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Review

Dioxygenases as O_2-dependent regulators of the hypoxic response pathway

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Abstract

A ubiquitous pathway by which mammalian cells sense and respond to changes in oxygen availability relies upon the hypoxic induction of a transcription factor, HIF. HIF in turn activates the expression of an assemblage of genes promoting compensatory shifts in the capacity for anaerobic metabolism, O_2 delivery, and other adaptive processes. The stability and activity of HIF are each regulated as a function of O_2. Both mechanisms are directly mediated by posttranslational modification of this transcription factor: hydroxylation of proline and asparagine residues, respectively. These modifications are performed by members of the Fe(II)- and 2-oxoglutarate-dependent dioxygenase family whose activities are directly and indirectly dependent on cellular O_2 levels. As such, these oxygenases fill a role as environmental and metabolic sensors, a paradigm that may extend to other biological pathways.

Keywords: Hypoxia; HIF; Dioxygenase; Prolyl hydroxylase; Asparaginyl hydroxylase; Oxygen sensing; FIH-1

Regulation of the hypoxic response pathway as a function of O_2 availability

The maintenance of metazoan oxygen homeostasis is mediated by a complex interplay of acute and chronic regulatory pathways that coordinate O_2 delivery and consumption. Insights into one such pathway evolved from studies of the regulation of the erythropoietin (Epo) gene. Transcription of the Epo gene is strongly induced in the kidneys in response to global hypoxia where its gene product promotes red blood cell maturation to increase systemic O_2 delivery. However, characterization of a putative hypoxic responsive element (HRE) mapped within the Epo gene revealed a big surprise: this enhancer element could respond to hypoxia in multiple cell lines, even those that did not express Epo [1]. This observation implied the existence of a conserved pathway by which cells could sense a reduction in O_2 availability and induce transcription. A biochemical purification approach ultimately led to the identification of a transcription factor, hypoxia inducible factor-1 (HIF-1), which specifically recognizes the Epo HRE [2]. Approximately 100 direct target genes have been identified to date for HIF that contribute to numerous developmental, physiological, and pathophysiological pathways [3]. As such, HIF and the factors that govern its regulation have garnered attention as possible drug targets for a number of disease states in which O_2 availability is compromised including myocardial and cerebral ischemia and cancer.

As shown in Fig. 1, HIF-1 is a heterodimer composed of a constitutive β subunit and an O_2-responsive α subunit, both members of the basic-helix–loop–helix (bHLH)-PAS family. Both the HIF-1α subunit and the related HIF-2α and HIF-3α paralogs appear to be regulated in a similar manner [4–6] though the unique phys-

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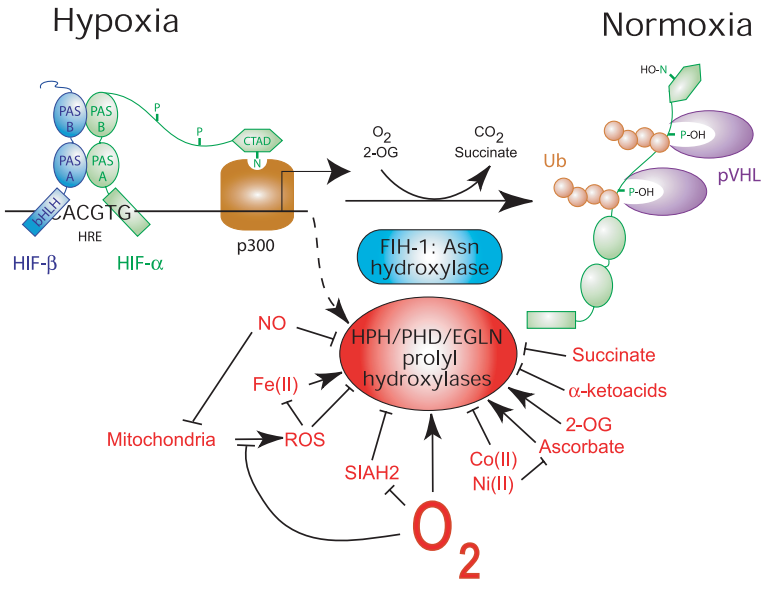

Fig. 1. Regulation of the mammalian hypoxic response pathway. When O_2 levels are low (hypoxia), HIF is both stable and competent to associate with transcriptional coactivators such as p300, resulting in induction of target genes containing HREs. When O_2 levels are high (normoxia), HIF prolyl and asparaginyl hydroxylases modify the HIF-α subunit to block coactivator recruitment and facilitate pVHL-mediated proteasomal degradation. The activities of the dioxygenases that perform these reactions are directly and indirectly affected by O_2 availability, metabolic intermediates, and feedback loops.

iological roles for these proteins remain under investigation. Two prominent mechanisms by which HIF responds to changes in O_2 availability have been identified, protein stability and coactivator recruitment. Though constitutively expressed, the α subunit is selectively targeted for rapid proteasomal degradation under normoxic conditions [7–10]. The product of the von Hippel–Lindau tumor suppressor gene (pVHL), a subunit of the responsible ubiquitin ligase complex [11–14], fails to recognize the O_2-dependent degradation domain (ODD) of the α subunit under hypoxic conditions, allowing HIF to accumulate [15,16]. Aside from its effects on HIF stability, O_2 also selectively abrogates the ability of the α subunit to recruit transcriptional coactivators such as CBP/p300 to its C-terminal transactivation domain (CTAD) [17–20].

Though tremendous progress was made with respect to HIF characterization, the nature of the underlying mechanism by which cellular O_2 levels were sensed and translated to changes in HIF induction remained a mystery [21]. An early hypothesis invoked a unique factor capable of directly binding O_2—a "monolithic" O_2 sensor that would bind O_2 via a heme-bound iron cofactor. While inhibition of HIF under normoxia was indeed compromised in cells upon exposure to iron chelators, divalent metals, and gases such as CO [22], no strong candidate sensor protein emerged and a second hypothesis gained momentum. This model proposed that O_2 levels were sensed indirectly through changes in metabolic status, redox state, and levels of reactive oxygen species (ROS), among others. These inputs would converge on HIF, at least in part, through their effects on various signal transduction pathways.

Recently a new paradigm has emerged following the breakthrough discovery that pVHL recognition of the HIF-α subunit was dependent upon specific posttranslational modifications within the ODD: hydroxylated proline residues [15,16,23] required to form H-bond interactions with pVHL [24,25]. Hydroxylation takes place only under normoxic conditions, and in its absence under hypoxia, the α subunit is stable. Soon after, it was reported that coactivator recruitment by the CTAD was likewise mediated by a similar posttranslational modification: hydroxylation of an asparagine residue [26]. Again, this site was only modified under normoxic conditions though in this case the hydroxyl group disrupts the interaction between the CTAD and coactivators.

These findings implied the existence of dedicated enzymes to perform these modifications and informed a new model for O_2 sensing and signaling. Proline and lysine residues within collagen are well-known sites for hydroxylation, modifications that facilitate proper folding. The enzymes that carry out these modifications are members of the Fe(II)- and 2-oxoglutarate (2-OG)-dependent family of dioxygenases [27]. O_2 is consumed as a substrate by these enzymes to effect hydroxylation. Though the procollagen-modifying enzymes did not accept the HIF-α subunit as a substrate, the HIF prolyl and asparaginyl hydroxylase activities present in cell extracts were also dependent on Fe(II), 2-OG, and O_2. By

virtue of their requirement for O_2 as a substrate, these hydroxylases became intriguing candidates for direct O_2 sensors in the hypoxic response pathway.

The enzymes responsible for both modifications have since been identified. In mammals, prolyl hydroxylation is performed by a family of three HIF prolyl hydroxylases variously named HPH-1/EGLN3/PHD3, HPH-2/EGLN1/PHD2, and HPH-3/EGLN2/PHD1 [28–30]. The three mammalian gene products share a highly conserved C-terminal catalytic domain adjacent to their divergent N-termini. Differences among the three enzymes have been reported for their tissue distribution, transcriptional regulation, subcellular localization, and their preferences for the two hydroxylation sites within the ODD (for review, see [31]). Additional physiological roles unique to each enzyme will no doubt emerge from mouse knockout studies currently underway. A single mammalian gene product is responsible for asparaginyl hydroxylation, factor inhibiting HIF-1 (FIH-1) [32–34]. Though there is much debate concerning the relative importance of the two modes of HIF regulation, it is becoming increasingly clear that the hydroxylases occupy nonredundant physiological roles that differ with respect to induction of individual HIF target genes under a variety of cellular O_2 tensions [35–38].

Structure of the HIF hydroxylases

With the responsible enzymes in hand, their role as biological O_2 sensors can now be properly analyzed. The HIF hydroxylases are all members of the cupin superfamily of proteins, a group characterized by a core of eight beta strands forming a "beta-jellyroll" domain [32]. In addition to the core domain, these proteins are decorated with a combination of strands and helices unique to each family member. Structures of FIH-1, a functional homodimer [33,39], have been determined by several groups [40–42]. FIH-1 consists of the eight-stranded core enveloped by six additional strands and eight helices. Moreover, the structures reveal FIH-1 in the presence or absence of a HIF peptide, cosubstrate, substrate analogs, or iron. The best resolved structure to date is a complex of FIH-1, iron, *N*-oxaloylglycine (NOG), and a peptide substrate comprising residues 786–826 of the HIF-1α CTAD (Fig. 2) [41]. The active site of this structure reveals the predicted His-X-Asp/Glu-X_n-His iron coordinating triad, a hallmark of 2-OG-dependent dioxygenases. A cosubstrate analog, NOG, binds in a similar manner to 2-OG with one C1 carboxyl oxygen and the C2 carbonyl oxygen coordinated to the Fe(II). The C5 carboxylate of NOG (or 2-OG) is stabilized by an interaction with FIH-1 residues Lys^{214}, Thr^{196}, and Tyr^{145}. Stabilization by a basic residue, Lys^{214}, is a common feature of 2-OG-dependent dioxygenases, and the equivalent residue for the HIF prolyl hydroxylases is predicted to be arginine. Interestingly, the additional stabilization of 2-OG by Thr^{196} and Tyr^{145} is unprecedented in this class of enzymes and may provide a means of inhibiting FIH-1 with selective competitors of 2-OG binding.

Mechanism of HIF hydroxylation by 2-OG-dependent dioxygenases

For both the HIF prolyl and asparaginyl hydroxylases, the general reaction mechanism entails utilization of

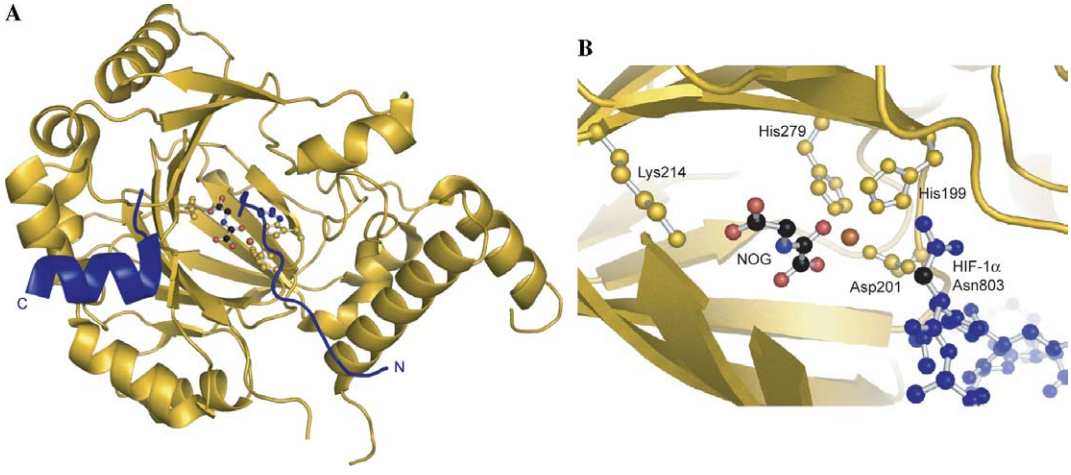

Fig. 2. The overall structure and active site of a complex of FIH-1, Fe(II) , NOG, and a HIF CTAD peptide are represented (PDB 1H2K). (A) Ribbon diagram showing FIH-1 in gold and the HIF CTAD peptide in blue. The iron and NOG are modeled as a sphere or ball-and-stick, and the N- and C-termini of the HIF CTAD are labeled. (B) The active site of FIH-1 with critical residues in FIH-1 and the target asparagine of the HIF CTAD depicted as gold and blue ball-and-stick models, respectively. Cβ of the target asparagine is black, iron is shown as an orange sphere while NOG is colored according to element type (carbon, black; nitrogen, blue, and oxygen, red). HIF-1α residues visible in the structure are ^{795}LTSYDCEVNAPI^{806} and ^{812}LLQGEELLRALD^{823}. The orientations of the panels are chosen for clarity and are not equivalent (figure generated using PyMOL).

molecular oxygen to convert 2-OG to succinate and CO_2 while effecting hydroxylation of the prime substrate, the HIF-α subunit [43–45]. Iron is responsible for the transfer of electrons to generate radical species and a highly reactive iron-oxo species (Fig. 3). The details of the proposed mechanism for both HIF hydroxylases involve the initial binding of Fe(II), 2-OG, HIF substrate, and O_2 followed by electron transfer from Fe(II) to the coordinated O_2 to yield Fe(III) and a superoxide radical. The superoxide radical subsequently attacks the carbonyl at position C2 of 2-OG forming a radical species that accepts an electron from iron. The resulting intermediate undergoes decarboxylation to produce succinate and CO_2. In the process of forming these products, O_2 is split with one oxygen atom moving to the succinate product while the other oxygen atom forms a highly reactive, putative Fe(IV)–oxo intermediate. This intermediate abstracts hydrogen from the α subunit at the target proline (CγH) or asparagine (CβH) residue [46]. An O–H bond is formed using one electron from the C–H bond and one from the former Fe=O, iron-oxo species. Concurrently, Fe(IV) undergoes a one electron reduction to generate an Fe(III) hydroxide. As a result, a radical is formed on the prime substrate, HIF. The final step involves the HIF radical attack on the iron-hydroxide resulting in the final hydroxylated HIF protein and Fe(II). Though this is the most likely mechanism based on current information regarding 2-OG dependent dioxygenases, all details have not been demonstrated directly for the HIF hydroxylases. The most compelling evidence for this model for the HIF hydroxylases comes from the identification of a substrate radical intermediate for the procollagen prolyl-4-hydroxylase [47].

A key to this mechanism involves the productive coupling of decarboxylation and hydrogen abstraction from the prime substrate, HIF. In the absence of substrate, this intermediate could abstract hydrogen from water, generating harmful hydroxyl radicals in the cell. Uncoupled decarboxylation of 2-OG has been reported for many 2-OG-dependent dioxygenases [48,49], and while uncoupling has not been observed for the HIF prolyl hydroxylases, uncoupling occurs in FIH-1 at ~1% of the maximum activity observed in the presence of the HIF substrate [36]. The details accounting for the minimal uncoupling by the HIF hydroxylases are unclear, but these observations are likely related to the as yet unexplained dependence on HIF substrate binding prior to the binding of O_2.

Determinants of substrate specificity for HIF hydroxylases

Multiple model HIF-1α peptides have been assayed for their ability to serve as a substrate for either the HPH or FIH-1 enzymes [24,36,50–53] (Table 1). From these studies, a peptide of 19 residues containing the consensus prolyl hydroxylation sequence LXXLAP is a suitable substrate with low micromolar K_m values [51]. However, various mutations can be tolerated at every position, other than the target proline, in these model peptides [50]. Interestingly, preferences have been observed for each HIF prolyl hydroxylase with respect to peptide size and the site within the ODD from which it was derived [51].

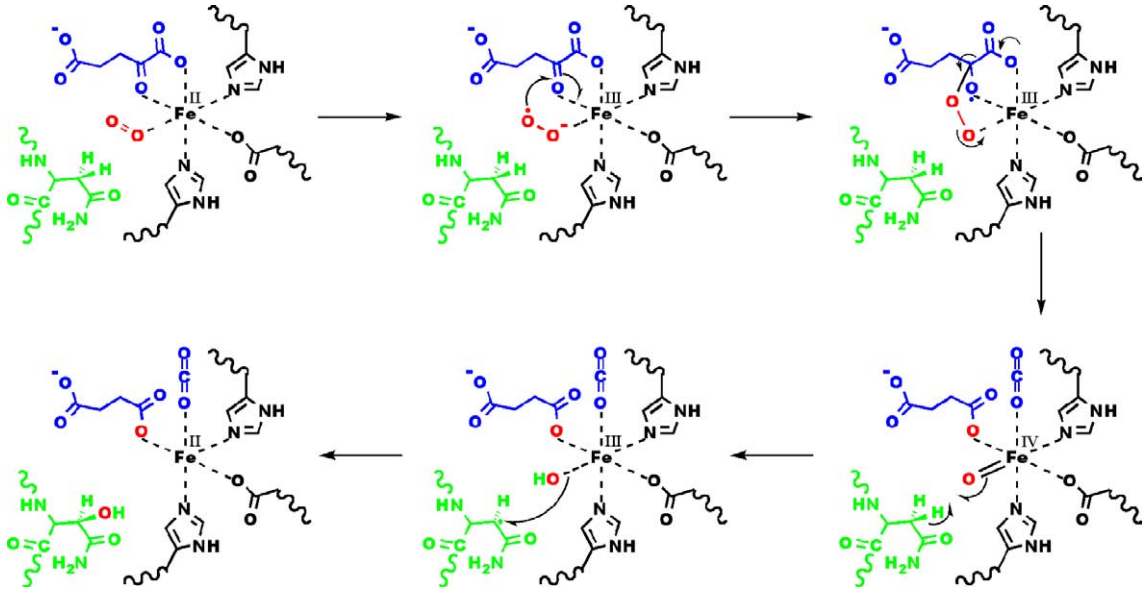

Fig. 3. Detailed proposed reaction mechanism for the HIF hydroxylases. The schematic is specific for the asparaginyl hydroxylase, FIH-1, but the model can be adapted to the prolyl hydroxylases by substitution of the substrate asparagine with proline. The substrates are colored as follows: O_2, red; HIF CTAD Asn, green; 2-OG, blue. See the main text for a comprehensive description of the model.

Table 1
Model peptide substrates for HIF hydroxylases[a]

HIF-1α peptide	Hydroxylase	K_m (μM)
^{556}DLDLEMLAPYIPMDDDFQL^{574}	HPH-1	7
	HPH-2	7
	HPH-3	8
^{788}DESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRAL^{822} [b]	FIH-1	100 ± 5

[a] See [36,51].
[b] Underlined residues are those visible in the FIH-1/HIF-1α CTAD peptide structure (Fig. 2).

The examination of FIH-1, the HIF asparaginyl hydroxylase, is greatly aided by the determination of a complex structure of FIH-1 with iron, NOG, and a HIF-1α peptide (Fig. 2) [41]. This structure shows two separate binding sites for HIF on FIH-1. The first binding site is comprised of HIF residues 795–806, which encompass the target asparagine, while the second binding site consists of residues 812–823. Using a model peptide spanning residues 788–822 (Table 1) as a reference, several deletion mutations were tested for their ability to bind to and act as substrates for FIH-1 [36]. Deletion of all residues within site 2 results in approximately a 10-fold decrease in maximal activity (V_{max}). In a subsequent study, only single alanine substitutions at the target Asn^{803} and the adjacent Val^{804} resulted in decreases in hydroxylation of the substrate [52]. A Val^{804} mutation likely affects the precise orientation of the substrate as it resides in a tight turn that gains more conformational flexibility when mutated to alanine. Of the various alanine scanning mutations only the Tyr798Ala substitution, which does not affect hydroxylation by FIH-1, is difficult to rationalize based on the structure as Tyr^{798} makes both a hydrogen bond and hydrophobic contacts with FIH-1 via the phenolic side chain [41]. However, this mutation may exert its effects on binding but not enzyme turnover rates.

Other than the HIF-α subunits, no other substrates have been conclusively identified for any of the HIF hydroxylases though it is widely speculated that other substrates exist. One likely candidate is Rpb1, the large subunit of RNA polymerase II and a substrate for pVHL that contains a hydroxylation site sharing the LXXLAP consensus [54]. A HIF-independent role for the *Drosophila* HIF prolyl hydroxylase homolog as a downstream effector of cyclin D/Cdk4 induced cell growth has also been proposed [55]. The nature of additional prolyl and asparaginyl hydroxylase substrates, and their regulation as a function of O_2 availability, will be of great future interest.

HIF hydroxylases: O_2 sensors?

By virtue of their substrate requirements, there is little doubt that hydroxylase inhibition under anoxic conditions is a direct consequence of the lack of O_2 availability. What about under hypoxic conditions though? The potential for the HIF hydroxylases to sense physiological levels of O_2 has been widely debated. While the procollagen-modifying enzymes have a strong affinity for O_2 and are active under conditions that induce a hypoxic response [51], early evidence provided support for an O_2 sensing role for the HIF hydroxylases. The K_m values measured from recombinant preparations of the three HIF prolyl hydroxylases as well as FIH-1 have been recently reported (Table 2) [36,51]. For the prolyl hydroxylases, these values approximate the expected O_2 concentrations encountered under ambient O_2 conditions (~200 μM), implying that minor changes in O_2 availability could substantially modulate hydroxylase activity. Indeed, in vitro analysis of the HIF prolyl hydroxylases shows a significant dependence of enzyme activity versus O_2 in a range spanning normal physiological levels [29]. The lower K_m of FIH-1 for O_2 implies that changes in O_2 levels must be more dramatic to alter FIH-1 activity. However, as available O_2 in tissues is much lower than the ambient levels [56,57], the possibility exists for O_2 regulation of FIH-1 over a narrower range. Combined, these findings support an O_2 sensing role for the hydroxylases over a broad, physiologically relevant, range of hypoxic O_2 concentrations.

Though these in vitro results are encouraging, demonstrating that the hydroxylases are bona fide O_2 sensors in vivo has not been straightforward. The reported K_m's imply a continuous drop in hydroxylase activity as O_2 falls from ambient levels that should be accompanied by a corresponding gradual increase in HIF stability. However, in cultured cells, little HIF accumulation is observed until O_2 levels fall below 5% [58]. Though it is possible that in vivo hydroxylase activity does not become immediately limiting or that purified recombinant enzymes lack components present in intact cells that alter their affinity for O_2, experimental evidence to reconcile these observations is wanting.

A competing model gaining experimental support indicates that mitochondria, the site of O_2 consumption during oxidative phosphorylation, are required for O_2 sensing and proper HIF induction under hypoxia [59–61]. It has long been reported that treatment of cells with various mitochondrial inhibitors could block HIF

Table 2
K_m values for HIF hydroxylases and cosubstrates with comparison to collagen prolyl hydroxylase

Hydroxylase	K_m (μM)			
	Fe(II)	2-Oxoglutarate	O_2	Ascorbate
FIH-1[a]	0.5 ± 0.2	25 ± 3	90 ± 20	260 ± 50
HPH-1[b]	ND	55	230	140
HPH-2[b]	ND	60	250	180
HPH-3[b]	ND	60	230	170
C-P4H-I[c]	2	20	40	300

[a] See [36].
[b] See [51].
[c] Collagen prolyl-4-hydroxylase [83].

induction under hypoxia [62]. These inhibitors include nitric oxide, an inhibitor of HIF induction initially proposed to compete with O_2 for binding at the HIF hydroxylase active site [63]. At least two models have been proposed to explain this observation. Under one scenario, inhibition of O_2 consumption in the mitochondria leads to a redistribution of O_2 within the cell, increasing the amount of O_2 available to the hydroxylases and other O_2-dependent enzymes [64]. A second, historically controversial, model involves the generation of ROS by the mitochondria under hypoxic conditions. These ROS are both necessary and sufficient to inhibit hydroxylase activity and to induce hypoxic HIF stabilization. Genetic and pharmacological inhibition of the electron transport pathway precludes ROS formation, blocking HIF accumulation under hypoxic, but not anoxic, conditions [59–61]. The precise mechanism by which ROS might inhibit the HIF prolyl hydroxylases requires additional study. While ROS might directly oxidize the enzyme-bound iron [65], indirect regulation of hydroxylase activity via signaling pathways responsive to ROS is also possible. Whether such regulation extends to FIH-1 has not yet been determined.

In addition to the direct and indirect effects of O_2 on enzyme activity, changes in O_2 availability can also influence expression levels of the HIF hydroxylases. Transcription of two of the HIF prolyl hydroxylase genes (HPH-1 and -2) is induced by HIF under hypoxia [29,66,67] creating a negative feedback loop that may serve to temper the magnitude of HIF induction as well as prime the cell for rapid HIF degradation upon reoxygenation. Conversely, it has been reported that the HPH-1 and -3 proteins are targeted for degradation under hypoxia, further enhancing HIF stabilization. This process mediated by Siah2, an E3 ubiquitin ligase found to be transcriptionally induced under hypoxia [68]. The interplay between these apparently competing pathways may provide a mechanism to adjust the relative levels of the three HIF prolyl hydroxylases, each of which appears to fulfill unique physiological roles by virtue of their differences in localization, substrate preferences, and O_2 responsiveness. The ability to fine tune the magnitude of HIF induction for the particular microenvironment of a given cell is likely of great importance as most tissues typically operate under borderline hypoxic conditions [57].

O_2-independent regulation of HIF hydroxylases

HIF hydroxylases employ both iron and ascorbate as cofactors while utilizing 2-oxoglutarate and O_2 as cosubstrates, each of which could be a potential target for regulation of these enzymes (Table 2). Removal of iron from the hydroxylases by many iron chelators can influence the enzymes in vitro and the hypoxic response in vivo [69]. Thus, the likelihood exists that any alteration of iron metabolism could also affect the HPH or FIH-1 enzymes. Similarly, the induction of the hypoxic response by divalent transition metals including Ni(II) and Co(II) was believed to occur by direct substitution of these metals for Fe(II) at the active site of the hydroxylases. While metal substitution has been seen for these enzymes in vitro [69], evidence is still lacking for metal substitution in vivo. Furthermore, metal ion movement within the cell is highly regulated with most ions bound to either small molecule or protein chaperones. One such small molecule chaperone is ascorbate, a critical cofactor for the HIF hydroxylases. New data have been presented for a model wherein addition of Ni(II) and Co(II) affects HIF hydroxylases by depleting the cellular stores of ascorbate [70,71]. While the data are in agreement with a model of ascorbate depletion, finer details of the precise amount and fluctuation of ascorbate in vivo could lend additional support [72].

A direct role for 2-OG availability as a regulator of HIF hydroxylase activity is likewise possible. Though 2-OG levels are generally presumed to be at such high levels that any changes in 2-OG would likely not affect HIF hydroxylase activity, these values may not accurately reflect nuclear and cytosolic 2-OG concentrations where the hydroxylases reside. Furthermore, the active site of this class of enzymes is rather promiscuous in accepting other small carboxylate compounds that may have profound effects on the activity of these enzymes. Increases in other metabolic 2-oxoacids, including pyruvate and oxaloacetate, can induce a hypoxic response presumably via binding to the active site of HIF hydroxylases in a manner analogous to 2-OG [73]. Activation of the hypoxic response has also been demonstrated using siRNA knockdown of succinate dehydrogenase, leading to an accumulation of succinate in the cell from approximately 120–450 mM. Higher levels of succinate are thought to decrease hydroxylase activity via product inhibition [74]. Whether a reduction in O_2's availability to serve as the terminal electron acceptor in the oxidative phosphorylation pathway might indirectly cause shifts in the concentrations of these metabolic intermediates within a physiologically

relevant range for hydroxylase regulation will require further study. Beyond the regulation of hydroxylase activity by O_2 and other metabolites, investigators are beginning to explore enzyme regulation through association with other protein factors [75,76] and via upstream signal transduction pathways [77,78].

Conclusions

Together these results inform our current model for O_2 sensing in the hypoxic response pathway (Fig. 1). Reminiscent of the monolithic sensor hypothesis, these oxygenases directly bind O_2. However, it is becoming increasingly clear that these enzymes also serve as focal points for the integration of multiple cellular inputs that indirectly convey cellular O_2 availability, redox status, and metabolic state.

Though members of the 2-OG-dependent dioxygenases had long been known to modify proteins, the discovery that hydroxylation could serve as a key regulatory modification, and that the responsible enzymes could themselves serve as cellular sensors, has added a compelling twist to their story. Bioinformatic approaches have revealed several additional proteins that structurally resemble the HIF hydroxylases, fueling speculation that such enzymes might play equally intriguing regulatory roles in other biological pathways. Aspartyl and asparaginyl hydroxylation of epidermal growth factor domains in several proteins [79] and prolyl hydroxylation of Skp1 [80], an adaptor protein present in several ubiquitin ligase complexes, have both been reported though the functional consequences of hydroxylation require further study. Recent evidence has indicated that hydroxylation might play a role in mediating the stability of iron response protein 2, a key posttranscriptional regulator of genes involved in iron homeostasis [81,82]. If true, it is tempting to imagine that the relevant hydroxylase might fill the role of cellular iron sensor in much the same way O_2 is sensed by the HIF hydroxylases. Still other candidate hydroxylase domains reside within putative DNA-binding proteins implicated as regulators of transcription. The identity of substrates for these enzymes, the nature of their regulation, and the consequences of the modifications they confer promise to be exciting discoveries on the horizon.

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