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Hypoxia Up-regulates Prolyl Hydroxylase Activity

A FEEDBACK MECHANISM THAT LIMITS HIF-1 RESPONSES DURING REOXYGENATION*

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The mechanism by which hypoxia induces gene transcription is now well established. Hypoxia reduces activity of prolyl hydroxylases (PHD) that hydroxylate specific proline residues in the oxygen-dependent degradation domain (ODD) of hypoxia-inducible factor-1 α (HIF-1 α). As a consequence, HIF-1 α accumulates and promotes hypoxic tolerance by activating gene transcription. This paper identifies the three forms of PHDs in rats and shows that a period of hypoxia selectively increases expression of PHD-2 mRNAs levels. We developed assays for PHD activity that used (i) the peptide-specific conversion of labeled 2-oxoglutarate into succinate and (ii) the binding of the von Hippel-Lindau protein to a glutathione S-transferase-ODD fusion protein. The two assays indicated a low enzymatic activity in normoxic and hypoxic cells and a rapid increase during reoxygenation. We also developed hydroxyproline-specific antibodies that recognized hydroxylated forms of a fusion protein (ODD-green fluorescent protein) that combined the ODD domain of HIF-1 α and the green fluorescent protein. Using this antibody, we demonstrated that reoxygenation induced a rapid hydroxylation of Pro-564, which was followed by a massive degradation of the proteins. The results suggest that a hypoxic up-regulation of PHD (presumably PHD-2) acts as a feedback mechanism to stop hypoxic responses in reoxygenated cells. We propose that proline hydroxylation might play a role in hypoxic preconditioning.

hypoxia-responsive element in the 3'-untranslated region of the erythropoietin gene (1). HIF-1 is composed of two subunits, HIF-1 α and the aryl hydrocarbon nuclear translocator, both of which belong to the large family of basic helix-loop-helix-*per-arrnt-sim* transcription factors (2). The mechanism of the hypoxic induction of HIF-1 α has recently been identified. Under normoxic conditions, specific HIF-1 α prolyl hydroxylases (PHD) hydroxylate two proline residues (Pro-402 and Pro-564) in the oxygen-dependent degradation (ODD) domain of HIF-1 α (3, 4). The von Hippel-Lindau protein (vHL) E3 ubiquitin ligase complex associates to hydroxylated proline residues and targets HIF-1 α to proteasomal degradation. Under hypoxic conditions, oxygen becomes rate-limiting for proline hydroxylation. As a consequence, HIF-1 α accumulates, migrates to the nucleus, and associates with the aryl hydrocarbon nuclear translocator and the complex interacts with hypoxia-responsive element of target genes (5, 6).

Less attention has been given to the situation in which hypoxic cells are reoxygenated. Tissue reoxygenation is not an unusual condition. It is frequently observed in pathological situations. For instance, newly formed blood vessels in tumors tend to be highly irregular and malformed. As a consequence, tumor cells often face transient changes in oxygen tension because of the irregular opening and closing of malformed vessels. Reoxygenation of cardiac or brain tissues after a period of ischemia has been extensively documented. Reperfusion has both deleterious and beneficial aspects. Reoxygenation is a major cause of cell damage via the production of reactive oxygen species. A short period of hypoxia followed by tissue reoxygenation is well known to protect the heart and the brain against further hypoxia. Two mechanisms have been proposed to account for hypoxic preconditioning. First, opening of K_{ATP} channels reduces cellular excitability and saves energy stores (7). Second, adenosine is released and exerts its protective effect via A1 receptor-mediated activation of protein kinase C (8). Hypoxic preconditioning is a major therapeutic and pharmacological target. Therefore, we examined the regulation of HIF-1 α during reoxygenation. For this purpose, we developed different biochemical assays for PHDs and antibodies directed against hydroxylated prolines to analyze protein prolyl hydroxylation during hypoxia and reoxygenation. Our results document a dramatic increase of PHD activity in freshly reoxygenated cells that probably serves to stop hypoxic signaling. They also provide direct evidence that hydroxylation of Pro-564 in the ODD domain of HIF-1 α controls the degradation of ODD-containing proteins during reoxygenation.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were from Promega. Culture medium and fetal calf serum were from Invitrogen. All of the chemicals were purchased from Sigma. [5-¹⁴C]2-OG and L-[³⁵S]methionine were purchased from Amersham Biosciences. [α -³²P]dCTP was from ICN.

Cells respond to reduced oxygen tensions by up-regulating the expression of genes involved in angiogenesis (e.g. vascular endothelial growth factor), erythropoiesis (e.g. erythropoietin), and glycolysis. The transcriptional activation of target genes is induced by a common transcription factor, hypoxia inducible factor-1 (HIF-1).¹ HIF-1 was first identified as a heterodimeric transactivator that recognizes a specific DNA sequence termed

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY229997 and AY228140.

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¹ The abbreviations used are: HIF, hypoxia-inducible factor; 2-OG, 2-oxoglutarate; GFP, green fluorescent protein; GST, glutathione S-transferase; ODD, oxygen-dependent degradation; PHD, prolyl hydroxylase; vHL, von Hippel-Lindau protein; PBS, phosphate-buffered saline.

Cell Lines and Culture Conditions—Rat C6 glioma cells were obtained from the American Type Culture Collection. Stable ODD-GFP transfectants were prepared as follows. A 222-bp DNA fragment corresponding to amino acids 530–603 (nucleotides 1616–1837) of human HIF-1 α (pcDNA3-HIF-1 α vector kindly provided by Dr. P. J. Ratcliffe) was PCR-amplified using the sense primer 5'-CCGCTCGAGGCCACC-ATGGAATCAAGTTGGAATTGGTA-3' and the reverse primer 5'-GCTCTAGACTGGAATACTGTAAC-5'. The product was digested by *Xho*I and *Xba*I and fused in-frame with the enhanced GFP of a *Xho*I/*Xba*I-digested pCI-eGFP2 plasmid. The product was digested by *Bgl*II and *Not*I and inserted into the *Bgl*II/*Not*I-digested pCDNA 3.1(+) vector (Invitrogen). C6 cells were transfected using a mixture of 10 μ g of plasmid DNA and 10 μ g of LipofectAMINE for 5 h at 37 °C. Stable transfectants were selected using 1.5 mg/ml G418 and cloned by limited dilution. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in 5% CO₂, 95% air in a humidified incubator. Hypoxic incubations (16 h at 2% O₂) were performed in a sealed "Bug-Box" anaerobic workstation (Ruskin Technologies).

Reverse Transcription-PCR and Northern Blot Analysis—Total RNA from normoxic and hypoxic C6 cells was isolated by the acid/phenol method of Chomczynski and Sacchi (9). RNA (2 μ g) was reverse-transcribed using the first strand cDNA synthesis kit (Amersham Biosciences). The following forward and reverse sets of primers were used. For PHD-1 (GenBank™ accession number NM_053208) (forward 5'-C-ACCATGGACAGCCCGTGCCAG-3' and reverse 5'-GGTAGGTGTAGT-TGGCTGTGATACTGGTACTTG-3'), for PHD-2 (GenBank™ accession number XM_134453) (forward 5'-ACCATGAACAAGCAGCGCATCTG-C-3' and reverse 5'-GACGCTTTGCTGACTGAATTGGGCTT-3'), and for PHD-3 (GenBank™ accession number NM_019371), forward 5'-G-CTTCTGTACTCTGGACAACTT-3' and reverse 5'-CGGCATCAAAAT-ACCAGACAGT-3'. PCR amplifications were carried out using 10 mM dNTP and 3 units of *Pfu* DNA polymerase (Promega). For PHD-1 and PHD-2, the PCR products were subcloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). PHD-1, PHD-2, and PHD-3 products were sequenced using the dideoxy terminator method.

For Northern blot analysis, samples of total RNA (10 μ g) were fractionated by denaturing electrophoresis through 1% agarose gel, capillary-blotted to charged nylon membrane (Zeta-Probe GT membrane, Bio-Rad) in 20 \times SSC buffer, and covalently linked by ultraviolet irradiation. Membranes were prehybridized at 65 °C for 1 h in 0.25 M sodium phosphate (pH 7.2), 7% SDS. Hybridization was performed in the same buffer containing 10⁶ cpm/ml α -³²P-labeled PCR-purified fragments for rat PHD-1, PHD-2, and PHD-3 probes (specific activity >10⁹ cpm/ μ g DNA). Membranes were washed twice in 25 mM sodium phosphate (pH 7.2), 5% SDS and once in 25 mM sodium phosphate (pH 7.2), 1% SDS for 30 min at 65 °C (each wash). Hybridization signals were quantitated using a PhosphorImager. α -³²P-labeled PCR-purified fragments and 18 S cDNA probe were prepared by using the Prime-a-Gene labeling system (Promega).

Antibodies—Pro-564 (LDLEALAPYIPADDDFQLRS)- and Hyp564 (LDLEALAHypYIPADDDFQLRS)-peptides were from Genaxis (Nimes, France). They corresponded to the 557–576 sequence of human HIF-1 α . Methionines were converted to alanines to avoid spurious oxidation events. Rabbit anti-Hyp564 antibodies were obtained from Genaxis and characterized using a radioimmunoassay. The Hyp564 peptide was iodinated using chloramine T and purified by high pressure liquid chromatography. The radioimmunoassay buffer was 150 mM NaCl, 1 g/liter bovine serum albumin, 0.1 g/liter sodium azide, and 10 mM potassium phosphate buffer (pH 7.5) supplemented with 20,000 cpm/ml iodinated peptide. After 16 h of incubation at 4 °C, antibody-peptide complexes were precipitated with polyethylene glycol. Mouse monoclonal antibodies were obtained from the following sources: HIF-1 α (Novus Biologicals), GFP (Roche Applied Science), α -tubulin (Sigma), and vHL (BD Biosciences). Horseradish peroxidase-coupled sheep anti-mouse and donkey anti-rabbit antibodies were from Jackson Immuno-research Laboratories.

Immunoblot Analysis—Cells were washed twice in ice-cold PBS and lysed in lysis buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 5 mM sodium fluoride) supplemented with protease inhibitors (Roche Applied Science). After 20 min at 4 °C with continuous rocking, extracts were centrifuged at 12,000 \times g for 10 min at 4 °C. Protein concentrations were determined by the Bradford protein assay. Protein extracts were electrophoresed and transferred to nitrocellulose membranes (Schleicher & Schuell) using standard procedures. The membranes were blocked with 5% (w/v) instant nonfat milk powder in PBS, 0.1% Tween 20 for 1 h at room

temperature followed by overnight incubation at 4 °C with primary monoclonal antibodies against HIF-1 α (1:1,000), Hyp564 (1:2,000), vHL (1:500), GFP (1:1,000), or α -tubulin (1:5,000). Blots were washed three times in PBS, 0.1% Tween 20 and incubated for 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse antibodies or donkey anti-rabbit antibodies (1:10,000) in PBS, 0.1% Tween 20. After extensive washes in PBS, 0.1% Tween 20, chemiluminescence detection was performed by incubating the membranes with 100 mM Tris-Cl (pH 8.5), 2.65 mM H₂O₂, 0.45 mM luminol, and 0.625 mM coumaric acid for 1 min followed by exposure to x-ray films (Eastman Kodak Co.).

HIF Peptide Hydroxylation Assay—Cell lysates were centrifuged at 3,000 \times g for 10 min to remove cellular debris and nuclei. Cell extracts (0.5 mg of protein/ml) were incubated in the reaction buffer (0.5 mM dithiothreitol, 50 μ M ammonium ferrous sulfate, 1 mM ascorbate, 2 mg/ml bovine serum albumin, 0.4 mg/ml catalase, 0.3 mM unlabeled 2-OG in 40 mM Tris-Cl at pH 7.5) supplemented with 50,000 dpm of [5-¹⁴C]2-OG and 100 μ M Pro-564 peptide. After 30 min of incubation at 37 °C, the radioactivity associated to succinate was determined as described previously (10). Experiments under hypoxia were performed in a hypoxic workstation.

Recombinant Proteins—GST-ODD fusion proteins were prepared as follows. pcDNA3-HIF-1 α was PCR-amplified with the forward 5'-GGAATTC AAGTTGGAATTGG-3' and reverse 5'-CCGCTCGAGCTG-GAATACTGTAAGTGT-3' sets of primers. The P564A mutant was generated following three rounds of PCR amplification. (i) The 1616–1741-bp fragment was PCR-amplified with the forward 5'-GGAATTC-AAGTTGGAATTGG-3' and the reverse 5'-GTCATCATCCATTGGGAT-ATAGGCAGCTAACATCTCCAAGTCTAA-3' sets of primers. (ii) The 1697–1837-bp fragment was amplified with the forward 5'-TTAGACT-TGGAGATGTTAGCTGCCTATATCCCAATGGATGATGAC-3' and the reverse 5'-CCGCTCGAGCTGGAATACTGTAAGTGT-3' sets of primers. (iii) The final PCR amplification was initiated in the presence of an equimolar ratio of the two PCR-purified products and followed by the addition of the forward 5'-GGAATTC AAGTTGGAATTGG-3' and the reverse 5'-CCGCTCGAGCTGGAATACTGTAAGTGT-3' primers. The ODD and the P564A ODD fragments were purified, digested with *Eco*RI and *Xho*I, and ligated into pGEX-4T-1 (Amersham Biosciences) to generate N-terminal GST-tagged fusion proteins. The two plasmids were checked by DNA sequencing. The constructs (pGEX-GST-ODD and pGEX-GST-ODD P564A) were transformed into *Escherichia coli* BL21(DE3). Cells were induced with 0.1 mM isopropyl β -D-thiogalactoside for 4 h and harvested by centrifugation at 4,000 \times g for 15 min and pelleted. Cells were sonicated on ice in lysis buffer (20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol) supplemented with protease inhibitors. After centrifugation at 100,000 \times g for 30 min, the clarified sonicates were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C. After four washes in PBS, the GST-tagged fusion proteins were eluted in 50 mM Tris-HCl, 10 mM reduced glutathione (pH 8). The integrity and yield of purified GST fusion proteins were assessed by SDS-PAGE. vHL was synthesized by using pcDNA3.1/V5-His-vHL as a template (a gift of Dr. S. L. McKnight) and the TNT coupled reticulocyte lysate system (Promega).

GST Pull-down Assay—Total cell extracts (0.5 mg of protein/ml) were incubated in reaction buffer (see above) supplemented with glutathione-purified GST-ODD and vHL. After 2 h, glutathione-Sepharose beads were washed three times with cold buffer (20 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). The bound proteins were eluted in SDS-running buffer and analyzed by SDS-PAGE. Bound vHL was visualized by autoradiography or by Western blotting.

RESULTS

Hypoxia Up-regulates Expression of PHD-2 mRNAs and PHD Proteins—HIF-PHDs belong to the extended family of 2-OG-dependent oxygenases that require Fe(II) and ascorbate as cofactors. Three forms of the enzymes are known in human and mouse genomes. Rat PHD-1, PHD-2, and PHD-3 (already known as SM-20) were cloned from rat C6 cell mRNAs and sequenced. Sequences of rat PHD-1 and PHD-2, which were novel, have been deposited in GenBank™ (accession numbers AY229997 and AY228140). Fig. 1A shows that mRNA species for the three forms of PHD were present in normoxic cells and that hypoxia specifically up-regulated expression of PHD-2 mRNAs. This expression was blocked by actinomycin D (5 μ g/ml). PHD-2 mRNA expression remained elevated for at

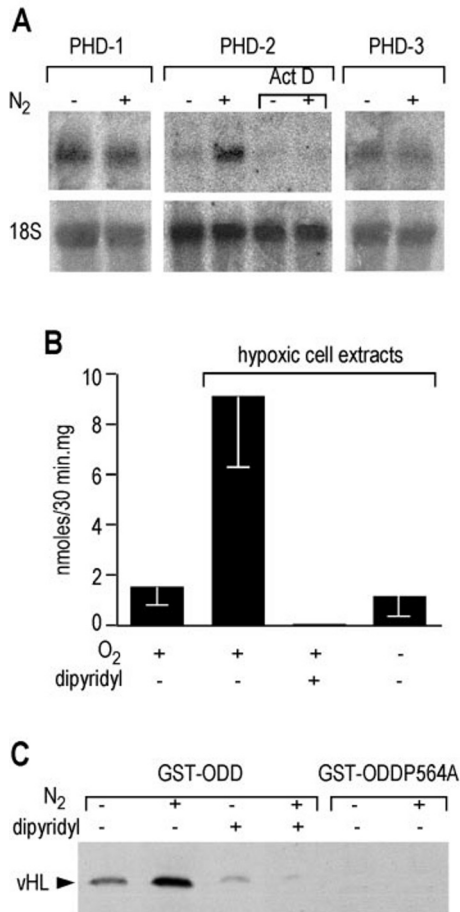


FIG. 1. Hypoxia up-regulated PHD-2 mRNA expression and PHD activity. *A*, Northern blot analysis of total RNA (10 µg/lane) extracted from C6 cells and exposed to normoxia and hypoxia. Actinomycin D (*Act D*) (5 µg/ml) was added as indicated. The blots were hybridized to PHD-1, PHD-2, PHD-3, or 18 S probes. Identical results were obtained in two other independent experiments. *B*, degradation of [¹⁴C]2-OG. Extracts from normoxic or hypoxic cells were prepared and used as a source of enzyme in a 2-OG degradation assay. The mean ± S.E. peptide-dependent activity ($n = 6$) is shown. Oxygen conditions during the assay are indicated in the figure. *C*, vHL binding assay. Bacterially expressed GST-ODD was *in vitro* hydroxylated by cell extracts prepared either from normoxic or hypoxic cells. Bound vHL was pulled-down and visualized by Western blots using an anti-vHL antibody. Experiments were performed in the absence or the presence of 0.1 mM 2,2'-dipyridyl and using GST-ODD or GST-ODD P564A proteins as substrates. Identical results were obtained in two other independent experiments.

least 1 h when hypoxic cells were reoxygenated (data not shown).

To determine whether the hypoxic induction of PHD-2 mRNA expression was accompanied by the synthesis of the corresponding protein, we used a newly devised PHD assay. Extracts of normoxic or hypoxic cells were incubated in the absence or the presence of a peptide substrate (Pro-564) that reproduced the 557–576 amino acid sequence of human HIF-1 α . The sequence comprised the Pro-564 residue that is hydroxylated by PHDs. Experiments were performed at normal oxygen tension, *i.e.* in the presence of unlimited amounts of oxygen. Under these conditions, our assay measured the maximum velocity of the enzyme, *i.e.* an estimation of the amount of active protein. The Pro-564 peptide-dependent activity was very low in extracts from normoxic cells (1.5 ± 0.7 nmol/30 min/mg protein). A 6-times larger enzyme activity was observed when hypoxic cell extracts were tested at ambient oxygen tension (Fig. 1*B*). The Pro-564 peptide-dependent activity

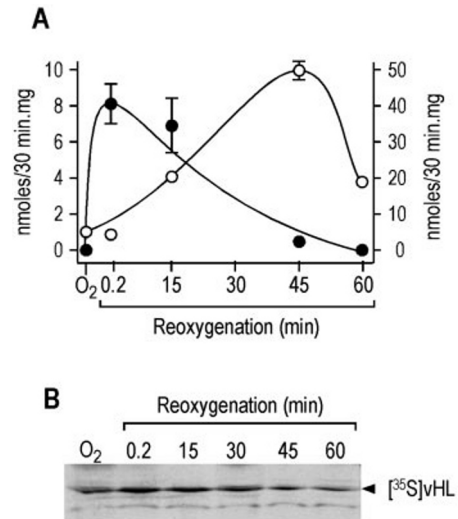


FIG. 2. Reoxygenation down-regulates expression of PHD. *A*, 2-OG degradation was analyzed in extracts from normoxic, hypoxic, and reoxygenated cells. Pro-564 peptide-dependent (●) and Pro-564 peptide-independent (○) activities were measured. Means ± S.E. ($n = 3$) in a representative experiment are shown. *B*, GST pull-down assay. GST-ODD was *in vitro* hydroxylated by cell extracts in the presence of cofactors and [³⁵S]vHL. The bound [³⁵S]vHL was pulled-down and visualized by autoradiography.

was inhibited by iron chelation using 2,2'-dipyridyl and was strictly dependent on the presence of oxygen. The basal activity measured in the absence of Pro-564 peptide substrate was insensitive to iron chelation (data not shown). It probably represented the activity of enzymes that degraded 2-OG but that were unrelated to HIF-PHDs. The up-regulation of PHD activity by hypoxia might be the result of the *de novo* synthesis or the unmasking of a latent enzyme activity. To distinguish between these two possibilities, we used actinomycin D to block transcription. Results indicated that actinomycin D (5 µg/ml) prevented the hypoxic induction of PHD activity (data not shown).

A second PHD assay used bacterially expressed GST-ODD fusion proteins as substrate and a pull-down assay relying on the ability of vHL to bind hydroxylated Pro-564 (Hyp564). The ODD sequence reproduced the 530–603 amino acid sequence of human HIF-1 α . Fig. 1*C* shows that incubation of GST-ODD with extracts from normoxic cells promoted vHL binding to GST-ODD. Binding was prevented by 2,2'-dipyridyl. As expected, it was not observed when a GST-ODD P564A fusion protein was used as a substrate. Fig. 1*C* further shows that extracts from hypoxic cells promoted a larger association of vHL with GST-ODD. Note that in the two *in vitro* assays, PHD activity was assayed at ambient oxygen, *i.e.* in the presence of unlimited amounts of oxygen. As a consequence, activities represented maximum velocities of the enzymes, *i.e.* enzyme concentrations. Indeed enzyme activities were lower in hypoxic cells because of the lack of oxygen. Taken together, these results indicated that hypoxia increased the levels of both PHD-2 mRNAs and PHD enzyme.

Fig. 2 analyzes changes in PHD activity during reoxygenation. It shows that the enzyme activity, measured using the 2-OG degradation assay (*panel A*) or the vHL binding assay (*panel B*), increased upon oxygenation and then decreased. Activity was back to basal levels after 45–60 min of reoxygenation. The peptide-independent activity was not modified by hypoxia. It increased slowly during reoxygenation (Fig. 2*A*).

Reoxygenation Destabilized ODD—To further analyze the effect of reoxygenation, we generated a chimeric protein consisting of the C-terminal part of the ODD domain of human

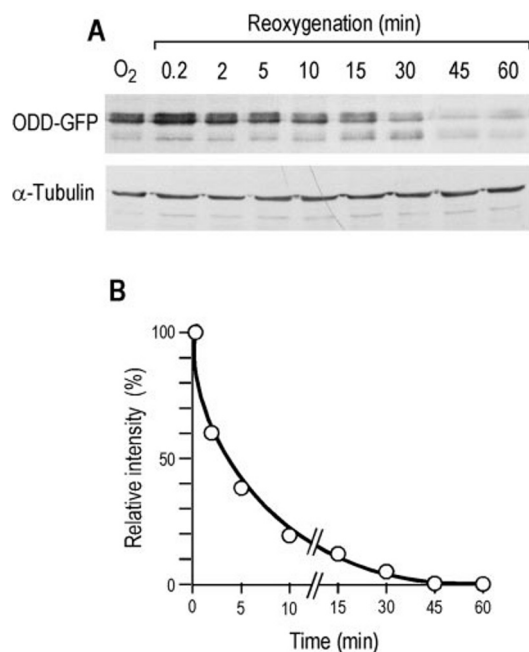


FIG. 3. Degradation of ODD-GFP during reoxygenation. *A*, Western blots showing expression of ODD-GFP (*top*) and α -tubulin (*bottom*) during reoxygenation of hypoxic cells. Control normoxic cells are shown for comparison. A representative experiment ($n = 3$) is shown. *B*, quantification of the data. The ODD-GFP signal was normalized to that of α -tubulin and plotted against time of reoxygenation. 100% represents maximum ODD-GFP to α -tubulin ratio observed in freshly reoxygenated cells (0.2 min).

HIF-1 α fused to GFP and used it as a reporter protein. The ODD sequence corresponded to the HIF-1 α (530–603) sequence and included Pro-564. Fig. 3A analyzes the degradation of ODD-GFP fusion protein during reoxygenation. It shows that ODD-GFP, which had accumulated during hypoxia, rapidly disappeared during reoxygenation. The ODD-GFP signal was quantitated and plotted against time. Fig. 3B indicated a half-life of ODD-GFP of ~ 3 min. It was much shorter than that of GFP (24 h) or of ODD-GFP (6 h) in the same cells (11). A decrease in the half-life of ODD-GFP from 6 h to 3 min suggested that the rate of degradation of the protein had increased 120-fold during reoxygenation. Similarly, we observed that hypoxia-stabilized HIF-1 α disappeared after only 5 min of reoxygenation (data not shown).

Identification of Hydroxylated Proteins in Cell Extracts—To examine the hydroxylation status of ODD-containing proteins during hypoxia and reoxygenation, we generated hydroxyproline-specific antibodies. The Hyp564 peptide was used to immunize a rabbit. The antibody recognized the Hyp564 peptide with a 300-times higher affinity than the non-hydroxylated peptide (data not shown). We then used Western blot and immunoprecipitation experiments to identify hydroxylated HIF-1 α in cell extracts. Labeling of endogenous HIF-1 α with the anti-Hyp564 antibody could not be demonstrated in extracts from normoxic, hypoxic, or reoxygenated cells. Thus, the data suggested that only unhydroxylated forms of HIF-1 α were present in these cells. This conclusion fully supports the hypothesis that hydroxylation of Pro-564 is essential for the proteasomal degradation of HIF-1 α (see “Discussion”). A weak labeling of HIF-1 α with the anti-Hyp564 antibody was only detected when normoxic cells were incubated in the presence of MG132 (10 μ M) to block the proteasomal degradation machinery (data not shown).

Fig. 4 analyzes the hydroxylation status of ODD-GFP using the anti-Hyp564 antibody. *Panel A* shows that hydroxylation of

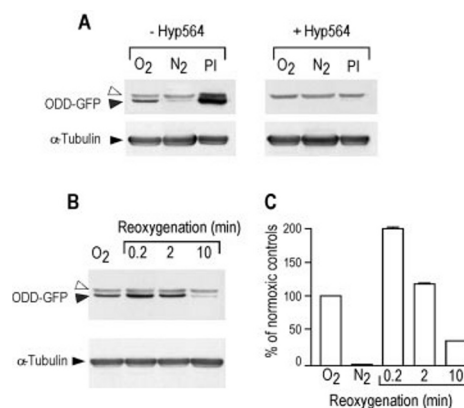


FIG. 4. Hydroxylation status of ODD-GFP during hypoxia and reoxygenation. Extracts from normoxic, hypoxic, or reoxygenated cells were subjected to Western blot analysis using the anti-Hyp564 (*top*) or anti α -tubulin (*bottom*) antibodies. *A*, the *white arrow* shows a nonspecific band that serves as a control for loading. Hypoxic cells were extracted in a hypoxic workstation to prevent hydroxylation of Pro-564 during extraction. Also shown is an extract from normoxic cells treated for 16 h with 10 μ M MG132 (*PI*) to block proteasomal degradation of ODD-GFP. The *right panel* shows the same experiment performed in the presence of 0.1 μ M Hyp564 peptide to neutralize specific antibodies. *B*, reoxygenation induces a transient accumulation of hydroxylated ODD-GFP. *C*, quantitative analysis of the data. The anti-Hyp564 signal was normalized to that of α -tubulin. 100% represents the ratio observed in control normoxic cells. Means \pm S.E. ($n = 3$) are shown.

ODD-GFP was increased in the presence of MG132. It was very low in hypoxic cells, as expected, since PHDs were inactive in the absence of oxygen (Fig. 1B). The specificity of the antibody was ascertained by the fact that an excess of Hyp564 peptide prevented the detection of the protein (Fig. 4A, *right panel*). Fig. 4B shows that hydroxylation of ODD-GFP increased during the first seconds of reoxygenation. Fig. 4C provides a quantitative analysis of the data. Maximum hydroxylation was observed during the first seconds of reoxygenation. It decreased nearly to control levels after 2 min and further disappeared. Note that the expression of ODD-GFP in reoxygenated cells also falls down below control normoxic levels (Fig. 3A). These results are the direct evidence that PHDs, which had accumulated during hypoxia (in a non-functional state since oxygen was limiting), forced the hydroxylation of Pro-564 and destabilized ODD-GFP when cells were reoxygenated.

DISCUSSION

Numerous antibodies have been raised against phosphorylated or acetylated protein motifs. They are important tools to visualize these post-translational modifications and to assess their functional significance. Here we generated antibodies against a synthetic peptide that mimicked the 557–576 sequence of human HIF-1 α and analyzed the *in vivo* hydroxylation of Pro-564 in the ODD domain of HIF-1 α . The antibody distinguished hydroxylated and native forms of the Pro-564 peptide but it was unable to recognize endogenous HIF-1 α in extracts from normoxic, hypoxic, or reoxygenated cells. This suggested that most of the HIF-1 α molecules were not hydroxylated. Chan *et al.* (12) recently reached the same conclusion using a different antibody. The absence of hydroxylated HIF-1 α molecules is fully consistent with the hypothesis that hydroxylation of Pro-564 targets HIF-1 α to the proteasome. Under normoxic conditions, HIF-1 α molecules are hydroxylated. They are rapidly degraded by the proteasome and cannot be detected. Under hypoxic conditions, the activity of PHDs is limited by the lack of oxygen. HIF-1 α escapes from degradation and accumulates under a non-hydroxylated form that is not recognized by the anti-Hyp564 antibody.

This paper also analyzes the hydroxylation status of Pro-564

in the context of an ODD-GFP fusion protein. Results indicated the presence of both native and hydroxylated forms of ODD-GFP in normoxic cells. The presence of a hydroxyproline in ODD-GFP but not in HIF-1 α suggested that hydroxylated ODD-GFP was degraded at a slower rate than hydroxylated HIF-1 α . This result was not surprising. Efficient targeting of HIF-1 α to the proteasome is now known to require hydroxylation of both Pro-402 and Pro-564 residues (13). The ODD sequence used in the ODD-GFP fusion protein only reproduced the 530–603 amino acid sequence of HIF-1 α and did not include the Pro-402 residue.

A major observation of this study is that a period of hypoxia up-regulates PHD enzyme levels. (i) Rat PHD-2 mRNAs are hypoxically induced (Fig. 1) as reported recently for human PHD-2 (14). (ii) Two different PHD assays documented the enzyme levels in extracts from hypoxic cells. (iii) Hydroxylated forms of an ODD-GFP fusion protein accumulate in reoxygenated cells. (iv) ODD-GFP is destabilized in reoxygenated cells. Thus, our results suggest the possibility that PHD-2 is responsible for the destabilization of ODD-GFP and HIF-1 α during reoxygenation. It has recently been proposed that an unknown factor is transcriptionally activated by HIF-1 α , targets HIF-1 α to the proteasome, and down-regulates HIF-1 α protein expression (15). Our results are fully consistent with this hypothesis and further suggest that PHD-2 might be this factor. A role for the up-regulation of PHD by hypoxia could be to stop hypoxic signaling in reoxygenated cells.

The largest PHD activity was observed in freshly reoxygenated cells. Activity then decreased and by 45 min reached low levels, similar to those observed in normoxic cells. This time course is consistent with the destabilization of ODD-GFP and HIF-1 α observed during reoxygenation. Increasing evidence suggests that HIF-1 α can be stabilized independently of prolyl hydroxylation (12), possibly via other post-transcriptional modifications. These include phosphorylations, asparagine hydroxylation (16, 17), and lysine acetylation (18). Results presented in Fig. 4 suggest that hydroxylation of Pro-564 is the major mechanism that down-regulates the expression of ODD-containing proteins during reoxygenation.

PHD activity assessed under conditions in which none of its substrates was limiting falls down within 1 h of reoxygenation (Fig. 2A). It is of interest to note that this decrease was not associated with a decrease in PHD-2 mRNA levels. Thus PHD activity in reoxygenated cells is probably controlled by post-transcriptional mechanisms. Note however that this regulation is probably of limited functional interest. Down-regulation of PHD activity in reoxygenated cells develops with a half-time of ~30 min, but by 5 min of reoxygenation, all of the substrates have been hydroxylated and targeted to the proteasomal degradation machinery.

It has recently been noticed that the substrate specificity of PHDs is quite low, thus suggesting that PHDs might have

other substrates in addition to HIF-1 α (19). It is also of interest to note that some functions of the SM-20 gene product in rats and the *Egl-9* gene product in *Caenorhabditis elegans* are unrelated to HIFs (14). More recently, it has been evidenced biochemically that proline hydroxylation of the large subunit of RNA polymerase II targeted the protein for ubiquitination (20). The observation that a large PHD activity accompanies reoxygenation raises the possibility that proline hydroxylations might play a role during reperfusion of hypoxic tissues. Whether a large PHD activity contributes to the beneficial (hypoxic preconditioning) or deleterious (cell damage) aspects of reoxygenation remains to be determined.

In conclusion, this study shows that hypoxia has two opposite actions on PHD. The lack of oxygen limits the enzymatic activity, but in contrast, it stimulates transcription. The major functional consequence of this dual regulation is that PHD activity reaches the highest levels during reoxygenation of hypoxic cells.

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