with the enhanced PHD2 expression under these conditions (Fig. 1). However, in addition to an increased amount of enzyme, another mechanism appeared to enhance PHD activity, which became evident when PHD activity was normalized to PHD2 protein levels (Fig. 1F). One likely explanation is that newly synthesized PHD2 complexes differ in their activity when built under hypoxia or NO treatment, e.g. caused by post-translational modifications. Alternatively, different interaction partners present in the cell extracts could affect its activity. In this respect it is of note that we previously observed a transient inhibition of PHDs by NO and subsequently a transient HIF-1α accumulation (26). The finding that NO induction of PHD2 generates enzymes with increased activity now suggests that newly formed PHDs are...
PHDs may have other substrates than HIFαs. This has been demonstrated for PHD1, which regulates the transcriptional activity of NFκB by hydroxylating IKKβ (38). However, the fact that substrate specificity was not observed for recombinant but for endogenous PHDs strongly suggests that cellular cofactors and/or the cellular environment is an important regulator of PHD activity.

**PHD2 Activity Is Cell Specifically Distributed within Cytoplasm and Nuclei**—One such factor could be the subcellular localization of the enzymes and their substrates. We previously observed that GFP fusions of PHD1–3 showed a typical distribution pattern in U-2OS cells; PHD2 was mainly localized in the cytoplasm, whereas PHD1 was found in the nucleus, and PHD3 was evenly distributed in both compartments (28). The GFP fusion proteins retained HIF-hydroxylase activity *in vitro* and were able to initiate degradation of endogenous HIF-1α independently of their different subcellular localization. Fractionation of U-2OS cells herein to determine subcellular distribution of endogenous PHD activity fully confirmed the pattern of PHD3 and PHD1 distribution (Fig. 2B). In contrast endogenous PHD2 exhibited preferential nuclear localization, which was not observed with the GFP fusion proteins (Fig. 2, A and B). Whereas PHD2 was shown to be mainly located in the cytoplasm of various normal tissues (29), increased nuclear PHD2 expression was observed in less differentiated phenotypes of cancer cells (30). A strong association of nuclear staining with higher grade tumors was observed, and most of the tumor regions with high PHD2 expression showed down-regulated HIF-1α (30). Similar to those observations, we found that increased nuclear PHD2 levels under hypoxia plus NO lead to a reduced nuclear HIF-1α accumulation compared with hypoxia alone (Fig. 2B), implying that hydroxylation of HIF-1α occurs in the nucleus. Determination of PHD2 activity confirmed nuclear PHD activity, which was higher than in the cytoplasmic compartment (Fig. 2, B–D). As already mentioned above, mechanisms other than the enzyme abundance appeared to enhance PHD activity (Fig. 1E). This holds true for nuclear PHD activity, which was higher when normalized to PHD2 protein levels than under control conditions. Higher normalized PHD2 activity may be due to alternative complex formation of PHD2 in the different compartments and under different conditions. Jokilehto et al. (30) observed a faster movement of PHD2-EGFP in the nucleus than in the cytoplasm, implying differential complex formation of PHD2. Furthermore Nakayama et al. (39) showed that at least for PHD3, lower mass complexes exhibit a higher specificity toward HIF-1α hydroxylation. It is tempting to speculate that lower mass complexes of PHD2 are responsible for the induced PHD2 activity in cell nuclei. Although heterodimeric higher mass complexes of PHD3 with PHD2 were correlated with a decrease in PHD activity (39), our PHD knockdown data exclude the possibility that interaction between PHD2 and PHD3 plays a significant role for PHD activity in U-2OS cells.

Our experiments to extend these finding to other cell lines indicate that different cell types contain different ratios of cytoplasmic to nuclear PHD activity (Fig. 3). The cell-specific subcellular distribution of HIF-hydroxylation capacity will be relevant for the efficiency of O2-dependent HIF degradation of the
**O$_2$-dependent Regulation of Nuclear PHD2 Activity**

In the investigated U-2OS osteosarcoma cell line and the DLD-1 colon carcinoma cell line, nuclear PHD2 activity was considerably higher than cytosolic activity (Figs. 2D and 3C), suggesting that nuclear PHD2 accounts for hydroxylation of HIF-1$\alpha$. In such cells PHD2 level and activity of the respective compartment will determine the HIF-1$\alpha$-pVHL binding and consequently will affect the efficiency of pVHL-dependent nuclear-cytoplasmic shuttling (40) as well as the pVHL-dependent ubiquitination and degradation by the proteasome (41). This is again in full agreement with the finding that compartment-specific degradation of HIF-1$\alpha$ is regulated in a cell-specific manner and depends on the content and activity of the components of the degradation pathway, e.g. the proteasome (42, 43).

_A Role for PHD2 as Nuclear Oxygen Sensor of HIF-1$\alpha$—_Finally we investigated O$_2$ dependence of cytosolic and nuclear PHD2 activity. Oxygen concentrations within the tissue are in the range from 30 to 100 $\mu$M, whereas measurements performed close to normoxic monolayer in culture revealed values from 0 to $\sim$170 $\mu$M O$_2$, depending on cell type (44). Furthermore, frequency distribution of O$_2$ partial pressures varied between normal breast or fibrocystic disease (median pO$_2$, 65 mm Hg $\sim$ 100 $\mu$M O$_2$) and breast cancers (median pO$_2$, 30 mm Hg $\sim$ 50 $\mu$M O$_2$) (45). We found that the PHD2 activities of both cytoplasm and nucleus was maximally in a range of 120–210 $\mu$M O$_2$. Below 120 $\mu$M O$_2$ PHD activity was reduced with a steep decrease in the activity curve between 30 and 80 $\mu$M O$_2$ (Figs. 4 and 5). The total PHD2 activity at any given O$_2$ concentration was dependent both on the treatment conditions (with higher PHD2 activity in cells treated with hypoxia or NO than under normoxia) and on the subcellular localization (with more PHD2 activity in the nucleus than in the cytoplasm; Fig. 4). Although this may be predicted because of respective amounts of PHD2 protein, our data clearly provide evidence for fully active enzymes functioning as O$_2$ sensors at different levels, depending on the circumstances. In contrast, the O$_2$-dependent regulation of PHD2 activity appeared to be independent of PHD2 protein levels, which is reflected by similar values of half-maximum PHD2 activity occurring between 41 ± 9.8 and 72 ± 16 $\mu$M O$_2$ (Table 1). This is in agreement with earlier reports on $K_{m,app.}$ (O$_2$) values of recombinant PHD2, which were in the range of 85–110 $\mu$M O$_2$ depending on the respective assay method and substrate length (14, 15). The strong O$_2$-dependent regulation below 100 $\mu$M O$_2$ shown for the cytosolic and nuclear PHD2 activity (with a mean half-maximum activity of nuclear PHD2 at 54 (±10) $\mu$M O$_2$ and for cytosolic PHD2 at 53 (±13) $\mu$M O$_2$) coincides with the physiological frequency distribution of O$_2$ concentrations in the tissue (36). Thus, O$_2$-dependent regulation of the cytosolic as well as the nuclear PHD2 activity perfectly matches the physiological concentrations of O$_2$ to which most cells are exposed (Fig. 5).

In summary, our data support the notion that PHD2 acts as the prominent oxygen sensor of the HIF pathway and is particularly active in the cell nucleus. Moreover, we show that the O$_2$ sensor system can be differentially induced in different cellular compartments to allow fine-tuning of the hypoxic response. Fig. 6 illustrates our hypothesis of how nuclear PHD2 activity contributes to oxygen sensing of the HIF$\alpha$ degradation pathway. Increased nuclear PHD2 activity becomes important especially under conditions of nuclear located HIF$\alpha$ (hypoxia, NO). Under such conditions HIF-dependent transcription and O$_2$-dependent regulation of its $\alpha$-subunit via PHD2 are coexistent processes that can affect each other. A, under normoxic conditions HIF$\alpha$ is not abundant (white-colored HIF$\alpha$) because synthesized HIF$\alpha$ is immediately hydroxylated by cytosolic or nuclear PHD2 and subjected to proteosomal degradation via pVHL. In consequence HIF-dependent gene expression is abolished, and therefore PHD2 levels and total PHD2 activity in compartments are low. With respect to the normoxic oxygen concentration PHD activity (half-maximum PHD2 activity O$_2$: 54 $\mu$M O$_2$) becomes maximal between 120 to 210 $\mu$M O$_2$. B, under hypoxic conditions or in the presence of a transient PHD inhibitor like NO, PHD2 activity (half-maximum PHD2 activity O$_2$: 54 $\mu$M O$_2$) of yet low PHD2 levels is down-regulated or inhibited, which leads to detectable HIF$\alpha$ accumulation (gray-colored HIF$\alpha$) in the nucleus. After HIF$\alpha$/$\beta$ dimerization, HIF activity leads to up-regulation of PHD2 gene expression. Enhanced PHD2 levels in the nucleus can compensate for low oxygen concentrations, and nuclear located HIF$\alpha$ is hydroxylated. Hydroxylated HIF$\alpha$ is subjected to pVHL-dependent proteosomal degradation in the nucleus or is transported via pVHL-dependent shuttle into the cytoplasm to be degraded. HIF$\alpha$, which remained in the cytoplasm (mostly not detectable; white-colored HIF$\alpha$) can be hydroxylated by increased cytosolic PHD2 levels.
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