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Review

Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases

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Abstract

Hypoxia-inducible factor 1 (HIF-1) functions as a master regulator of oxygen homeostasis by mediating a wide range of cellular and systemic adaptive physiological responses to reduced oxygen availability. In this review, we will summarize recent progress in elucidating the molecular mechanisms of HIF-1 activation, focusing on the role of oxygen-dependent prolyl and asparaginyl hydroxylases in hypoxia signal transduction.

Keywords: Hypoxia-inducible factor 1; Hypoxia; Hydroxylation; Transcriptional regulation; Dioxygenase; Oxygen sensing

Oxygen homeostasis

For all organisms, changes in O_2 concentration represent a fundamental physiologic stimulus. Particularly in vertebrate animals, either deficiency or excess of O_2 elicits acute (rapid-onset and short-term) and chronic (delayed-onset and long-term) responses. Acute responses often entail changes in the activity of preexisting proteins, such as ion channels that mediate adaptive changes in cell excitability, contractility, and secretory activity. Chronic responses usually involve changes in gene expression and the gene products modulate the character of the acute responses. A remarkable variety of molecular mechanisms that control the activity of prokaryotic and yeast transcription factors in response to changes in the environmental O_2 concentration have been described [1–3]. To insure adequate oxygen availability and at the same time minimize the influence of oxygen free radicals, i.e., to maintain O_2 homeostasis, a variety of adaptive physiological mechanisms have evolved to maintain oxygen concentrations within a narrow range.

Mammals regulate oxygen delivery and consumption through a combination of cellular and systemic processes. For example, when oxygen is limiting, individual cells decrease oxidative phosphorylation and rely on glycolysis as the primary means of ATP production. To facilitate this switch to glycolysis cells up-regulate the expression of genes encoding glycolytic enzymes and glucose transporters. Other hypoxic responses monitor global oxygen levels and effect system-wide responses that modulate tissue O_2 availability. For instance, the hypoxic induction of the hormone erythropoietin (EPO) by the kidney stimulates red blood cell production to increase the oxygen carrying capacity of the blood. Tissues and cells experiencing reduced oxygen supply, e.g., wounds, increase the levels of the angiogenic cytokine vascular endothelial growth factor (VEGF). VEGF then acts on endothelial cells to stimulate the proliferation of new blood vessels, which in turn help maintain an adequate supply of oxygen. On the other hand, in many disease states including cancer, stroke, and heart attack, the oxygen delivery systems can become dysregulated and hypoxia becomes a major component of the pathophysiology of these diseases [4].

EPO expression was studied to elucidate the molecular mechanisms associated with the induction of hypoxia

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responsive genes and from these investigations hypoxia-inducible factor 1 (HIF-1) was identified as a key transcriptional regulator of the *EPO* gene [5,6]. Subsequent research has revealed that a large number of other hypoxia-inducible genes are also regulated by HIF-1 under hypoxic conditions, indicating that HIF-1 functions as a master transcriptional regulator of the adaptive response to hypoxia [7,8]. In addition, a growing number of roles for HIF-1 in the regulation of gene expression under non-hypoxic conditions are being identified [4].

HIF-1

The discovery of HIF-1 as a global regulator of O_2 homeostasis initially resulted from analysis of the molecular mechanisms by which *EPO* gene transcription was activated in response to hypoxia. A small (50-bp) DNA sequence that functions as a hypoxia response element (HRE) was identified in the 3′-flanking region of the human *EPO* gene. HIF-1 was identified and purified as a nuclear factor that was induced in hypoxic cells and bound to the HRE [5,6,13]. HIF-1 is a heterodimeric transcription factor composed of a HIF-1α subunit and a HIF-1β subunit [13,14]. HIF-1β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) because it was previously shown to dimerize with the aryl hydrocarbon receptor after activation of the latter by binding aryl hydrocarbons such as dioxin [9]. In contrast, human HIF-1α was a previously unidentified protein. Comparison of complementary DNAs encoding human, mouse, and rat HIF-1α reveals 90% amino acid sequence identity. Both HIF-1 subunits are members of the basic helix–loop–helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family of transcription factors. Each subunit contains two PAS domains, designated PAS-A and PAS-B. The HLH and PAS domains mediate heterodimer formation between the α- and β-subunits which is necessary for DNA binding by the basic domains [10].

In mammals, three genes have been shown to encode HIF-α subunits that appear to be regulated in a similar manner. The HIF-1α protein is ubiquitously expressed, whereas its paralogs, HIF-2α (also known as endothelial PAS domain protein 1, HIF-1-like factor, HIF-related factor, and member of PAS superfamily 2) [11] and HIF-3α [12], have more restricted expression patterns. HIF-1β is generally found to be constitutively expressed and insensitive to changes in oxygen availability, whereas the levels of the α-subunits are acutely regulated in response to hypoxia. Although there is evidence for hypoxic induction of HIF-1α mRNA levels in some cell types, the predominant O_2-dependent regulation of HIF-1α is mediated by post-translational mechanisms.

Regulation of HIF-1α protein stability

A major goal in the field has been to understand the molecular mechanism by which cells are able to sense O_2 levels and transduce the signal of reduced O_2 levels to HIF-1. It has been reported that O_2 levels can affect the protein stability, subcellular localization, DNA binding capacity, and transcriptional potency of HIF-1. While the HIF-1α subunits may be subject to various levels of regulation by O_2 including transcription and translation, it has been the recent analysis of HIF-1α protein stability and transactivation potency that have provided the greatest insights into the molecular mechanism of O_2-dependent HIF-1 regulation.

Initial biochemical analysis of HIF-1α revealed that this protein was subject to rapid turnover (half-life less than 5 min) under non-hypoxic conditions (20% O_2), whereas hypoxia (e.g., 1% O_2) inhibited degradation leading to the accumulation of HIF-1α [15]. Treatment with proteasomal inhibitors including MG132 and *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal (Cbz-LLL) revealed that HIF-1α was degraded by the ubiquitin–proteasome pathway under non-hypoxic conditions [15–18]. Subsequent studies identified a domain of approximately 200 amino acids (residues 401–603) that is necessary and sufficient for O_2-regulated expression and removal of the domain rendered HIF-1α stable under non-hypoxic conditions [15,18].

The von Hippel–Lindau (VHL) syndrome is characterized by predisposition to develop tumors that are highly vascularized due to the constitutive expression of a large number of hypoxia-inducible genes including *VEGF*. Because *VEGF* and other hypoxia-inducible genes are known HIF-1 targets, these observations raised the question of whether VHL disease and HIF-1 were somehow related. Tumor cells which lacked the VHL protein expressed high levels of HIF-1α and HIF-2α protein under non-hypoxic conditions [19]. O_2-regulated HIF-1 activity and target gene expression under non-hypoxic conditions can be restored by transfection of a wild-type VHL expression plasmid. Further analysis demonstrated that VHL physically interacted with HIF-1α and that VHL was part of a multiprotein ubiquitin ligase capable of ubiquitinating HIF-1α subunits and targeting them for destruction by the proteasome [20,21]. Along with hypoxia, iron chelating agents such as desferrioxamine were also able to block the interaction of HIF-1α with VHL, suggesting a requirement for iron in the degradation of HIF-1α [19].

An important breakthrough came with the finding that VHL recognition of the HIF-1α subunit was dependent on the hydroxylation of conserved proline residues within HIF-1α under non-hypoxic conditions [22–24]. Hydroxylation and subsequent VHL-mediated ubiquitination of HIF-1α is suppressed under hypoxic conditions, resulting in HIF-1α protein accumulation even without changes in HIF-1α mRNA levels (Fig. 1). These data strongly suggested the involvement of enzymes with prolyl hydroxylase activity in the hypoxic response pathway. This was supported by the finding that HIF-1α modification was dependent on both Fe(II) and α-ketoglutarate (also known as 2-oxoglutarate), similar to the well-characterized hydroxy-

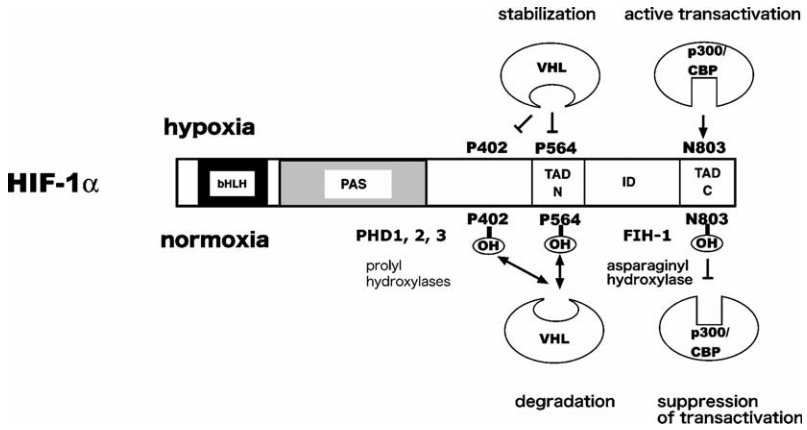

Fig. 1. HIF-1α is a substrate for both prolyl and asparaginyl hydroxylases the enzymatic activity of which is dependent on Fe(II), α-ketoglutarate, ascorbate, and O_2. Under normoxic conditions, the proline and asparagine residues are hydroxylated by specific dioxygenases. These hydroxylations regulate the stability and transcriptional activity of the HIF-1α subunit. TAD-N, transactivation domain N-terminal; ID, inhibitory domain; TAD-C, C-terminal transactivation domain; PHD, prolyl hydroxylase domain-containing protein; and FIH-1, factor-inhibiting HIF-1.

Table 1
Comparison of the HIF-1α hydroxylases with type I procollagen-4-hydroxylase

Gene	Synonyms	Substrate		K_m		
		Pro-402	Pro-564	O_2 (μM)	α-Ketoglutarate (μM)	Ascorbate (μM)
EGLN1	PHD2/HPH-2	+	+	230	60	170
EGLN2	PHD1/HPH-3	+	+	250	60	180
EGLN3	PHD3/HPH-1	−	+	230	55	140
HIF1AN	FIH-1	Asp-803		90	25	260
C-P4H-I				40	20	300

lases that modify procollagen in the endoplasmic reticulum. A family of three HIF-1α prolyl hydroxylases (designated prolyl hydroxylase domain-containing proteins (PHDs) 1, 2, and 3) was identified using genomic screening and a subsequent biochemical purification of hydroxylase activity (see Table 1) [25,26]. Inactivation of these enzymes in cell lines by RNA interference promotes HIF-1α stability and HIF-1 activity, verifying their role in the pathway [27].

HIF-1 transactivation

In addition to the domain regulating protein stability, HIF-1α contains two transactivation domains, TAD-N (residues 531–575) and TAD-C (residues 786–826), which bind coactivators including CBP, p300, steroid receptor coactivator (SRC)-1, and transcriptional intermediary factor-2 [28–30]. Residues 576–785 comprise an inhibitory domain the deletion of which increases transactivation domain function especially under non-hypoxic conditions [28]. Transactivation domain function is negatively regulated by O_2 independently of protein stability. Exposure of cells to hypoxia or the iron chelator desferrioxamine induces both HIF-1α protein stabilization and transactivation [28,29], suggesting the existence of a common molecular pathway. Using the yeast two-hybrid assay to screen for proteins that interact with the HIF-1α inhibitory domain and modulate its biological activity, FIH-1 (factor inhibiting HIF-1), a negative regulator of HIF-1 transactivation domain function, was identified [31].

A conserved asparagine residue in TAD-C (Asn-803 in human HIF-1α) was found to be hydroxylated under non-hypoxic conditions by mass spectrometry analysis and the hydroxylation inhibited the binding of HIF-1α to the coactivators CBP and p300 [32]. The HIF-1α asparaginyl hydroxylase was found to be FIH-1 [33,34]. Asn-803 is located approximately 20–30 residues carboxyl-terminal to the putative FIH-1 binding region of HIF-1α [31]. This suggests that for FIH-1 to efficiently hydroxylate the asparagine residue in HIF-1 it may need to bind to a region in HIF-1 adjacent to the asparagine motif. To support this notion it has been demonstrated that binding of p300 is not enhanced by the hydroxylase inhibitor dimethyloxalylglycine, or iron chelator, when the putative FIH-1 binding region is removed [35].

Properties of HIF-1α hydroxylases

Both PHDs and FIH-1 enzymes possesses a double-stranded β-helix (“jelly roll”) core and Fe(II)-binding residues common to members of the dioxygenase family [36,37] that includes the collagen prolyl 4-hydroxylases (C-P4Hs) [38]. To study the details of the catalytic properties of the enzymes, all three human PHD enzymes and FIH-1 were expressed as recombinant proteins in insect cells using an assay based on measurement of $^{14}CO_2$ released during the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate, adopting methods similar to those used for C-P4Hs [39,40]. From these studies, the biochem-

ical properties of PHDs and FIH-1 including the kinetic constants for the co-substrates have been clarified in vitro (Table 1).

Substrate specificity

In HIF-1α, at least one of two critical proline residues, Pro-402 and Pro-564, is hydroxylated under normoxic conditions [25,42]. The resulting 4-hydroxyproline residue is essential for the binding of HIF-1α to the VHL E3-ubiquitin ligase complex. The proline residues hydroxylated in HIF-1α and HIF-2α are all present in the context of an Pro-Xxx-Xxx-Leu-Ala-Pro consensus sequence. The human type I and type II C-P4Hs cannot hydroxylate a 19-residue synthetic peptide corresponding to the sequence around Pro-564. All the PHDs hydroxylate the peptide from the C-terminal hydroxylation site of HIF-1α and HIF-2α (containing Pro-564 in human HIF-1α). K_m values of the three PHDs for the peptide are very similar. PHD1 and PHD2 have higher K_m values for a peptide containing the N-terminal hydroxylation site (Pro-402 of human HIF-1α) and this peptide is not hydroxylated by PHD3 [39]. In contrast, the peptide from N-terminal hydroxylation site of HIF-2α and HIF-3α is a good substrate for all the PHDs [39].

The three PHD enzymes retained partial activity even in the absence of added Fe(II), suggesting that the iron atom may be more tightly bound to the PHDs than is the case for the C-P4Hs [43]. It is therefore not possible to determine the K_m values for Fe(II). The K_m values of the three PHDs for α-ketoglutarate were about 3-fold and those for ascorbate about half relative to those of type I C-P4H, and there were no significant differences in these values between the three enzymes. FIH-1 catalyzes hydroxylation of the β-carbon of Asn-803. FIH-1 has a more stringent specificity for substrate peptides [40]. Structural studies indicate that an FIH-1 homodimer forms in solution and is essential for FIH-1 activity [41].

O_2-dependency of enzymatic activity

A striking difference between the two classes of prolyl 4-hydroxylases was found in the K_m values for O_2, which were determined here for both the PHDs and the type I C-P4H in the same experiments. The K_m values of the PHDs were slightly above the concentration of dissolved O_2 in air (about 200 μM), and there were no significant differences between the three enzymes [44,45]. The K_m of the type I C-P4H was much lower, only about one-sixth of these values. In contrast, the K_m of FIH-1 for O_2 is less than half that of the PHD family members, suggesting that a hypoxic window might exist in which HIF-1α would be stable due to the absence of prolyl hydroxylation and yet would be transcriptionally inactive due to hydroxylation on Asn-803 [40].

The expression of PHD2 and PHD3, but not PHD1, is induced by hypoxia, suggesting a role for these enzymes in a negative feedback pathway responsible for enhanced degradation of HIF-1α after re-oxygenation. Differences between the PHDs are seen in their intracellular localization patterns when overexpressed as tagged proteins [46,47]. Human PHD1 is predominantly localized to the nucleus, whereas PHD2 is present in the cytoplasm. Human PHD3 distributes evenly in both compartments. Like PHD2, FIH-1 appears to be largely cytoplasmic [47].

These differences in substrate specificity, intracellular distribution, and inducibility by hypoxia imply distinct functions for the three PHDs. However, the physiological impact of this diversity is less clear. A study using RNA interference to suppress the expression of each PHD family member in various cell lines revealed that silencing of PHD1 or PHD3 did not promote HIF-1α accumulation under non-hypoxic conditions, whereas silencing of PHD2 expression resulted in HIF-1α accumulation and a reduced rate of HIF-1α turnover following reoxygenation [27].

Although there is no room for argument that the prolyl and asparaginyl hydroxylases are essential components of the physiological oxygen-sensing pathway in cells, HIF-1 is also regulated by additional O_2-independent molecular processes. There are several reports demonstrating that exposure of cells to certain nitric oxide (NO) donors or gaseous NO modulates HIF-1 activity. Treatment of cells with *S*-nitrosoglutathione (GSNO), DETA-NO, or NOC18 induces HIF-1 activity under non-hypoxic conditions [48,49]. In contrast, sodium nitroprusside (SNP) inhibits hypoxia-induced HIF-1 activation [50–54]. The molecular mechanisms that regulate HIF-1α expression and transactivation in response to NO donors are under active investigation but appear to involve effects on both HIF-1α synthesis and stability.

HIF-1 not only accumulates in response to hypoxia but also in response to the activation of a number of cellular signaling pathways. It is thought that many of these signaling pathways stimulate the translation of HIF-1α mRNA into protein [55–57]. Signaling via the HER2 and IGF-1 receptor tyrosine kinases induces HIF-1 expression by an O_2-independent mechanism. HER2 activation increases the rate of HIF-1α protein synthesis via phosphatidylinositol 3-kinase (PI3K) and the downstream serine-threonine kinases AKT (protein kinase B) and mTOR (mammalian target of rapamycin; also known as FKBP/rapamycin-associated protein) [56]. IGF-1-induced HIF-1α protein synthesis is dependent upon both the PI3K and MAPK pathways [57]. mTOR phosphorylates and activates the translational regulatory proteins eIF-4E-binding protein 1 (4E-BP1) and p70 S6 kinase ($p70^{S6K}$). Phosphorylation of 4E-BP1 disrupts its inhibitory interaction with eukaryotic initiation factor 4E (eIF-4E), whereas activated $p70^{S6K}$ phosphorylates the 40S ribosomal protein S6. Moreover, it was recently suggested that the action of the RasV12 and v-Src pathways may be mediated in part through inhibition of HIF-1α prolyl hydroxylation [58]. However, there is a report that constitutively activated c-Src leads to HIF-1α protein accumulation due to enhanced rate of

HIF-1α protein synthesis and not due to reduced HIF-1α degradation [59]. The hydroxylases themselves remain potential targets for rapid regulation by upstream signaling pathways such as phosphorylation, oxidation, and acetylation.

Hypoxia leads to elevated levels of PHD2 and PHD3 mRNA expression in a HIF-1-dependent manner [27]. On the other hand, the half-life of PHD1 and PHD3 is regulated by O_2 concentration. Interestingly, these enzymes are also degraded by the ubiquitin–proteasome system. In contrast to HIF-1α, the stability of PHD1 and PHD3 is decreased in hypoxic cells [60]. Siah1 and Siah2, two mammalian homologues of the *Drosophila* seven-in-absentia RING-finger protein, target PHD1 and PHD3 for proteasome-mediated degradation under hypoxic conditions [61]. Siah proteins possess E3-ubiquitin ligase activity, and both PHD1 and PHD3 are demonstrated substrates of Siah1 and Siah2 [60].

In addition to interacting with the TAD-C region, FIH-1 has also been shown to interact with VHL [31]. The significance of the interaction is still to be investigated in detail. Interestingly, both FIH-1 and VHL have been shown to interact with chromatin modifying histone deacetylase (HDAC) enzymes, which are known to play an important role in gene repression [31]. Together, these observations raise the possibility that FIH-1 may have multiple roles other than just regulating the TAD-C of HIF-1α and HIF-2α [31]. The transcriptional co-repressor protein ING4 was recently shown to interact with PHD2 [62], although it is not clear how this would regulate HIF-1 activity in an O_2-dependent manner since PHD2 appears to interact with HIF-1α constitutively.

Summary

HIF-1 activity is regulated by the PHD and FIH-1 dioxygenases in an O_2-dependent manner. The affinities of three recombinant prolyl hydroxylases for O_2 were reported to be about 200 μM [33]. These values are near the concentration of O_2 in the atmosphere. For HIF-1 activation, these enzymes appear to function as the core engines that drive the O_2 sensing machinery. On the other hand, many other intracellular signaling processes such as redox and phosphorylation have been implicated in HIF-1-dependent gene expression. These signaling pathways do not appear to be directly regulated by hydroxylases, suggesting the existence of additional pathways that signal changes in the cellular O_2 concentration. The mitochondrial respiratory chain has been proposed to act as an O_2 sensor that regulates HIF-1-dependent gene expression during hypoxia through activation of oxidant-dependent intracellular signaling [63–66]. The integration by the hydroxylation machinery of signals from the mitochondria may play a critical role in regulating HIF-1 activity. Further research in this area is likely to establish new connections between energy metabolism, redox state, oxygen homeostasis, and cell survival.

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