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-1α (HIF-1α). As a consequence, HIF-1α 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-1α 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.

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). HIF-1 was first identified as a heterodimeric transactivator that recognizes a specific DNA sequence termed hypoxia-responsive element in the 3‘-untranslated region of the erythropoietin gene (1). HIF-1 is composed of two subunits, HIF-1α and the aryl hydrocarbon nuclear translocator, both of which belong to the large family of basic helix-loop-helix-per- arnin-sim transcription factors (2). The mechanism of the hypoxic up-regulation of HIF-1α has been extensively documented. Under normoxic conditions, specific HIF-1α proline hydroxylases (PHD) hydroxylate two proline residues (Pro-402 and Pro-564) in the oxygen-dependent degradation (ODD) domain of HIF-1α (3, 4). The von Hippel-Lindau protein (vHL) E3 ubiquitin ligase complex associates to hydroxylated proline residues and targets HIF-1α to proteosomal degradation. Under hypoxic conditions, oxygen becomes rate-limiting for proline hydroxylation. As a consequence, HIF-1α 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 \( K_{\text{ATP}} \) 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-1α during HIF-1α has. 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-1α 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-\(^{14}\)C]2-OG and \( [35\)S]methionine were purchased from Amersham Biosciences. \([\alpha-\(^{32}\)P]dCTP was from ICN.

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HIF Peptide Hydroxylation Assay—Cell lysates were centrifuged at 3,000 × g for 10 min to remove cellular debris and nuclei. Cell extracts (0.5 mg/ml) were incubated with the membranes with 100 μM dithiothreitol, 50 μM ammonium ferrous sulfate, 1 mM ascorbate, 2 mg/ml bovine serum albumin, 0.4 mg/ml catalase, 0.3 mM unlabeled Tris (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 Hydroxylation Activity during Reoxygenation

Recombinant Proteins—GST-ODD fusion proteins were prepared as follows. pcDNA4-HIF-1α was PCR-amplified with the forward 5′-GAATTCGATGTAATCTGTCGTTGTTGGATG-3′ and reverse 5′-CCGCTCGAGCTGGAATACTGTAACTGT-3′ sets of primers. The P564A mutant was generated with equimolar rounds of PCR amplification. The 1871–1741 bp fragment was PCR-amplified with the forward 5′-GAATTCGATGTAATCTGTCGTTGTTGGATG-3′ and reverse 5′-GTGTTAGATTGTTGTTGTTGGATG-3′ sets of primers. The ODD and the P564A ODD fragments were purified, digested with EcoRI and XhoI (5,5′-dig BamHI), and ligated into pGEX-4T-1 (Amersham Biosciences). vHL was expressed in BL21 (DE3). Cells were induced with 0.1 mM isopropyl β-D-thiogalactoside for 4 h and harvested by centrifugation at 4,000 × g for 15 min and pelleted. Cell lysates were centrifuged at 100,000 × 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/I/V5-His as a template (a gift of Dr. S. L. McKnight) and the Tet coupled reticuloocyte lysate system (Promega).

HIF 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 μg/ml). PHD-2 mRNA expression remained elevated for at
Phylogen Hydroxylase Activity during Reoxygenation

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 hybridized to PHD-1, PHD-2, PHD-3, or 18 S probes. Identical results were obtained in two other independent experiments. 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 presence of unlimiting amounts of oxygen. As a consequence, activities were lower in hypoxic (Fig. 2) panels A and 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. 2A).

Reoxygenation Destabilized ODD—To further analyze the effect of reoxygenation, we generated a chimera of human HIF-1α. 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 unlimiting 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/min/mg protein). A 6-times larger enzyme activity was observed when hypoxic cell extracts were tested at ambient oxygen tension (Fig. 1B). The Pro-564 peptide-dependent activity 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-1α. Fig. 1C 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 unlimiting 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. 2A).
HIF-1α fused to GFP and used it as a reporter protein. The ODD sequence corresponded to the HIF-1α-(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-1α 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-1α in cell extracts. Labeling of endogenous HIF-1α 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-1α were present in these cells. This conclusion fully supports the hypothesis that hydroxylation of Pro-564 is essential for the proteasomal degradation of HIF-1α (see “Discussion”). A weak labeling of HIF-1α with the anti-Hyp564 antibody was only detected when normoxic cells were incubated in the presence of MG132 (10 μM) 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 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-1α and analyzed the in vivo hydroxylation of Pro-564 in the ODD domain of HIF-1α. The antibody distinguished hydroxylated and native forms of the Pro-564 peptide but it was unable to recognize endogenous HIF-1α in extracts from normoxic, hypoxic, or reoxygenated cells. This suggested that most of the HIF-1α molecules were not hydroxylated. Chan et al. (12) recently reached the same conclusion using a different antibody. The absence of hydroxylated HIF-1α molecules is fully consistent with the hypothesis that hydroxylation of Pro-564 targets HIF-1α to the proteasome. Under normoxic conditions, HIF-1α 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-1α 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-1α suggested that hydroxylated ODD-GFP was degraded at a slower rate than hydroxylated HIF-1α. This result was not surprising. Efficient targeting of HIF-1α to the proteasome is now known to require hydroxylations 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-1α 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-1α during reoxygenation. It has recently been proposed that an unknown factor is transcriptionally activated by HIF-1α and targets HIF-1α to the proteasome, and down-regulates HIF-1α 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 hydroxylating 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-1α observed during reoxygenation. Increasing evidence suggests that HIF-1α 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-1α (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 (hypo preconditoning) 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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