

Review Article

Hypoxia-inducible factor prolyl hydroxylases as targets for neuroprotection by “antioxidant” metal chelators: From ferroptosis to stroke



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ABSTRACT

Neurologic conditions including stroke, Alzheimer disease, Parkinson disease, and Huntington disease are leading causes of death and long-term disability in the United States, and efforts to develop novel therapeutics for these conditions have historically had poor success in translating from bench to bedside. Hypoxia-inducible factor (HIF)-1 α mediates a broad, evolutionarily conserved, endogenous adaptive program to hypoxia, and manipulation of components of the HIF pathway is neuroprotective in a number of human neurological diseases and experimental models. In this review, we discuss molecular components of one aspect of hypoxic adaptation in detail and provide perspective on which targets within this pathway seem to be ripest for preventing and repairing neurodegeneration. Further, we highlight the role of HIF prolyl hydroxylases as emerging targets for the salutary effects of metal chelators on ferroptosis in vitro as well in animal models of neurological diseases.

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Adaptive mechanisms to stress: combating neurodegeneration

Over the past 20 years, our laboratory has used two primary approaches to understand therapeutic strategies for preventing oxidative stress-mediated neurodegeneration. First, we have studied the endogenous mechanisms that lead to or prevent cell death induced by depletion of the antioxidant glutathione in primary neurons in vitro. Detailed biochemical analysis of this form of death, recently termed ferroptosis [1], has revealed novel redox-regulated transcriptional pathways that can compensate for persistent glutathione depletion and oxidative stress to foster cell survival [2,3]. In parallel, investigations into the mechanism of classical “antioxidants” in our in vitro model have surprisingly revealed remarkable and recurrent bias toward transcriptional adaptive mechanisms often involving more than 100 genes [4–7]. In this review, we discuss evidence from these distinct investigations and how that evidence has led us to focus our attention on an evolutionarily conserved adaptive response and its role in neuroprotection.

Oxygen-dependent metabolism

It is thought that self-replicating RNA became enclosed in a phospholipid membrane about 3.8 billion years ago, forming the basic unit of life called the cell (reviewed by [8]). These cells were able to obtain the energy required to replicate themselves and perform other necessary tasks from the sea of organic molecules from which they originated. However, such a self-limiting situation drove cells to evolve mechanisms through which they could autonomously generate, store, and use energy.

The principal pathways of energy metabolism are remarkably similar in all present-day cells, indicating that they emerged early in the process of evolution and have been conserved. For instance, all cells use ATP as the currency of energy to drive the synthesis of cell constituents and to carry out other energy-requiring activities. The mechanisms by which cells generate ATP are thought to have evolved sequentially: first glycolysis, then photosynthesis, and finally oxidative metabolism. In glycolysis, glucose is broken down to produce 2 ATP molecules. In photosynthesis, energy from the sun is harvested to produce glucose, which creates molecular oxygen (O_2) as a by-product and has caused that oxygen to become abundant in the earth’s atmosphere. In oxidative metabolism, oxygen is used to break down glucose much more efficiently than in glycolysis, yielding 36 ATP molecules instead of 2.

Because of this efficiency, almost all present-day cells—including archaea, bacteria, and eukaryotic cells in humans—use oxidative reactions as their principal source of energy. For cells to rely on oxygen for metabolism, they need to be able to sense a decrease in oxygen availability—a phenomenon called hypoxia—and to then trigger a response program that helps them to cope with that decrease. One simple way to do so is to have sensor enzymes that use oxygen to inhibit the response program; when oxygen supply drops, the sensors are inhibited and the response program is then rapidly activated.

Cells as simple as bacteria express prolyl 4-hydroxylase domain-containing enzymes (PHDs) that use oxygen to add a hydroxyl group to proline residues on specific substrate proteins. This hydroxylation is a highly evolutionarily conserved sensory mechanism for oxygen;

in fact, prolyl 4-hydroxylation is the single most prevalent posttranslational modification in humans [9]. In all animal cells, even those of the simplex animal, *Trichoplax adhaerens*, one of the proteins subject to prolyl 4-hydroxylation is called hypoxia-inducible factor (HIF) [10].

Hypoxia-inducible factor

HIF was first identified as a transcriptional activator that binds to the hypoxia-response element (HRE) in the promoter region of erythropoietin and has since been shown to coordinate many evolutionarily conserved adaptive responses to hypoxia [11]. HIF target genes include VEGF, EPO, GLUT1, PFK1, BNIP3, neuroglobin, and dozens of other genes that work in concert at cellular, local tissue, and systemic levels, to restore oxygen delivery, to enhance glucose uptake and glycolysis, to reduce mitochondrial content and the rate of oxidative phosphorylation, and to regulate cell survival either by promoting adaptation or by engaging programmed cell death under prolonged or severe stress conditions. HIF-1 regulates gene expression not only by direct binding to target gene promoters at the consensus sequence (5'-RCGTG-3'), but also by counting among its targets numerous transcription factors, histone demethylases, and microRNAs that can in turn affect gene expression [69].

HIF is a heterodimer consisting of a constitutively present β subunit and a short-lived, oxygen-regulated α subunit. HIF family members are diagrammed in Fig. 1. Three α isoforms have been

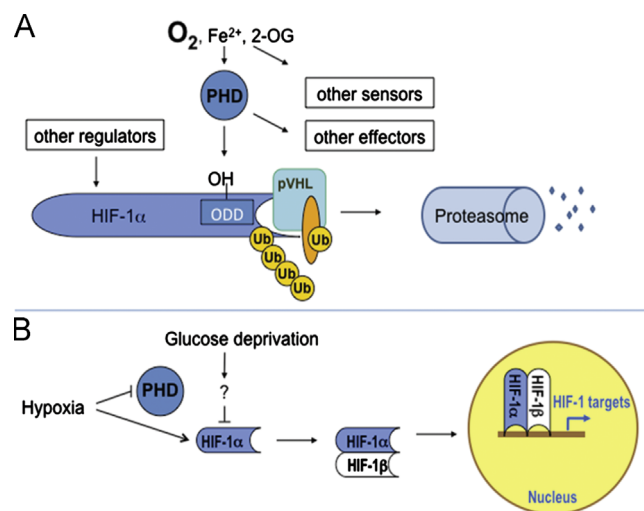


Fig. 1. Schematic of oxygen-dependent HIF-1 α regulation. (A) Under normoxic conditions, PHDs hydroxylate P564 on HIF-1 α , allowing it to be recognized by the E3 ubiquitin ligase von Hippel-Lindau protein (VHL), ubiquitinated, and targeted for proteasomal degradation. As members of a large family of iron- and 2-oxoglutarate-dependent dioxygenases, PHDs integrate multiple signals of metabolic homeostasis and are one of many such sensors; further, PHDs have HIF-independent substrates, and HIF protein levels and transcriptional activity are regulated in many PHD-independent ways. (B) Under hypoxia, PHDs are inhibited, allowing HIF-1 α to elude degradation, dimerize with its β partner in the nucleus, bind transcriptional coactivators and hypoxia-response elements in promoter regions of target genes, and enhance transcription rates. Glucose deprivation has been reported to decrease hypoxic stabilization of HIF-1 α ; the mechanisms by which this occurs are unclear.

identified in humans, and HIF-1 α is highly evolutionarily conserved, sharing approximately 90% homology with mouse and rat. All α and β subunits contain an N-terminus basic helix–loop–helix domain for DNA binding and a Per-ARNT-Sim (PAS) domain for dimerization. HIF-1 α and -2 α subunits contain two oxygen-dependent degradation domains and a C-terminal transactivation domain (C-TAD) that binds the coactivators CBP/p300; HIF-3 α is truncated to lack the C-TAD and thus is speculated to act as a competitive inhibitor of HIF-1 α and HIF-2 α .

HIF subunits do not only activate transcription of HRE-regulated genes, though. HIF-2 α was recently shown to bind the RNA-binding protein RBM4 and the cap-binding eIF4E2 to initiate translation of select mRNAs; this process occurs under conditions of hypoxia in which the normal cap-dependent translation machinery is inhibited [12]. HIF-1 α binds to the transcription factor Sp1 to block activation of the gene encoding the mismatch DNA repair protein MSH2 and also binds to the Notch intracellular domain to potentiate transcriptional activation of Notch target genes [69]. HIF- β subunits, first identified as the aryl hydrocarbon receptor nuclear translocator, are necessary for the xenobiotic response mediated by the aryl hydrocarbon receptor.

HIF-1 α is the most widely studied of the α isoforms and is expressed in all human cell types, whereas HIF-2 α shows more restricted expression. Both HIF-1 α and HIF-2 α show some selectivity in their cassettes of target genes. For example, HIF-1 α preferentially induces glycolytic enzyme genes, whereas HIF-2 α induces several genes involved in neurological disorders and cancer invasion [13]. HIF-1 α appears to mediate acute responses to hypoxia, whereas HIF-2 α is activated primarily during chronic hypoxia.

Constitutive deletion of the *Hif1 α* gene in mice causes lethality by day 11 of embryonic development, resulting from cardiovascular malformation and defective cephalic vascularization, indicating that HIF-1 α is essential for embryonic vascularization. Neural-cell-specific HIF-1 α -deficient mice exhibit hydrocephalus accompanied by a reduction in neural cells and an impairment of spatial memory, indicating that expression of HIF-1 α in neural cells is essential for normal development of the brain.

Oxygen-dependent degradation of HIF-1 α

HIF- α protein half-life is regulated by oxygen-dependent degradation (Fig. 1A). Under normoxic conditions, PHDs hydroxylate P564 on HIF-1 α , allowing the domain to be recognized by Von Hippel-Lindau (VHL) protein, an E3 ubiquitin ligase, and thereby targeted for degradation by the 26s proteasome [14–16]. Under hypoxia, a decrease in PHD activity leads to HIF-1 α accumulation, heterodimerization with β subunits, recruitment of the histone acetyltransferases p300 and CBP, and transactivation of target gene expression [17].

A number of other proteins contribute to the canonical PHD–VHL–proteasome degradative pathway. ARD1 acetylates lysine532 of HIF-1 α , located in the oxygen-dependent degradation (ODD) domain. This modification appears to enhance recruitment of VHL, but is not required for HIF-1 α degradation under normal conditions [18]. VHL binds hydroxylated PHD in complex with elongin B, elongin C, Cul2, and Rbx1, all of which are required for ubiquitination [19].

HIF PHDs

PHD enzymes are highly conserved, iron-dependent, 2-oxoglutarate-dependent dioxygenases. PHDs are the primary oxygen sensors that keep HIF-1 α protein levels low during

normoxia and allow the protein to be rapidly stabilized upon hypoxia [15,16]. Pharmacological and molecular studies have demonstrated that PHD inhibition is broadly neuroprotective and mediates the salutary effects of iron-chelating drugs. FDA-approved drugs that inhibit PHDs have been identified and are poised for clinical trials [20,78,111].

PHDs, also known as Egl 9 homologs (EGLNs), exist in three isoforms named PHD1 (EGLN2), PHD2 (EGLN1), and PHD3 (EGLN3). They are part of a superfamily of iron-dependent, 2-oxoglutarate-dependent dioxygenases; other members of this family include the collagen prolyl hydroxylases, which regulate the extracellular matrix, and the jumonji-domain-containing histone demethylases, which regulate gene expression through chromatin structure modifications. PHDs hydroxylate both P564 and P402 on HIF-1 α , but under conditions of normoxia, P564 is hydroxylated before P402 and primarily regulates oxygen-dependent degradation [21]. PHD2 is the most abundant PHD and the most important in setting steady-state levels of HIF-1 α subunits [22]. PHDs, especially PHD2 and PHD3, are transcriptionally upregulated by HIF-1 and are therefore important not only for basal regulation of HIF but also for feedback inhibition during prolonged hypoxia or rapid degradation upon reoxygenation [23]. Although all three PHD isoforms recognize the LXXLAP motif, they show some isoform-selective preferences for flanking regions on substrates, which provides some direction for attempts to develop isoform-specific inhibitors [24].

Several isoform-specific, HIF-independent functions of the PHDs have been identified. Some alternative substrates have been identified that show selectivity among the PHD isoforms. The β 2-adrenergic receptor, a G-protein-coupled receptor important for cardiac function, is hydroxylated specifically by PHD3, ubiquitinated by VHL, and proteasomally degraded under hypoxia [25]. Rbp1, the large and enzymatically active subunit of RNA polymerase II, is recruited to DNA under oxidative stress conditions in a manner requiring its hydroxylation at Pro1465 and subsequent nondegradative ubiquitination by VHL; PHD1 is necessary for Pro1465 hydroxylation, whereas PHD2 inhibits this hydroxylation [26]. PHD1 also specifically regulates cyclin D1 in a hydroxylase-dependent, transcription-dependent, HIF-independent manner: PHD1 inactivation decreases cyclin D1 levels and suppresses mammary gland cell proliferation and tumor formation in vivo [27].

In deed, PHDs are not only gatekeepers for the oxygen-dependent degradation of HIF-1 α , but are also integrated sensors of cellular metabolism [28]. Proline hydroxylation is enzymatically coupled to the decarboxylation of 2-oxoglutarate (2-OG), a process that yields succinate and CO₂. PHD activity therefore requires 2-OG and is inhibited in the presence of high concentrations of tricarboxylic acid cycle intermediates such as pyruvate, isocitrate, oxaloacetate, succinate, or fumarate. Because PHD enzyme activity also requires iron, PHDs serve moreover as sensors of iron homeostasis [29].

Although it is well established that PHDs, particularly PHD2, are critical for normoxic degradation of HIF-1 α , it remains debated exactly how hypoxia inhibits PHD activity. At least three distinct models have been proposed to explain this phenomenon. The first model proposes that PHD enzymatic activity could be reduced as substrate levels drop; this is a simple model in which PHDs are the primary oxygen sensors regulating HIF and is consistent with K_m for PHD observed in vitro [30]. The second model argues that O₂ consumption by an intact electron transport chain redistributes O₂ intracellularly into mitochondria [31,32]. Models 1 and 2 are not mutually exclusive, however, because subcellular O₂ gradients could shift the apparent K_m of PHDs for O₂ in vivo. The third model purports that mitochondrial peroxide production increases under hypoxia and that H₂O₂ dismutated from O₂⁻ produced at Reiske proteins is a specific signaling

molecule released from mitochondria to inhibit PHDs [117]. Work by several groups suggests that the stabilization of HIF-1 α by hypoxia requires signaling through mitochondria and intracellular kinases, in contrast to HIF-1 α stabilization by anoxia or by PHD-inhibiting iron chelators [33,34]. Models 2 and 3 invoke conflicting data on whether mitochondrial reactive oxygen species (ROS) production is necessary for or irrelevant to hypoxic HIF stabilization.

Von Hippel-Lindau protein

VHL was first identified as a tumor-suppressor gene that is mutated in Von Hippel-Lindau syndrome, a dominantly inherited familial cancer syndrome that precipitates a variety of malignant and benign tumors. The most common of these tumors are central nervous system hemangioblastomas (benign, highly vascularized tumors), pheochromocytomas (tumors originating from the chromaffin cells of the adrenal glands), and clear-cell renal-cell carcinomas (ccRCCs). Somatic mutations affecting VHL are also seen in the majority of cases of sporadic ccRCC.

VHL was shown to mediate HIF-1 α degradation, which explains the angiogenic phenotype of many VHL-null tumors [35]. VHL was subsequently shown to have a number of substrates other than HIF- α , including fibronectin and collagen IV α 2 chain in the extracellular matrix, microtubules, the RNA polymerase II subunits Rbp1 and Rbp7, atypical protein kinase C λ , VHL-interacting deubiquitinating enzymes VDU-1 and VDU-2, p27, p53, and Jade-1 [36]. VHL-mediated ubiquitination does not always lead to substrate degradation, though; VHL stabilizes p53 and Jade-1, for example [37].

The ubiquitin–proteasome degradation pathway

An ATP-dependent proteolytic system independent of lysosomes was first described in reticulocytes [38], and the Nobel Prize in Chemistry 2004 was awarded jointly to Aaron Ciechanover, Avram Herschko, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation.

Proteins are targeted for proteasomal degradation by the covalent addition of at least four ubiquitin molecules to a lysine residue on the client protein [39]. The addition of ubiquitin requires three enzymatic steps. First, a ubiquitin-activating enzyme (E1) couples the hydrolysis of ATP with adenylation of a ubiquitin molecule [40]. This adenylylated ubiquitin is then transferred to a cysteine of a second enzyme, ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin ligase (E3) recognizes the specific protein to be ubiquitinated and transfers the ubiquitin from E2 to the target protein. Addition of further ubiquitin groups can be mediated by an E3 or an E4 polyubiquitin chain conjugation factor. This conjugation is significant, because the way in which ubiquitin chains are constructed can determine the fate of ubiquitinated proteins [41]. Polyubiquitinated proteins are recognized by the 19S regulatory cap, then deubiquitinated and unfolded, so that they can be translocated through a narrow gate into the central channel of the 20S catalytic core of the proteasome. The β subunits of the 20S core act as proteases to degrade client proteins, releasing short peptides typically 7–9 amino acids in length.

ATP is required at several steps of the ubiquitin–proteasome degradative pathway (reviewed by [42]). First, ATP hydrolysis is required for activation of ubiquitin by E1, but not for later steps of ubiquitin conjugation. ATP binding, but not hydrolysis, is required for the assembly of the 26S proteasome from the 19S and 20S particles, as well as for target protein binding by the 19S subunit,

gate opening, translocation, and proteolysis. ATP hydrolysis is required for the unfolding of substrate proteins and, in some cases but not all, for proteolysis and deubiquitination [43,44].

ATP is required for proteasomal degradation and can also be depleted under conditions of ischemia (when blood supply drops, leading to a scarcity of oxygen, glucose, and other energy substrates). Nevertheless, it is worth noting that proteasomal degradation continues during brief ischemia and can even mediate rapid preconditioning tolerance to later harmful ischemia [45]. In contrast, prolonged ischemia can inactivate proteasomal degradation, leading to the accumulation of ubiquitinated proteins. Such accumulation may contribute to cell stress or form aggregates that may in turn be degraded by the autophagosome/lysosome pathway [45]. A major regulator of ATP homeostasis, AMPK, is negatively regulated by the proteasome such that inhibition of proteasomal degradation can stimulate the energy conservation program mediated by AMPK [46].

Regulation of HIF-1 α by ODD-independent mechanisms

HIF-1 α synthesis, stability, and ability to activate target gene transcription can be regulated through many known ODD-independent mechanisms under various physiological and pathological conditions.

Regulation of HIF-1 α activity

Once HIF-1 α is stabilized, its ability to bind p300/CBP is negatively regulated by an O $_2$ and Fe $^{2+}$ -dependent asparaginyl hydroxylase, a factor inhibiting HIF, which targets Asn803 within the C-TAD [47]. In addition to CPB and p300, other histone acetyltransferases (HATs) that coactivate transcription have been shown to interact with and potentiate HIF, including SRC1 and TIF2. On the other hand, the HAT CITED2 competes with HIF-1 for binding to p300/CBP and is transcriptionally upregulated in hypoxia by HIF-1, and thus may provide a negative feedback on HIF-1 activity during extended hypoxia [48]. CITED2 is also a negative regulator of NF- κ B-mediated gene transcription and thus may serve a general role in feedback regulation for homeostatic programs.

HIF-1 α phosphorylation by casein kinase 1 δ at Ser247 within the PAS domain inhibits dimerization with HIF-1 β and subsequent transcriptional activity, without affecting HIF-1 α stability or nuclear accumulation [49]. HIF-1 α is phosphorylated by p42/p44 mitogen-activated protein kinases (MAPKs 1/3) at Ser641/643, and this phosphorylation enhances HIF-1-dependent nuclear localization and gene transcription [50,51]. The regulatory-associated protein of mTOR (raptor) interacts with HIF-1 α at a mammalian target of rapamycin (mTOR) signaling motif located in the N-terminus of HIF-1 α . Raptor facilitates binding of HIF-1 α to the coactivator CBP/p300, thus enhancing HIF-1 α -mediated transcription [52]. S-nitrosylation of Cys800 and the redox-responsive protein Ref-1/APE-1 also promote HIF-1 transcriptional activity in a manner dependent on p300 [53–55].

ODD-independent regulation of HIF-1 α protein stability

The half-life of HIF-1 α is regulated in an O $_2$ -independent manner by the competitive binding of either the heat shock protein 90 (HSP90) or the receptor of activated protein kinase C (RACK1) to the PAS domain of HIF-1 α . RACK1 is an E3 ubiquitin ligase that interacts with elongin C via a binding site that shows significant sequence similarity to VHL, thereby promoting HIF-1 α ubiquitination and degradation in a manner that is independent of PHD2 and VHL [56]. RACK1-mediated HIF-1 α degradation can

be blocked not only by HSP90, but also by calcineurin, a calcium-activated phosphatase that dephosphorylates RACK1, thus blocking its dimerization and binding to elongin C, suggesting that HIF-1 α can be stabilized by increases in intracellular calcium [57].

GSK3 can directly phosphorylate Ser551, Thr555, and Ser589 within the ODD domain, leading to recognition by a ubiquitin ligase called F-box and WD protein and targeting HIF-1 α for proteasomal degradation independent of prolyl hydroxylation and VHL binding [58,59]. HIF-associated factor, an E3 ligase for HIF-1 α (but not HIF-2 α), plays an important role in oxygen-independent HIF-1 α regulation in cancer by degrading HIF-1 α in response to growth factor signaling, and perhaps other stimuli, even in the presence of hypoxia [60]. The small ubiquitin-related modifier (SUMO) can be conjugated to HIF-1 α at Lys391 and Lys477 and enhance HIF-1 α stability [61], but the mechanism by which this stabilization occurs and the physiological or pathological context in which SUMOylation regulates HIF-1 have not been determined. In response to oxidative stress, a small ubiquitin-like protein called neural precursor cell expressed developmentally downregulated 8 (NEDD8) becomes conjugated to the PAS domain of HIF-1 α and stabilizes HIF-1 α [109]. The role of ROS in hypoxic HIF-1 α stabilization is controversial.

Regulation of HIF-1 α transcription and translation

HIF-1 α is regulated by positive and negative feedback loops; in addition to hypoxic induction of PHD3 described above, *Hif1 α* itself is among the target genes of HIF-1 α . Hypoxic induction of HIF-1 α mRNA requires the activity of the PI3K/Akt pathway but not the ERK1/2 pathway [62].

NF- κ B, a major mediator of immune responses, binds to the promoter of HIF-1 α and increases HIF-1 α mRNA levels under hypoxia [62]. Cross-talk between NF- κ B and HIF-1 α is complex; for example, NF- κ B has long been known to be induced by hypoxia [63], and more recently it was shown that NF- κ B protein levels are increased under hypoxia downstream of HIF PHD1 hydroxylation of IKK β [64].

The NO donors NOC18 and *S*-nitrosoglutathione induce HIF-1 α expression and transcriptional activity without inhibiting HIF-1 α hydroxylation, ubiquitination, and degradation, indicating an effect on HIF-1 α protein synthesis that was confirmed by pulse labeling studies and shown to require PI3K and MAPK signaling through the translational regulatory proteins eukaryotic translation initiation factor 4E-binding protein (4E-BP1), p70 S6 kinase, and eIF-4E [65]. The tumor suppressor p53 binds HIF-1 α and promotes its degradation in hypoxia via Mdm-2-mediated ubiquitination and proteasomal degradation [66]. This can be overcome by Jun activation domain-binding protein-1, which was previously known as a coactivator of the AP-1 transcription factor before it was shown to activate HIF-1 transcriptional activity by interfering with p53 binding to HIF-1 [67].

Growth factor signaling boosts the rate of translation of HIF-1 α and a select group of other mRNAs (reviewed by [68]). Receptor tyrosine kinases in the plasma membrane are activated by extracellular growth factor ligand binding, which initiates two parallel cascades of phosphorylation activation: PI3K \rightarrow Akt/PKB \rightarrow mTOR and RAS \rightarrow MEK \rightarrow ERK. mTOR and ERK converge on two mechanisms of promoting translation: activation of S6K and subsequent activation of the ribosomal protein S6, as well as inhibition of 4E-BP1, to disinhibit cap-dependent translation mediated by eIF-4E. Additionally, ERK activates MNK, which directly phosphorylates eIF-4E to increase its activity. It is not known precisely how mTOR signaling promotes the translation of HIF-1 α , but HIF-1 α is likely to be particularly sensitive to fluctuations in the rate of protein synthesis because of its short half-life (approximately 5 min) under normoxic conditions. The

many levels of regulation on HIF-1 α , and its involvement in cellular responses to stimuli other than hypoxia, such as inflammation and growth factor signaling, all support the notion that HIF-1 α is a critical central regulator of adaptive transcriptional responses.

HIF PHD inhibition as a neuroprotective strategy

Because HIF-1 α is primarily degraded under normoxia in a manner requiring PHDs, HIF can be pharmacologically induced by structurally diverse small-molecule PHD inhibitors. HIF PHD inhibition consistently produces neuroprotection in diverse neurological disease models, including stroke [5,69], Alzheimer disease [70,71], allergic encephalomyelitis [72], Parkinson disease [73], oxidative stress [74], excitotoxicity [75], trophic factor deprivation [76], and mitochondrial dysfunction [77] (Table 1). PHDs have been identified as the critical targets of iron chelators such as desferoxamine that are clinically beneficial in many neurological disorders and that recapitulate the neuroprotective effects of hypoxic preconditioning on later ischemic challenge [5]. Novel PHD inhibitors poised for clinical trials have been identified through high-throughput screening of FDA-approved drug libraries [78]. PHD inhibition produces neuroprotection via both HIF-dependent and HIF-independent mechanisms [79]. Thus, PHD inhibition is a promising therapeutic approach that engages multiple downstream effector pathways, of which HIF is one.

Prolyl hydroxylation is substantially more sensitive than asparaginyl hydroxylation to inhibition by iron chelators [80]. Therefore, FDA-approved iron chelators or more specific PHD inhibitors could therapeutically activate the many downstream effectors of PHD signaling. There is also potential for off-target effects upon pathophysiological or pharmacological HIF manipulation. For these reasons, probing the mechanisms by which HIF is regulated by ischemia and by candidate drugs is as important for safety and efficacy as measuring HIF activity itself. Identifying the physiological, pathological, and pharmacological conditions permissive to HIF stabilization and their mechanism of action is an important first step in understanding the therapeutic benefits or potential side effects of manipulating HIF signaling.

HIF PHD inhibitors abrogate ferroptosis, a novel form of nonapoptotic death

Recently a novel form of iron-dependent, nonapoptotic death was defined by Brent Stockwell and coworkers [1]. Using Ras mutant cancer cells, the group elegantly demonstrated that cancer cell death induced by the chemotherapeutic agent erastin had unique morphological, biochemical, and genetic features compared to cell death induced by the nonspecific tyrosine kinase inhibitor staurosporine, the ROS hydrogen peroxide, or the autophagy inducer rapamycin. Specifically, ferroptosis could be blocked by iron chelators (desferoxamine), ERK signaling pathway inhibitors (e.g., U0126), and cycloheximide, a protein synthesis inhibitor. Moreover, an unbiased short interfering RNA screen involving 1087 genes identified 6 genes that are required for erastin-induced ferroptosis: ribosomal protein L8 (*RPL8*), iron-response element binding protein 2 (*IREB2*), ATP synthase Fo complex subunit C3 (*ATP5G3*), citrate synthase (*CS*), tetratricopeptide repeat domain 35 (*TTCC35*), and acetyl-CoA synthetase family member (*ACSF2*) (Table 2). In this model, cell death occurs downstream of inhibition of the plasma membrane transporter for cystine, Xc⁻. Inhibition of cystine transport leads to depletion of antioxidant levels via starvation of cysteine, the rate-limiting amino acid precursor in glutathione synthesis. Erastin thus

Table 1

Small molecules with HIF PHD inhibitory activity are neuroprotective in a host of models of neurological conditions in vitro and in vivo.

Neurological disease	HIF PHD inhibitor	In vitro model	In vivo model	Ref.
Stroke	Deferoxamine (DFO)	Oxygen glucose deprivation (OGD)		[119–121]
		Glutathione depletion model		[5,74,84,122]
		NMDA toxicity		[123,124]
		Nerve growth factor (NGF) deprivation		[125]
			Hypoxia–ischemic injury	[126]
			Middle cerebral artery occlusion (MCAO)	[5,98,119,127–130]
	Ethyl-3,4-dihydroxy benzoate (DHB)	Glutathione depletion model		[5,84,122]
		H ₂ O ₂ -induced oxidative injury		[131]
		NGF deprivation		[76]
			MCAO	[5,98]
	Dimethyl-oxalyl glycine (DMOG)	OGD		[132]
		Glutathione depletion model		[122]
	NGF deprivation		[76]	
		MCAO	[69,132]	
Parkinson disease	DFO	6-Hydroxydopamine (6-OHDA)		[133]
		1-Methyl-4-phenylpyridinium (MPP ⁺)		[99]
		Rotenone		[99]
	DHB	MPP ⁺	6-OHDA)	[134]
			1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	[79]
DMOG	MPP ⁺		[79,135]	
Alzheimer disease	DFO	SHSY-5Y cells overexpressing the human APP		[79]
		Swedish mutation		[136]
			APP/PS1 mice	[136]
		P301L mice	[137]	
Huntington disease	DFO	3-Nitropropionic acid		[77,138]
	DMOG			[77]
Friedreich ataxia	DFO	H ₂ O ₂ -induced oxidative injury in FRDA fibroblasts		[139]
		Frataxin expression in FRDA lymphoblasts		[140]

Desferoxamine inhibits HIF PHD activity by depleting iron, a critical cofactor for these and other iron-, 2-oxoglutarate-, and oxygen-dependent dioxygenases. Dihydroxybenzoic acid and dimethylaloxalylglycine are competitive inhibitors of 2-oxoglutarate, a cosubstrate in the HIF PHD catalytic cycle.

Table 2

Features of ferroptosis.

Characteristic feature	Occurrence
Morphology	Smaller mitochondria with increased membrane density
Cell death mechanism	Iron-dependent ROS accumulation
Precursor requirement	Requires initial lipid precursor
Possible cell death rescue mechanism	Iron chelation and genetic inhibition of cellular iron uptake
Regulatory genes	<i>RPL8, IREB2, ATP5G3, CS, TTC35, ACSF2</i>
Reported inducers	RSLs such as erastin and RSL-3
Characterized inhibitor	Ferrostatin-1
Other inhibitory compounds	Iron chelator DFO, antioxidant Trolox, MEK inhibitor U0126, protein synthesis inhibitor cycloheximide
Reported incidences of ferroptosis	Glutamate-induced neurotoxicity and cell death of certain cancer cells
Bioenergetics failure (a necrotic feature)	No
Cytoplasmic and organelle swelling (a necrotic feature)	No
Plasma membrane rupture (a necrotic feature)	No
Apoptotic bodies (an apoptotic feature)	No
Chromatin condensation (an apoptotic feature)	No
Formation of double membrane vesicle (an autophagic cell death feature)	No

Data were taken from Ref. [1]. Ferroptosis seems to be a biochemically, morphologically, and genetically distinct form of cell death that is sensitive to iron chelation in cancer cells. Although ferroptosis has not been characterized fully in neurons, our studies suggest that several agents known to block ferroptosis in cancer cells also block ferroptosis in primary neurons (e.g., ERK inhibitors, cycloheximide, and iron chelators). Our studies suggest that iron chelators target the HIF prolyl hydroxylases to inhibit ferroptosis [84,118].

induces oxidative stress, which ultimately leads to death. The findings are intriguing because it is well established that iron chelators [74], ERK signaling pathway inhibitors [81,82], and cycloheximide [83] can abrogate oxidative glutamate toxicity in neurons. In the neuronal paradigm, glutamate, like erastin, inhibits cystine uptake, leading to an iron-dependent, protein synthesis-dependent form of cell death. And although it is as yet unclear whether the genes necessary for erastin-induced ferroptosis also are required for oxidative glutamate toxicity in neurons, we have shown that an on-target effect of iron chelators in preventing oxidative glutamate toxicity (probably a neuronal form of ferroptosis) is the HIF prolyl hydroxylases [5], specifically HIF prolyl hydroxylase 1 [84]. Thus we would argue that the salutary effects of iron chelators in preventing ferroptosis can be assigned to inhibition of a specific family of iron-, 2-oxoglutarate-, and oxygen-dependent dioxygenases, the HIF prolyl hydroxylases, and not to direct inhibition of Fenton chemistry or reactive oxygen species formation.

Cerebral ischemia

Epidemiology and pathology of cerebral ischemia

Stroke is a leading cause of death and long-term disability in the United States [85]. Every year, about 795,000 people in the United States have a stroke, and about 23% of people who survive a stroke eventually have another [85]. In 2010, the estimated cost of stroke in the United States was \$53.9 billion, including the cost of health care services, medications, and lost productivity [85]. Cerebral ischemia is the most common form of stroke, accounting for approximately 87% of all strokes. Cerebral ischemia is defined as insufficient blood flow to the brain to supply an adequate amount of oxygen and nutrients. Cerebral ischemia may result from occlusion or constriction of blood vessels. At present, there is only one FDA-approved drug treatment for cerebral ischemia: the thrombolytic agent tissue plasminogen activator, which is recommended for iv administration only within the first 4.5 h after the onset of stroke symptoms.

Unlike hypoxia, in which cells can maintain ATP levels by shifting metabolism away from mitochondrial respiration and toward glycolysis, ischemia involves a reduction in glucose supply. Glucose is generally understood to be the obligatory energy substrate for the brain; in an intact brain, astrocytes take up glucose and utilize it for glycolysis and then shuttle pyruvate and lactate into neurons, which then use these substrates for oxidative phosphorylation [86]. More recently it has been shown that ketone bodies can also be used by the brain as energy substrates and, indeed, are taken up by the brain at an increased rate during cerebral ischemia [87,88]. Nonetheless, in the ischemic core where oxygen and glucose supplies are lowest, ATP may be severely depleted within minutes; in a rat model of forebrain ischemia, within 10 min glucose concentration dropped from 3.64 to 0.21 $\mu\text{M/g}$ and ATP concentration dropped from 2.64 to 0.18 $\mu\text{M/g}$, with corresponding increases in lactate and AMP [89].

ATP depletion in the ischemic core causes rapid necrotic cell death. Although many ATP-requiring processes such as gene transcription, DNA repair, protein synthesis and trafficking, and proteasomal degradation can be shut down briefly without causing lasting harm, neurons need a constant supply of ATP to maintain ionic gradients that support membrane polarization. Loss of membrane potential leads to excessive glutamate release, activation of extrasynaptic NMDA receptors, aberrant calcium influx, mitochondrial damage, and downstream intracellular signaling events culminating in excitotoxic cell death (reviewed by

[90]). In addition to glutamate-mediated mechanisms, a number of other ion channels can contribute to ion dyshomeostasis and activate cell death pathways, including sodium–calcium exchangers, hemichannels, volume-regulated anion channels, acid-sensing channels, transient receptor potential channels, and nonselective cation channels [91]. Aberrant calcium influx can cause not only excitotoxic release of neurotransmitters but also activation of calcium-dependent intracellular death pathways (e.g., through calpain) and mitochondrial permeability transition leading to caspase-dependent apoptotic cell death.

In the tissue surrounding a focal ischemic core, termed the penumbra, collateral blood flow produces gradients of oxygen and glucose that allow cells to avoid rapid energetic crisis and necrotic cell death, but many of these cells die in the 24–72 h after the ischemic event. This death is largely apoptotic rather than necrotic and follows a complex cascade of excitotoxicity, inflammation, and oxidative stress [92]. Current strategies for developing novel therapeutics to reduce the loss of brain tissue and motor/cognitive function are focused on restoring homeostasis and promoting neuronal survival within the penumbral regions. In experimental models of cerebral ischemia, hypoxic preconditioning has been shown to be extremely effective in reducing infarct volume and behavioral deficits ([93]; reviewed by [94]). Elucidating the mechanisms by which hypoxic preconditioning affords protection has been a major focus of ischemia research. Endogenous sensors of hypoxia and their downstream effectors of broad, evolutionarily conserved adaptive responses to hypoxia are prime targets for therapeutic manipulation [95]. Key among these targets is the transcriptional activator HIF.

HIF-1 α in cerebral ischemia

HIF-1 α protein levels are increased in mouse and primate brains after experimental cerebral ischemia [96,97], but it is unclear whether HIF-1 α limits or contributes to ischemic pathology.

In vivo models of cerebral ischemia show that HIF-1 α can prevent injury [98] and improve functional recovery [99] or increase infarct and edema volume [100,101]. In a rat model of focal ischemia, a biphasic induction of HIF-1 α was observed both in vivo and in vitro at 1–12 h after stroke and then again after 48 h, and selective inhibition of HIF-1 α at the early but not the late time point was neuroprotective [102]. In vitro models of cerebral ischemia also show that HIF-1 α either protects or exacerbates cell death via expression of target genes that encode both prosurvival and proapoptotic proteins [103,104] and demonstrate divergent roles of HIF-1 α in distinct CNS cell types [105].

Hypoxia and ischemia activate other stress response pathways that engage in cross-talk with the HIF pathway, including endoplasmic reticulum (ER) stress-induced activation of the unfolded protein response and suppression of protein synthesis [106]. Accumulation of misfolded proteins in the ER lumen leads to PERK-mediated phosphorylation of eIF2 α , inhibiting protein synthesis to prevent further accumulation of unfolded proteins in the ER. Therefore the rates of translation of HIF-1 α , its target genes, and its interactors are subject to additional layers of regulation under hypoxic or ischemic conditions. In vitro studies using oxygen and glucose deprivation have indicated that glucose is required for hypoxic HIF-1 α stabilization in human mesangial and hepatoma (Hep3B) cells [107] and in human renal carcinoma (UOK262) cells [108]. In considering how glucose deprivation could curtail hypoxic HIF-1 α stabilization, these researchers considered that glucose not only supports ATP production through glycolysis and mitochondrial oxidative phosphorylation, but also supplies the pentose phosphate pathway (also known as the hexose monophosphate shunt), which produces NADPH. They proposed a model by which glucose fuels NADPH production

through the pentose phosphate pathway/hexose monophosphate shunt to provide a substrate for NADPH-dependent oxidases, which in turn produce reactive oxygen species that inhibit prolyl hydroxylases. According to this model, glucose deprivation enhances HIF-1 α degradation by relieving a ROS-mediated inhibition of PHD enzyme activity. However, it remains unclear (1) whether PHDs in fact mediate the effects of glucose and (2) whether this model is applicable to neuronal cells. More recent work demonstrated that NEDD8 conjugation to the PAS domain mediates ROS-induced HIF stability, calling into question whether proline hydroxylation is truly the target through which glucose deprivation affects HIF-1 α levels [109].

Quantitative mechanistic reporters for HIF-1 α as a surrogate for dynamically monitoring PHD activity in cells

Although the potential therapeutic value of HIF PHD inhibition and HIF-1 α in cerebral ischemia has been widely appreciated for over a decade, prior studies of HIF-1 α regulation in neurons have been limited by the difficulty of performing quantitative analysis of immunoblotting employing commercially available HIF-1 α antibodies. This is particularly true in neuronal cells, in which HIF-1 α levels are very low, and complicates the study of post-transcriptional modifications of HIF-1 α that are key for understanding the mechanisms by which it is regulated. Further, given the neuroprotective properties of prolyl hydroxylase inhibition, a practical, quantitative reporter for PHD enzyme activity in vivo would be extremely valuable. To address this gap, we have developed a sensitive, specific reporter to quantitatively examine distinct parts of the canonical HIF degradation pathway in human neuronal cells, representing a substantial departure from the status quo, namely the approach of looking only at HIF-1 α levels and HIF-1-mediated gene transcription [110].

HRE-promoter-driven luciferase reporters have been widely used to measure HIF transactivation of target gene expression. For example, HRE-luciferase expressed in an immortalized mouse striatal neuron cell line, HT22, was used for high-throughput screening of drugs that activate HIF-mediated transcription [111]. These constructs are valuable tools for assaying an endpoint of HIF activation, but do not provide mechanistic insight regarding HIF regulation. For measuring HIF-1 α protein accumulation, several reporters containing luciferase and green fluorescent protein (GFP) have been developed [112–114]. The main advantage of using luciferase rather than GFP is the substantial increase in sensitivity with an enzymatic assay; luciferase is 3 orders of magnitude more sensitive and has a correspondingly much wider dynamic range of linearity than fluorescent proteins. Quantitative assays of luciferase activity can be confirmed with immunoblotting with a highly specific and sensitive commercially available monoclonal antibody against firefly luciferase that is much more quantitative than currently available antibodies against HIF-1 α .

A full-length HIF-1 α reporter such as that used by Moroz et al. [113], which includes the transactivation domain, introduces the confound of exaggerating HIF-mediated gene transcription, thus engaging feedback loops that may alter HIF-1 α regulation. In contrast, a reporter containing only the ODD domain lacks the dimerization and DNA-binding domains, allowing it to report on the oxygen-dependent regulation of HIF-1 α protein levels without interfering with endogenous HIF signaling. Some studies have inferred PHD activity from HIF-1 α levels without directly assaying PHD enzyme activity [107,108]. This is problematic given that HIF-1 α protein stability is regulated in PHD-independent ways. PHD enzymatic assays employing GST-fusion constructs have been performed in cell lysates from HEK293 cells [115], but not in neuronal cells. In vitro assays of PHD enzyme activity have

been developed, but require large amounts of recombinant enzyme and expensive reagents (reviewed by [116]). Therefore it would be highly valuable to be able to biochemically detect not only stabilization but prolyl hydroxylation and ubiquitination of a HIF-1 α reporter to identify mechanisms by which HIF-1 α is stabilized under given experimental conditions.

A fusion protein containing the oxygen-dependent degradation domain of HIF-1 α (aa 530–652) fused to firefly luciferase under control of the cytomegalovirus promoter has been previously used as a reporter for HIF-1 α stabilization in high-throughput screening for novel activators of the hypoxic response [78] and for in vivo imaging [112,117]. These studies employing the ODD-luc reporter have looked only at accumulation of the reporter as measured by luciferase activity assay, without investigating the mechanism by which it accumulates or is degraded. We have used the ODD-luc reporter as a quantitative reporter for the oxygen-dependent degradation of HIF-1 α in SH-SY5Y human neuroblastoma cells, taking advantage of luciferase activity assays and the wider dynamic range of luciferase antibodies relative to antibodies for endogenous HIF-1 α . We have confirmed that ODD-luc is regulated by PHD, VHL, and the proteasome in a manner similar to that of endogenous HIF and found that ODD-luc is a practical, specific, and quantitative reporter for the oxygen-dependent degradation of HIF in human neuronal cells [110]. Further, immunoblotting for P564 hydroxylation on ODD-luc provides a sensitive and quantitative direct assay of PHD enzyme activity in human neuronal cells. We expect this reporter to provide important information regarding PHD activity in vitro and in vivo after ischemia (and in other neurological disorders) and we expect that it will facilitate strategies to stabilize HIF in ischemia. These efforts will provide additional, needed clarification regarding the salutary and deleterious roles of HIF in ischemia and how HIF PHD inhibition can be used to bias outcomes favorably.

Concluding remarks

In this review, we have tried to highlight the important evolutionary role that hypoxic adaptation has played in metazoans. We have reviewed the molecular mechanisms subserving posttranscriptional and transcriptional adaptation to hypoxia, focusing primarily on the nervous system. Finally, we highlight the fact that HIF activation and HIF prolyl hydroxylase inhibition are not interchangeable and the potential roles for inhibitors of the HIF prolyl hydroxylases in a range of neurological conditions, particularly stroke.

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