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Hypoxia Inducible Factor Prolyl 4-Hydroxylase Enzymes: Center Stage in the Battle Against Hypoxia, Metabolic Compromise and Oxidative Stress

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Abstract

Studies of adaptive mechanisms to hypoxia led to the discovery of the transcription factor called hypoxia inducible factor (HIF). HIF is a ubiquitously expressed, heterodimeric transcription factor that regulates a cassette of genes that can provide compensation for hypoxia, metabolic compromise, and oxidative stress including erythropoietin, vascular endothelial growth factor, or glycolytic enzymes. Diseases associated with oxygen deprivation and consequent metabolic compromise such as stroke or Alzheimer's disease may result from inadequate engagement of adaptive signaling pathways that culminate in HIF activation. The discovery that HIF stability and activation are governed by a family of dioxygenases called HIF prolyl 4 hydroxylases (PHDs) identified a new target to augment the transcriptional activity of HIF and thus the adaptive machinery that governs neuroprotection. PHDs lose activity when cells are deprived of oxygen, iron or 2-oxoglutarate. Inhibition of PHD activity triggers the cellular homeostatic response to oxygen and glucose deprivation by stabilizing HIF and other proteins. Herein, we discuss the possible role of PHDs in regulation of both HIF-dependent and -independent cell survival pathways in the nervous system with particular attention to the co-substrate requirements for these enzymes. The emergence of neuroprotective therapies that modulate genes capable of combating metabolic compromise is an affirmation of elegant studies done by John Blass and colleagues over the past five decades implicating altered metabolism in neurodegeneration.

Keywords

Hypoxia inducible factor; Prolyl 4-hydroxylase; Transcriptional regulation; Neuroprotection; Iron chelation

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Special issue dedicated to John P. Blass.

Introduction

'Transcriptional regulation' is a phenomena by which the amount and timing of appearance of the functional product of a gene is controlled. Processes critical to survival and death of cells such as metabolism are tightly regulated at the transcriptional level via intrinsic as well as extrinsic factors. The intrinsic transcription-machinery involves proteins called 'transcription factors' that bind to specific sites in DNA and recruit the basal transcriptional machinery, thereby regulating the process of gene expression. The regulation of activity of transcription factors is influenced by their protein levels or by post-translational modifications such as phosphorylation or acetylation. Signals that regulate transcription factor stability or activity are generated under physiological and pathological conditions. One of the key regulators of transcriptional activities of cells is oxygen.

Oxygen deprivation is a central feature of a number of neurological conditions including stroke, spinal cord injury and traumatic brain injury. Animals require oxygen to survive, and have evolved diverse mechanisms to sense and respond to low oxygen tensions. When faced with globally low blood oxygen levels (hypoxia), humans and other mammals reflexively engage a number of systemic physiological changes including an increase in the lung ventilation rate to restore normal oxygen tensions to vital organs. In addition to systemic homeostatic mechanisms to combat global hypoxia, organisms have also evolved cell intrinsic, homeostatic mechanisms that allow them to deal with both regional and global hypoxia [1,2]. The ability of a single cell to gauge the oxygen concentrations in its microenvironment and to protect itself through internal regulation is a fundamental process in species of animals that depend critically on oxygen to maintain cellular function [3]. The adaptation to a sustained reduction in oxygen availability necessitates changes in gene expression, which would be predicted to lead to reduced oxygen consumption and increased oxygen delivery, and provide a means of counteracting the detrimental effects of hypoxia [3,4]. This review will describe the cell intrinsic signaling pathways that are engaged to activate adaptive genetic responses to hypoxia in the brain and how these pathways may be manipulated for therapeutic advantage. Reduction or blockage of blood supply to the brain tissues, in conditions such as stroke, leads to a decrease in the supply of components such as glucose and oxygen necessary for the regular function of brain. After a few minutes without oxygen and glucose the cells of the brain begin to die. Among the cells that die are nerve cells. These nerve cells are responsible for controlling various parts and processes within the body. When these nerve cells cannot function properly, the body parts they are responsible for controlling also cannot function properly.

Unfortunately, dead brain cells are not spontaneously replenished and the loss of function caused by the death of these cells can be permanent. The severity and extent of the residual damage depends on the type of stroke, as well as the size and location. However there are circumstances when instead of being permanently damaged, the brain cells may be "in shock" or "bruised". When this is the case they may start functioning properly again after they have recovered and some lost abilities may return. To facilitate this recovery the most challenging task for researchers is to identify the pathways and/or molecules that are involved in the process and regulate the recovery mechanisms and to design tools that will help manipulate the activity of these pathways in favor of recovery. One of the main hypothesis that is tested in our laboratory is that the therapeutic manipulation of endogenous transcriptional programs of neuroprotection and repair will facilitate recovery after stroke. Our studies have led us to focus on the adaptive responses to hypoxia and hypoxia-ischemia as a strategy for accomplishing this goal.

The identification of the HIF transcription system by Wang and Semenza [5] was a milestone in our understanding of oxygen physiology. Since then the HIF system has emerged as a key

regulatory system of responses to hypoxia at both local and systemic levels. It is believed that approximately 1–1.5% of the genome is transcriptionally regulated by hypoxia and many of these genes are known to be regulated by HIF-1α. This remarkable protein or its homologs (e.g., HIF-2α, HIF-3α) appear to be synthesized constitutively in all of the cells of our body and constitutively degraded. Under conditions of hypoxia, hypoglycemia or hypoxia/hypoglycemia, an elegant series of molecular events are triggered that result in the stabilization of HIF proteins. The undegraded HIF-1α protein is free to dimerize with its constitutively expressed partner, HIF-1β Together HIF-1α and HIF-1β bind to specific hypoxia response elements in the promoters of a host of genes (e.g., erythropoietin, vascular endothelial growth factor, glycolytic enzymes) and activate expression of these genes (Fig. 1). These genes mediate biological changes (e.g., increased O_2 delivery, increased angiogenesis, increased anaerobic glycolysis) that facilitate adaptation to hypoxia and associated metabolic compromise.

In addition to its role in combating hypoxia, it is reasonable to propose that HIF-dependent gene expression can provide resistance to oxidative stress. This is because many of the genes regulated by HIF-1 or HIF-2 (e.g., erythropoietin, VEGF, MnSOD) can by themselves prevent oxidative stress-induced death [6-10]. Moreover, since activation of the HIF pathway is associated with a shift away from oxidative energy metabolism towards anaerobic energy metabolism, it is likely that this is accompanied by a decrease in mitochondrial free radical production [11,12]. The putative ability of HIF directed transcription to fortify antioxidant defenses and to reduce oxidant load is intriguing given the compelling evidence for the role of oxidative stress in the destructive consequences of stroke. Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of oxidants, potentially leading to damage” [13]. Recent evidence has pointed out that reactive oxygen species (ROS) generation occurs at multiple time points after stroke [14-16]. Oxidative stress in stroke develops as a result of excitotoxicity and inflammation leading to necrosis or apoptosis [17]. Pharmacological and genetic strategies that reduce oxidative injury also decrease brain damage. Accordingly, oxidative stress is an established target for drug development in stroke. The protection of many cellular components may be achieved by reducing oxidative damage by treating with antioxidants [18]. Activation of HIF by small molecule “drugs” potentially represents a distinct therapeutic strategy for treating oxidative stress. The advantage of this strategy as compared prior “antioxidants” is the ability of a single drug to activate more than seventy genes that could provide adaptation to ischemia and oxidative stress [19,20]. However, the specificity of these pharmacological agents remains to be the main limitation factor in their use as therapeutic agents.

As mentioned above, one of the best-described pathways involved in adaptation to hypoxia, an important component of ischemia, includes the transcription factor, HIF-1. Studies on the expression of HIF-1 and its target genes in adult rat brain have shown that after focal cerebral ischemia, mRNAs encoding HIF-1α, glucose transporter-1 and several glycolytic enzymes including lactate dehydrogenase were up-regulated in the areas around the infarction [21]. HIF and its target genes are induced 7.5 h after the onset of ischemia and increased further at 19 and 24 h [22]. Similar findings were obtained in a rat neonatal stroke model [23]. These findings along with parallel studies in HIF-1α knockout animals indicate that HIF-1α is an essential component in changing the transcriptional repertoire of tissues as oxygen levels drop in animal models. Recent studies indicate that individuals with a certain HIF polymorphism are more likely to have a stroke [24]. These studies suggest that HIF-1 is an important target for stroke drug development in humans.

Regulation of hypoxic adaptation by HIF prolyl 4-hydroxylases

HIF is an oxygen-labile protein that becomes stabilized in neurons response to hypoxia, iron chelators and divalent cations such as cobalt chloride ($CoCl_2$) [25]. The levels of HIF-1α are

regulated by the activity of a group of enzymes called HIF-prolyl 4-hydroxylases (PHDs) (for general nomenclature, please refer to Table 1). These enzymes use iron, oxygen and 2-oxoglutarate to hydroxylate proline residues (pro-564 or pro-402) (amino acid positions designated in humans) in the HIF-1α molecule, allowing it to bind to the von Hippel-Lindau (pVHL)-E3 ubiquitin ligase complex, von Hippel-Lindau (pVHL) and undergo proteasomal degradation. So far three different iso-forms of PHDs have been identified, HIF-prolyl 4-hydroxylase-1:2 and 3 [27]. They share homology in the C-terminal catalytic domain but have significant difference in N-terminal sequences (discussed in detail in section 5).

The discovery of PHDs identified a new aspect of HIF regulation and indeed PHDs have become an attractive target for controlling HIF activity for therapeutic purposes. Pharmacological inhibition of these enzymes show that they can serve as a potential therapeutic target for the treatment of conditions that occur as a consequence of ischemia in a host of organs including the brain [27-34]. We previously used an in vitro model of oxidative stress to correlate the protective effects of iron chelators with their ability to activate HIF-1 [25]. In this scheme, iron chelators inhibit the activity of PHDs. Inhibition of PHDs prevents the attachment of an OH group (hydroxylation) to phylogenetically conserved proline residues at amino acid 402 and 564 in the protein HIF-1α [35]. Unhydroxylated HIF-1α does not bind the E3 ubiquitin ligase, von Hippel-Lindau protein [29,30,36]. HIF-1α is thus not ubiquitinated and degraded by the proteasome. Once stabilized, nuclear HIF-1α can heterodimerize with its partner, HIF-1β, bind to the pentanucleotide hypoxia response element (RCGTG) [37] in gene regulatory regions and increase the expression of established protective genes including vascular endothelial growth factor (VEGF) and erythropoietin (Epo) [2,38]. According to this model, the protective effects of iron chelators may include inhibition of iron-dependent PHDs. In a more recent study conducted in our laboratory we have used small molecules and peptides that don't bind iron, but do inhibit the PHDs to show that PHD inhibition is sufficient to inhibit neuronal death due to oxidative stress in vitro and permanent focal ischemia in vivo [31].

Proline hydroxylation

Reaction mechanism

HIF Prolyl 4-hydroxylase enzymes (PHDs) belong to a superfamily of 2-oxoglutarate dependent hydroxylases, which employ non-heme iron in the catalytic moiety [39]. They require oxygen in the form of dioxygen, with one oxygen atom being incorporated in the prolyl residue, and the other into 2-oxoglutarate, yielding succinate and CO_2 (Fig. 2). The 2-oxoglutarate is stoichiometrically decarboxylated during hydroxylation, with one atom of the O_2 molecule being incorporated into the succinate and the other into the hydroxyl group formed on the proline residue. Past studies have made it possible to elucidate the reaction mechanism and structural features of the catalytic sites of dioxygenase enzymes [40-43]. The catalytic mechanism of these enzymes can be divided into two half-reactions: initial generation of the hydroxylating species, and its subsequent utilization for 4-hydroxyproline formation [40,41]. Fe^{2+} is located in a pocket coordinated with the enzyme by three side-chains with two histidines and an aspartate forming the catalytic triad [41,44,45] (Fig. 3). Molecular oxygen is bound to the Fe^{2+} end-on in an axial position interacting with the dioxygen unit [41] (Fig. 3). One of the electron-rich orbitals of the dioxygen unit is directed to the electron-depleted orbital at the C_2 of the 2-oxoglutarate bound to the iron (Fig. 3A), so that the C_2 undergoes rehybridization and forms a covalent bond with the noncoordinated atom of the dioxygen unit. This weakens both the C–C bond in the 2-oxoglutarate and the O–O bond in the dioxygen [40,41]. Decarboxylation then takes place simultaneously with the cleavage of the O–O bond leading to the formation of succinate. At the same time, a ferryl ion is formed, which hydroxylates a proline residue in the peptide substrate in the second half-reaction (Fig. 3B and C) [40,41].

Although the PHD-mediated hydroxylation reaction is inherently dependent on ambient oxygen pressure, providing a molecular basis for the oxygen-sensing function of these enzymes [46], inhibition at other cofactor binding sites by 2-oxoglutarate analogs or iron chelators is equally capable of inhibiting the activity of the enzyme under normoxic conditions [31]. The sensitivity of the PHD activities in response to changing levels of fundamental molecules, like oxygen, 2-oxoglutarate and iron, is a very interesting and useful scheme for engaging cellular adaptive responses. A change in the cellular levels of oxygen, iron or 2-oxoglutarate could have deleterious consequences. The resulting interruption in the activity of prolyl 4-hydroxylases leads to the activation of rescue mechanisms that help the cell survive. This awards the PHD activity a potentially central role in the scheme of neuroprotection due to hypoxia, iron or 2-oxoglutarate deficiencies and makes them a specific controllable target to achieve this goal.

Oxygen

HIF Prolyl 4-hydroxylases appear to play a very important role in ensuring tight regulation of oxygen homeostasis in the brain to avoid metabolic compromise. The striking sensitivity of PHDs to graded levels of oxygen in vitro, mirrors the progressive increase in HIF-1 protein stability and transactivation activity observed when cells are subjected to graded hypoxia [27,47]. The arterial pO_2 has been reported to be around 90 mm Hg. PHDs have been found to have a striking low O_2 affinity of 178 mm Hg- above the dissolved normobaric partial pressure of O_2 in the air. The affinity of PHDs for O_2 is much lower compared to collagen prolyl-4-hydroxylases that exhibit a K_m of about 28 mm Hg [28]. A more recent study using different lengths of the HIF C- and N-terminal peptides shows about 40% change in the affinity of different isoforms of PHDs for oxygen [48]. This low affinity allows the PHDs to operate in a highly sensitive manner, in which small changes in oxygen concentration result in pronounced changes in enzymatic reaction velocity and thus HIF-1α turnover. Studies have shown that HIF-1α levels are generally low in rodent tissues under physiological conditions and are substantially increased in response to systemic hypoxia or tissue ischemia [49,50]. Interestingly, HIF-1α levels remain low even in regions of these animals such as the renal medulla, which are characterized by low oxygen tensions. Furthermore, the extent and time course of induction as well as cell-type-specific expression of HIF-1α varies, suggesting that individual, cell-specific thresholds for activation of the response may exist. Disturbances in oxygen availability have been implicated in the central nervous system (CNS) pathology of a number of disorders including stroke, head trauma, neoplasia, vascular malformations and chronic neurodegenerative diseases. Specific redox sensitive transcription factor systems including HIF-1, nuclear factor kB (NF-kB), activator protein 1 (AP-1) and early growth response protein-1 (EGR-1), have been described that respond to changes in pO_2 or an excess in reactive oxygen species (ROS) by activation of appropriate target gene expression [51,52]. As mentioned above, the sensitivity of PHD to ambient oxygen levels in cells suggests a possible connection between these disorders and the activity of PHDs that needs to be explored.

Iron

In addition to pO_2, PHD activity is regulated by the presence of ferrous iron (Fig. 3) [53]. Indeed, it had been known for some time that iron chelators or $CoCl_2$ function as hypoxia "mimics". Both agents stabilize HIF-1α and induce the expression of HIF target genes. Of note, they also can protect immature cortical neurons from oxidative stress [25,31,54]. The identification of PHDs as enzymes that contain a jellyroll motif with conserved iron and 2-oxoglutarate binding residues provided a ready explanation for the ability of iron chelators and $CoCl_2$ to stabilize HIF. Treatment with $CoCl_2$ also leads to stabilization of HIF-1α and a marked up-regulation of its target genes [25,55]. Accordingly, this may be due to at least three mechanisms: partial inhibition of PHDs by depletion of ascorbate, which is required to maintain the PHDs in an active state, and/or direct binding of cobalt to HIF-1α, which may prevent its

degradation by VHL-dependent and VHL-independent pathways. However, there have been recent reports that show that the enzyme retained a significant amount of activity after treatment with iron chelators such as Desferrioxamine (DFO). It was shown that only about 40–60% of the PHD activity was inhibited in vitro by DFO [55]. The studies show that the K_m values for iron for PHDs are about 0.03–0.1 μM, significantly below the normal labile pool of iron (0.3–0.8 μM) [56]. Since there is a striking increase in not only HIF-1 protein stabilization but its target genes after DFO treatment, it is necessary to evaluate the decrease in the activity of these enzymes that is required to stabilize HIF-1 and downstream target genes in vivo. Using in vivo imaging system using animals expressing reporters coding for luminescent proteins such as luciferase will provide a better understanding of the effects of the pharmacological inhibitors of these enzymes in vivo [57].

Indeed HIF-1α is regulated in an iron- and redox-sensitive fashion by the HIF-prolyl 4-hydroxylases (PHDs). Oxidation of Fe^{2+} to Fe^{3+} may underlie the ability of ROS to promote HIF-1α buildup via inactivation of these enzymes under physiological conditions and in cancer [58]. Reports suggest that cysteine and histidine can also modulate iron redox status in a manner similar to ascorbate (Fig. 4) [60-62] and the relative cellular levels of these substances or of glutathione could thus play a significant role in determining the basal HIF-1α degradation rate of many cells. Deficiencies in iron reducing capacity may possibly produce greater PHD inactivation in neoplastic cells than in normal cells [63]. Treatment of embryonic cortical neurons with molecules that chelate iron such as DFO leads to inhibition of activity of the PHDs and HIF-1 upregulation [31] Fig. 4).

Studies show that cancer cells deficient of JunD, a transcription factor involved in cell proliferation, generate excessive ROS and accumulate HIF-1 in an ascorbate- and cysteine-reversible manner [60]. A similar mechanism may underlie HIF-1 activation by ROS produced during cytokine signaling [64] or radiation therapy [65]. Similarly, redox regulatory systems such as Thioredoxin or Ref-1 have been shown to induce HIF-1 stabilization and transactivation function [66-70].

2-oxoglutarate

As shown in Fig. 3, the PHDs require a molecule of 2-oxoglutarate as a co-substrate to catalyze the transfer of a hydroxyl group at the proline 564 and 402 on HIF-1 protein. Structural analogue of 2-oxoglutarate such as dimethyl-oxalyl-glycine (DMOG) inhibit the activity of these enzymes. Normal levels of 2-oxoglutarate in various tissues range from 50 to 230 μM [71], reports suggest that similar to oxygen, the affinity of PHDs for 2-oxoglutarate is very low i.e., ~60 μM as compared to that of collagen prolyl 4-hydroxylases that is 20 μM [55]. This low affinity provided high sensitivity towards changes in the levels of this co-substrate that might occur due to defects in other metabolic pathways such as Krebs cycle. Defects in the Krebs cycle are likely to influence 2-oxoglutarate levels compromising PHD function. Recent reports show that some other metabolic intermediates of TCA cycle such as pyruvate and oxaloacetate also effect PHD function in an isoform dependent manner [72]. Accordingly, pyruvate and oxaloacetate can activate HIF by binding reversibly to the 2-oxoglutarate site of two distinct isoforms of HIF-1 prolyl 4-hydroxylases, PHD2 and PHD3, at concentrations comparable to their normal physiological levels (Fig. 4). Both pyruvate and oxaloacetate can also induce expression of prolyl 4-hydroxylase isoforms 1 and 2 genes in human glioblastoma cells as a mechanism for limiting hypoxia-inducible gene expression and also for adapting to re-oxygenation. There are contradictory reports regarding the effects of other TCA cycle metabolites such as fumarate and succinate on HIF-1 activation [59,73-75]. Little is known about the involvement of PHDs in pathological settings such as neoplasia or neurological disorders. However, reports suggest that PHD may be a target of growth factor signaling pathways and/or oncogenic transformation. It has been shown that certain oncogenes such as

ras and *src* induce HIF-1 under normoxia by inhibiting prolyl hydroxylation on Pro 564 [76]. Recent findings also suggest a possible link between PHD function, tumorigenesis and neurodegeneration. Accordingly, the two ubiquitously expressed mitochondrial enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH), catalyze sequential steps in the Krebs cycle. Interestingly, mutations in the autosomally encoded enzyme and enzyme subunits are associated with hereditary predispositions to various tumors [77-80]. Thus, both SDH and FH act as tumor suppressor genes. In contrast, homozygous germline mutations in SDH subunits cause mitochondriopathies such as Leigh's syndrome, which affect the central nervous system and skeletal muscles. The underlying mechanism through which loss of function leads to neoplasia or neurodegeneration remains unclear [81]. It is therefore likely that defects in the Krebs cycle influence 2-oxoglutarate levels or other metabolic intermediates compromising PHD function.

PHDs—beyond HIF-1

The above discussion suggests that it is reasonable to hypothesize that PHDs act as iron- and redoxsensors. It also strengthens the idea that PHDs are contributing towards the cell's fate via multiple mechanisms. There is indirect evidence that PHDs interact with molecules other than HIF-1 such as iron regulatory protein-2 (IRP2), RNA polymerase II (RNA pol II), mitogen-activated protein kinase organizer-1 (MORG-1) and other isoforms of HIF itself.

PHDs and other HIFs

HIF-2*α* and HIF-3*α* are the other isoforms of HIF-1*α* [82-84]. Like HIF-1*α*, HIF-2*α* (also known as endothelial PAS protein-1, EPAS-1) is also upregulated by hypoxia and activates transcription via binding of hypoxia response elements (HREs) and is regulated in the same way as HIF-1 [76,83,85-88]. The HIF-1*α* and HIF-2*α* proteins share only 48% identity, but they both contain the conserved oxygen degradation domain [89,90], and are regulated by prolyl 4-hydroxylases. There is increasing evidence that HIF-1*α* and HIF-2*α* may have different functions [91-94]. For example, the targeted inactivation of HIF-1*α* and HIF-2*α* genes in embryonic stem cells results in different responses to various stimuli, such as hypoxia and hypoglycemia [95] and HIF-1$\alpha^{-/-}$ and HIF-2$\alpha^{-/-}$ knock out mouse embryos have different and variable phenotypes [96-98]. HIF-3*α* has high similarity with HIF-1*α* and HIF-2*α* in the regulatory oxygen degradation domains (ODD), but lacks structures for transactivation that are found in the C-terminus of HIF-1*α* and HIF-2*α*. The activity of HIF-3*α* is upregulated in low oxygen, it dimerises with HIF-*β*/ARNT and binds to the HRE core sequence, but interestingly, HIF-3*α* appears to suppress hypoxia-inducible HIF-mediated gene expression in the human kidney and could therefore be a negative regulator of hypoxia-inducible gene expression [99,100]. These findings create a very interesting scenario with reference to the consequences of PHD activity. The differences in the functions of different isoforms of HIF*α* regulated by the prolyl 4-hydroxylases indicates that PHDs work via multiple molecules/pathways depending on the stimuli and physiological state of the cell that define the overall outcome of the event.

PHDs and IRP2

Oxidative stress is a hallmark of neurodegenerative disorders [101], such as Parkinson's and Alzheimer's diseases, where iron accumulates pathologically in the substantia nigra [102] or in senile plaques [103], respectively. Even though it is still not clear whether this is a primary cause or a secondary effect, iron is increasingly being considered as a pathogenic cofactor [104,105]. IRP2 is regulated in response to the iron and oxygen supply. It is synthesized de novo under conditions of iron starvation, [106] remains stable in iron-starved or hypoxic cells, [107] and undergoes proteasomal degradation in iron-replete and normoxic cells [108]. Both iron and oxygen are required for the activity of PHDs. Pharmacological data suggest that a

pathway for iron-mediated degradation of IRP2 requires the activity of PHDs [109-111], the co-substrate analogue dimethyl-oxalyl-glycine (DMOG) can efficiently inhibit the iron-dependent degradation of IRP2 in cells pretreated with DFO [109,112]. The reports show that when human embryonic kidney cells are treated with an iron chelator, DFO, the IRP2 is stabilized, this effect is diminished by the addition of iron. However, addition of DMOG, a PHD inhibitor, diminishes the iron-induced degradation of IRP2. Accordingly, the pathways for iron- and oxygen-dependent degradation of IRP2 and HIF-1 share remarkable similarities [107]. However, DMOG fails to inhibit IRP2 degradation in cells not pretreated with DFO suggesting that this pathway operates in conjunction with the levels of the labile iron pool (LIP) and is activated only when previously iron-depleted cells are exposed to iron. These findings provide the groundwork for the identification of components of the pathway, including the 2-oxoglutarate-dependent enzymes in future.

PHDs and RNA Pol II

The von Hippel-Lindau (pVHL) protein regulates the accumulation of HIF-1 by acting as an E3 ubiquitin ligase complex, [29,30,36,113], and thereby regulates the expression of hypoxia-inducible genes. The pVHL complex can function as an E3 ligase, which targets the hyperphosphorylated large subunit (Rpb1) of RNA polymerase II for ubiquitination and degradation [114]. Importantly, binding of pVHL to the full-size Rpb1 requires hydroxylation of proline-1465 within Rpb1 and phosphorylation of the carboxy terminal domain (CTD) [114-116]. Immobilized Rpb1 peptide (amino acids 1440–1475) containing the hydroxylated proline binds to pVHL, whereas the nonhydroxylated peptide does not bind pVHL as shown by the Kornberg laboratory [117]. This reaction occurs in a manner similar to HIF-1 regulation. However, it acquires pVHL-binding properties after incubation with PC12 cell extracts in the presence of Fe^{2+}, ascorbic acid, and 2-oxoglutarate, cofactors required for PHD activity [114]. The hydroxylated, but not the nonhydroxylated, peptide competes with HIF for pVHL binding [114]. These data provide biochemical evidence that Rpb1 can be modified by proline hydroxylation. However, it is unclear whether proline hydroxylation requires CTD phosphorylation. Comparison of the sequence of the HIF-1α oxygen dependent degradation domain (ODDD) with representative libraries of protein structures revealed an identical region with similarities to a fragment of Rpb1 and the adjacent Rpb6 subunit. An almost 50-amino acid Rpb1 counterpart is 30% identical to HIF-1α and contains the L(XY)LAP motif, including proline at position 1465 similar to the proline residue at position 564 in HIF-1α. Computational models and experimental observations demonstrate significant structural similarity between the ODDD of HIF-1 and Rpb1/Rpb6. It is therefore reasonable to predict that the stability and thus function of RNA pol II in regulation of gene expression requires proline hydroxylation. The direct interaction between the PHDs and the Rbp1 and Rbp6 is thus an important piece of information that has yet to be provided by any group.

PHDs and MORG-1

The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK)-1 and ERK-2 are components of an evolutionarily conserved protein kinase cascade that participates in the regulation of various cellular processes, including gene expression, growth, differentiation, and apoptosis. A number of “scaffolding proteins”, both yeast and mammalian, were shown to associate with and enhance functional interaction of the components of MAPK pathways, although their physiological and molecular functions are incompletely understood. MAPK organizer (MORG)-1, which interacts with several components of the ERK cascade and functions as a scaffold protein linking ERK responses to specific agonists. Recent evidence shows that MORG-1 interacts with PHD3 and decreases HIF-1 mediated reporter gene induction [118]. It is interesting that PHD3 has been recently been implicated in the survival of sympathetic neurons following growth factor deprivation [119]. The physiological significance of MORG-1 interaction with PHD3 remains to be elucidated, however, as this

report shows an interaction between the non-conserved region of PHD3 and a novel interactor MORG-1, it presents an important example that suggests yet another possible mechanism/means to modify the activity of PHDs in a very isoform specific manner since the three isoforms differ in their non-conserved regions. Such interactions also broaden the search for novel molecules that might be interacting with PHDs and effecting their activities, but do not contain potential hydroxylation sites in the primary structure. Detailed study of these novel interactions will not only identify novel pathways that are involved in the PHD regulated biology. The direct effect of these interaction on the overall well being and survival of neurons will not only uncover new pathways and targets for PHDs but will also help define the precise role of distinct PHD isoforms in neuroprotection.

PHDs and SIAH 2

Changes in oxygen affect PHD transcription and stability in addition to directly affecting PHD enzymatic activity [27,120,121]. Hypoxia also induces the accumulation of the ring finger protein seven in absentia (Drosophila) homolog 2 (SIAH2) [122] possibly in a HIF-independent manner [123]. SIAH2 is highly conserved mammalian homolog of drosophila seven in absentia (SIAH), which possesses potent RING finger E3 ubiquitin ligase activities. This activity is implicated in the degradation of diverse proteins including DCC, β-catenin, N-CoR, c-myb, Numb, and TRAF2 [124-129]. Analysis of steady-state levels of exogenously expressed PHD3 revealed almost undetectable expression in $SIAH2^{+/+}$ cells, whereas it was clearly detected in $SIAH2^{-/-}$ cells [123], showing that SIAH2 negatively regulates PHD3 levels. The same report shows that SIAH2 poly ubiquitylates, and hence tags PHD1 and PHD3 for destruction [123]. The regulation of PHDs by the SIAH proteins indicates a feedback mechanism of PHD regulation that controls the activity of PHD3. A more recent study [130] shows that the isoform specific interaction of Siah2 with the PHD3 is due to the differences in the structure of the three molecules. The amino terminal domains of PHD1 and PHD2 limit their direct interaction with SIAH2 and that PHD3 can form complexes which include homo- and hetero-dimers/multimers, and that assembly of PHD3 into complexes affects its activity towards HIF-1α and susceptibility for degradation by SIAH2.

Isoforms of HIF-Prolyl 4-hydroxylase enzymes

To date three isozymes of the mammalian prolyl 4-hydroxylases specific to HIF-1α have been identified: PHD1, PHD2 and PHD3 [27] (corresponding to human HPH3, HPH2 and HPH1 [26] and *C. elegans* EGLN2, EGLN1, EGLN3 [131] (Table 1). All three orthologs appear to be a product of gene duplication as there is only one gene in *C. elegans* (EGL-9) [27] and *D. melanogaster* (CG1114) [132]. The three isoforms differ in expression regulation, tissue distribution, cellular localization, and ability to hydroxylate HIF-1α (Table 2) [38,123]. They share homology in the C-terminal catalytic domain but have significant difference in N-terminal sequences [133-135].

PHD1 is a 43.6 kDa constitutively expressed protein that has no response to hypoxia or $CoCl_2$ at mRNA level [136] but is induced by estrogen and stimulates cell proliferation [137]. PHD1 has high level of expression in the testis with low level expression in the kidney, liver and heart [138-140]. It was found that this protein localizes exclusively to the nucleus [134]. As mentioned before, there are two oxygen dependent domains (ODD) in HIF-1α. A C-terminal ODD (CODD) (around proline 402) and an N-terminal ODD (NODD) (around proline 564). PHD1 hydroxylates both N- and C-terminal ODD in HIF-1α. Two isoforms of PHD1 with molecular weights of 43 and 40 kDa, respectively are generated by alternative translational initiation [141]. Both PHD1 isoforms have similar HIF-1α prolyl 4-hydroxylase activity. Although it is commonly accepted that PHD1 is not transcriptionally upregulated by low oxygen tensions, a recent report shows upregulation of PHD1 mRNA in HT22 cells as well as in brains of hypoxic mice [142].

PHD2 is a 46 kDa protein whose expression can be regulated by hypoxia, DFO or $CoCl_2$, all established inhibitors of prolyl 4-hydroxylase activity [134,143]. Basal expression levels are high in heart and moderate in brain. The majority of PHD2 is localized to the cytoplasm with lower levels in the nucleus [134]. The dominant cytoplasmic localization of PHD2 in human osteosarcoma cells (U2OS) and human hepatoma cells (Hep3B) suggest about active exclusion of this isoform from nucleus [134]. Groulx and Lee provide evidence that the degradation of HIF-1α is initiated by strictly nuclear prolyl hydroxylation and is completed by the export of HIF-1α-pVHL complexes [144]. PHD2 hydroxylates both N-and C-terminal ODD in HIF-1α Studies have shown that although all three isoforms hydroxylate specific prolines in HIF subunits in a strongly conserved LXXLAP sequence motif (where X indicates any amino acid and P indicates the hydroxyl acceptor proline), mutation in the amino acid residues in the vicinity of these amino acids was least tolerated by PHD2 thus showing the highest specific activity toward the primary hydroxylation site of HIF-1α [27,133,134]. PHD2 has also been shown to have more influence over HIF-1α than HIF-2α [145]. Only inhibition of PHD2 but not PHD1 or PHD3 by siRNA is sufficient to upregulate HIF-1α in normoxia in various cell types such as HeLa (human cervical carcinoma cell line), CAL27 (human tongue squamous cell carcinoma cell line), HaCAT (Human keratinocyte cell line), HT29 (colon adenocarcinoma cell line), CAL51 (breast cancer cell line), RCC4/pVHL (RCC4 stably transfected with pcDNA3pVHL), WM9 (melanoma cell line) and FHN (primary fibroblasts) [146]. On the other hand, inhibition of HIF-1α by siRNA also leads to a loss of upregulation of PHD2 and PHD3 in hypoxia [147]. A hypoxia response element (the binding site for HIF-1) has been found in the human PHD2 gene. It is located at 412 bp upstream of start codon [148]. Analysis of PHD2 mRNA expression level in different organs of rat following hypoxia (8 h at 8% oxygen) showed its reduction in brain, whereas in heart and kidney there were no changes in the PHD2 mRNA levels. Liver on the other hand showed an increase in the mRNA levels of PHD2 [140]. Interestingly, the brain was the only organ where PHD2 mRNAs (and PHD3 mRNA in two out of three rats) were downregulated after 8 h systemic hypoxia. This does not appear to be a toxic effect since levels of the other genes analyzed were preserved or enhanced. The mechanism of this PHD downregulation is unknown, but it could potentially lead to enhanced HIF accumulation, mediating possible cerebral protective effects [140].

The third identified isoform, PHD3 is a 27.3 kDa protein. HRE consensus sequences in a region of 40 kb around the human EGLN3 gene have been identified [149]. PHD3 expression can be upregulated by hypoxia or the hypoxia mimetics, DFO or $CoCl_2$. It is distributed evenly in nucleus and cytoplasm [134]. Despite it being an oxygen-dependent enzyme, PHD3 retains significant activity under conditions of hypoxia with greater activity towards HIF-2α [145] as opposed to HIF-1α, similar observation has been reported for PHD1. PHD3 preferentially hydroxylates CODD PHD3 hydroxylates the CODD but not NODD [27,28,145,150]. In addition to full length PHD3 (27 kDa) at least two alternative slice forms have been found with predicted molecular weight 17 kDa and 24 kDa, respectively [151]. Expression patterns in the tissue show that the full length of PHD3 is detected as major product and the 17-kDa protein is expressed in the same tissue as a minor product. This small splice version has no prolyl 4-hydroxylase activity. In contrast the alternative splice variant that encodes a 24-kDa protein preserves prolyl 4-hydroxylase activity. It is interesting that the 24 kDa protein has been found only in primary cancer tissues [151]. Both active versions of PHD3 in Hep3B cells are localized to the nucleus. An interesting homolog for PHD3 reported in the literature is SM-20. SM-20 is a growth factor regulated gene in muscle cells that can promote cell death in neurons [131, 152,153]. The extremely high degree of sequence identity between SM-20 and PHD3 indicates that they represent orthologous proteins in rats, mouse and humans [131].

Conclusion

The above discussion identifies some fundamental gaps in our knowledge of how PHD enzymes operate in brain: Are the neuroprotective effect of PHD inhibition observed after treatment with global inhibitors of PHDs mainly driven by inhibition of one isoform? If so which one? What is the subcellular localization of that isoform in brain? Is it localized in cellular compartments abundant in HIF-1? As discussed above, each one of these isoforms has distinct roles and specificity, depending upon their subcellular localization in different organs and cell types, however very little is known about these details in different regions and cell types in brain. Moreover the role of these enzymes in regulating mechanisms (other than HIF stabilization) that might be involved in the process of cell survival is also garnering support. Growing awareness of other substrates such as IRP2 [109] and RNA polymerase [114] or the RNA polymerase heavy subunit whose stability can be regulated by a hydroxylation reaction suggests that other plausible schemes in addition to, or exclusive of HIF-1 that may account for protection by HIF prolyl 4-hydroxylase inhibition. Identification of the isoform(s) of prolyl 4-hydroxylase enzymes that is neuroprotective and its novel target(s) to pinpoint the pathways that are regulated during this neuroprotection. This will allow researchers to design tools/molecules that will facilitate the manipulation of the activity and function of the relevant PHD isoform(s) as a therapeutic target in stroke and other diseases associated with metabolic compromise such as Alzheimer's disease. As metabolic compromise is firmly rooted in the pathophysiology of neurodegeneration, PHD inhibitors are poised to take center stage in CNS therapeutics. However, one important missing link for this approach is the risk associated with chronic administration of PHD inhibitors. For instance, transcriptional regulation of cell survival pathways need to be strictly controlled to eliminate risk of tumor growth. Further investigations are required to understand the risks as well as the possibilities to eliminate the risk by combinatorial therapy approaches.

Abbreviations

AP-1, Activator protein 1
CO_2, Carbon dioxide
$CoCl_2$, Cobalt chloride
CODD, Carboxy-terminal oxygen degradation domain
DFO, Desferrioxamine
DMOG, Dimethyl-oxalyl-glycine
EGR-1, Early growth response protein-1
ERK, Extracellular signal-regulated kinase
Fe^{2+}, Ferrous
Fe^{3+}, Ferric
FH, Fumarate hydratase
HIF, Hypoxia inducible factor
HIF-1α, Hypoxia inducible factor-1 alpha
HIF-1β, Hypoxia inducible factor-1 beta
HRE, Hypoxia response elements
IRP2, Iron regulatory protein-2
LIP, Labile iron pool
MnSOD, Manganese Superoxide Dismutase
MORG-1, Mitogen-activated protein kinase organizer-1
NF-kB, Nuclear factor kappa B
NODD, N-terminal oxygen degradation domain
O_2, Oxygen
ODD, Oxygen degradation domain
ODDD, Oxygen dependent degradation domain

PHD, prolyl 4-hydroxylase domain
pVHL, von Hippel-Lindau protein
RNA pol II, RNA polymerase II
ROS, Reactive Oxygen Species
SIAH2, Seven in absentia homolog 2
SDH, Succinate dehydrogenase
TCA, Tricarboxylic acid
VEGF, Vascular endothelial growth factor

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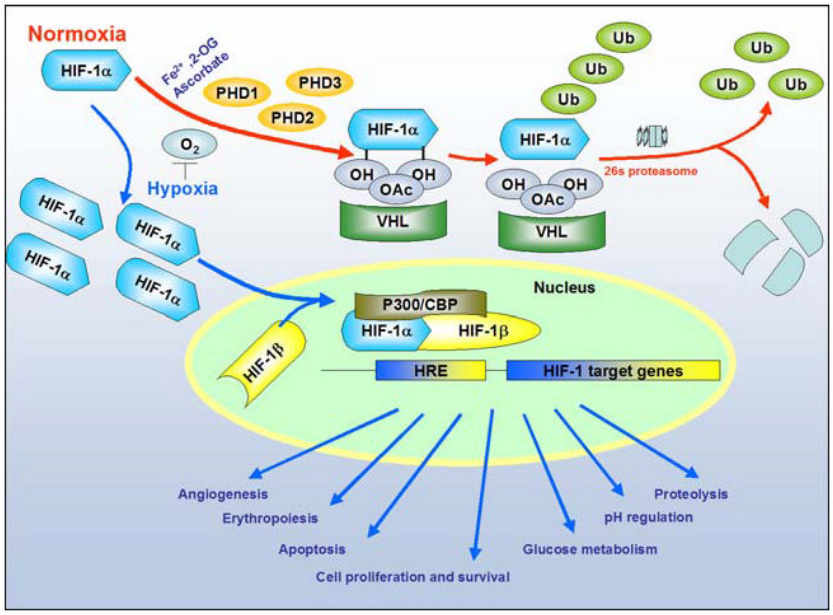

Fig. 1.

Cellular response to hypoxia: Levels of Hypoxia Inducible Factor-1 (HIF-1) are regulated by cellular oxygen by proline hydroxylation. The reaction is catalyzed by the enzymes prolyl 4-hydroxylases (PHD1:2 and 3). Under normoxia (red arrows), the intracellular level of HIF-1*α* is kept low by rapid ubiquitination and subsequent proteasomal degradation via recruitment of von Hippel-Lindau protein (pVHL), which depend on the hydroxylation of proline residues. In contrast, under hypoxia (blue arrows), both the intracellular level and the transcriptional activity of HIF-1*α* increase as a result of suppressed PHD activities. Consequently, HIF-1*α* forms a heterodimer with HIF-1*β* and changes the transcriptional rates of HIF-1-regulated genes under hypoxia (NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article)

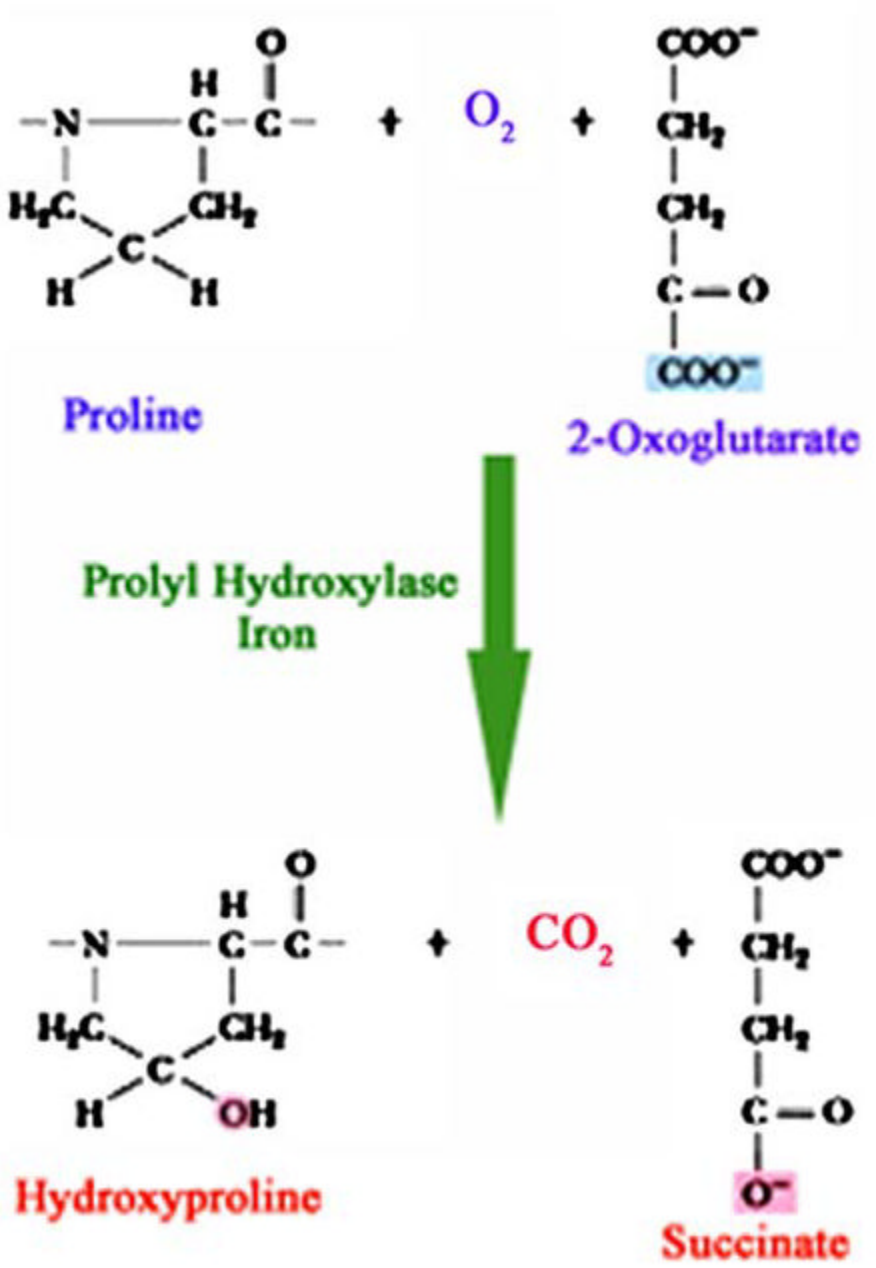

Fig. 2.
Schematic representation of the reaction catalyzed by prolyl 4-hydroxylases. The 2-oxoglutarate is stoichiometrically decarboxylated during the hydroxylation reaction in the presence of dioxygen and iron, resulting in the generation of carbon dioxide (CO_2) and succinate

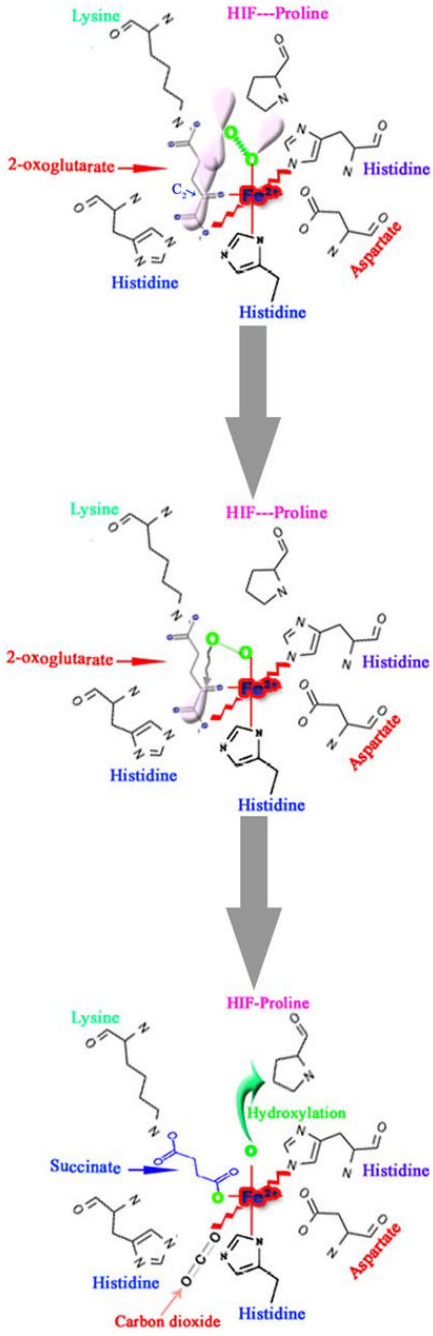

Fig. 3.

Schematic representation of the first half-reaction of prolyl 4-hydroxylase that takes place within the coordination sphere of the catalytic site iron (Fe^{2+}) and the critical residues at the co-substrate binding sites. The Fe^{2+} is coordinated with the enzyme by two histidines (His) and an aspartate (Asp). The 2-oxoglutarate-binding site can be divided into two main subsites; subsite I consists of a Lysine residue (Lys), which ionically binds the C_5 carboxyl group of 2-oxoglutarate, while subsite II consists of two cis-positioned equatorial coordination sites of enzyme-bound Fe^{2+} and is chelated by the C_1 carboxyl and C_2 oxo functions. Molecular oxygen is assumed to be bound end-on in an axial position, producing a dioxygen unit. (**A**) One of the electron-rich orbitals of the dioxygen is directed to the electron-depleted orbital at the C_2 of

the 2-oxoglutarate bound to the iron. (**B**) Nucleophilic attack on C_2 generates a tetrahedral intermediate, with loss of the double bond in the dioxygen unit and of double bond characteristics in the oxo-acid moiety. (**C**) Elimination of CO_2 coincides with the formation of succinate and a ferryl ion, which hydroxylates a proline residue in the peptide substrate in the second half-reaction. An additional amino acid in the vicinity of the catalytic triad is an important residue histidine, probably being involved in both coordination of the C_1 carboxyl group of 2-oxoglutarate to Fe^{2+} and cleavage of the tetrahedral ferryl intermediate (Modified from Kivirikko et al. [43])

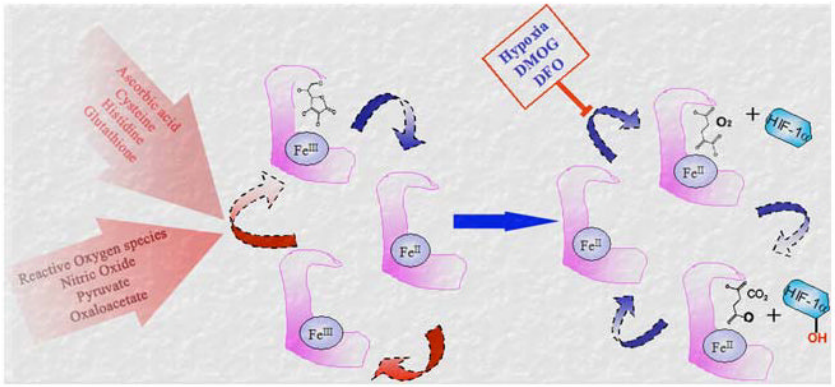

Fig. 4.

Regulation of redox state of iron at the catalytic site of the prolyl 4-hydroxylase. Ferrous (Fe^{2+}) is required for the catalytic activity of the enzyme. The oxidation of Fe^{3+} to Fe^{2+} is facilitated by ascorbate, cysteine, Histidine or glutathione and is reversibly inhibited by reactive oxygen species, nitric oxide, pyruvate and oxaloacetate. Hypoxia, 2-oxoglutarate analogue dimethyl-oxalyl-glycine (DMOG) and iron chelator Desferrioxamine (DFO) inhibit the enzyme activity by reducing the availability of co-substrates at the catalytic site (Modified from Lu et al. [59])

Table 1

Nomenclature of Prolyl 4-hydroxylase enzymes [26]

Nomenclature 1	Nomenclature 2	Nomenclature 3
PHD1	HPH3	EGLN2
PHD2	HPH2	EGLN1
PHD3	HPH1	EGLN3

Table 2

Summary of the physiological properties of the three HIF prolyl 4-hydroxylases indicating their ability to modify Pro402/564 (designated position in human HIF), intracellular localization (N, nuclear; C, cytosolic), ability to mediate HIF-1 stability as determined by RNA interference, induction of hydroxylase mRNA expression in response to hypoxia, and tissue expression patterns [26]

Gene	Size kDa	Substrate		Localization	RNAi	Hypoxia induction	Tissue distribution
		p402	p564				
EGLN1/HPH-2/PHD2	46	+	+	C/ > N	+ + +	+	liver = heart > brain = kidney > lung
EGLN2/HPH-3/PHD1	43.6	+	+	N	+	−	Testis > liver > kidney = heart = brain
EGLN3/HPH-1/PHD3	27.3	−	+	C/N	−	+	Heart > liver > brain > kidney > lung