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MULTI-AUTHOR REVIEW

Hypoxia, hypoxia-inducible factors (HIF), HIF hydroxylases and oxygen sensing

James D. Webb · Mathew L. Coleman · Christopher W. Pugh

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Abstract This article outlines the need for a homeostatic response to alterations in cellular oxygenation. It describes work on erythropoietin control that led to the discovery of the hypoxia-inducible transcription factor (HIF-1) and the parallel recognition that this system was responsive to a widespread oxygen-sensing mechanism. Subsequently, multiple HIF isoforms have been shown to have overlapping but non-redundant functions, controlling expression of genes involved in diverse processes such as angiogenesis, vascular tone, metal transport, glycolysis, mitochondrial function, cell growth and survival. The major role of prolyl and asparaginyl hydroxylation in regulating HIFs is described, as well as the identification of PHD1-3 and FIH as the oxygen-sensing enzymes responsible for these hydroxylations. Current understanding of other processes that modulate overall HIF activity, including influences from other signalling mechanisms such as kinases and nitric oxide levels, and the existence of a variety of feedback loops are outlined. The effects of some mutations in this pathway are documented as is knowledge of other substrates for these enzymes. The importance of PHD1-3 and FIH, and the large family of 2-oxoglutarate and iron(II)-dependent dioxygenases of which they are a part, in biology and medicine are discussed (part of a multi-author review).

Keywords Hypoxia · HIF · Prolyl hydroxylase · Asparaginyl hydroxylase · Oxygen sensing · PHD · FIH

J. D. Webb · M. L. Coleman · C. W. Pugh (✉)
Henry Wellcome Building for Molecular Physiology, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7BN, UK
e-mail: cpugh@well.ox.ac.uk

Introduction

Oxygen is the most abundant element in our planet as a whole. The majority of life has evolved over a period during which atmospheric oxygen has risen from very low levels to current levels of just under 21%, with perhaps a higher peak in the dinosaur era. For the majority of eukaryotic life forms oxygen has become the preferred terminal electron acceptor in metabolism, whereas many prokaryotes can utilise alternative electron acceptors. However, oxygen is a highly reactive element, and activated oxygen species are extremely toxic, used to benefit in bacterial killing by professional phagocytes, but with the potential for detrimental effects, including DNA, RNA and protein damage resulting in cell death. Oxygen supply and consumption, therefore, need to be tightly balanced to ensure optimal usage of this critically important element.

Mechanisms for regulating behaviour in response to changes in oxygen availability have been identified in both unicellular and multicellular organisms. Unicellular organisms are exposed to ambient levels of oxygen and have to adjust their metabolism accordingly. Environmental oxygen levels may vary in a diurnal pattern due to light-driven photosynthesis; the ambient level may be affected by the rate at which oxygen is consumed by the organism when the rate of supply is limited. Those organisms in which oxygen is the ultimate electron acceptor in energy metabolism can only tolerate anoxia, the total lack of oxygen, for very brief periods. In contrast, hypoxia, a reduction in oxygen availability, is an important stimulus for a variety of adaptive responses. Several regulatory mechanisms have been dissected, examples including ArcA, Fnr, OxyR, SoxR and FixL in various bacteria and Hap1 (Cyp1) in *Saccharomyces cerevisiae* [1–6].

A challenge for complex multicellular organisms, such as man, is to ensure optimal oxygen availability (and the disposal of waste products from oxygen consumption) is maintained to every cell in the organism throughout development and post-natal life. Tight linkage must therefore exist between oxygen availability and growth during development, and oxygen availability and metabolism in adulthood. In man, the placenta and the entire cardiovascular and respiratory systems are devoted to this purpose. These systems are controlled by neuronal, hormonal and autocrine mechanisms, allowing the organism to defend against both too little and too much oxygen being available. Responses to hypoxia can be triggered by very modest variations in blood oxygen supply, such as the effects of donating a unit of blood or moderate changes in altitude, and deviations in oxygen supply and demand are either causes or consequences of the vast majority of diseases. Adaptive responses can occur at the level of individual cells, individual tissues and organs, the organism as a whole, and ultimately over prolonged periods of time within and between species (see articles by Joseph, Calbet and Lundby in this issue). Many of the adaptive responses require changes in expression of multiple genes, sometimes across several cell types, implying the need for common master regulators and hierarchical control systems. Sensing mechanisms are required that can control oxygen delivery and consumption mechanisms, but the time frame required for different adaptive responses to hypoxia will vary enormously, needing to be very short when controlling the acute ventilatory response, but much longer-term in determining the morphogenesis of entire tissues, organs or organisms. Since oxygen can only reach the most distal parts of an organism by transfer down a concentration gradient, the oxygen level experienced by different cells in the body varies substantially, although that experienced by a particular cell type in a particular tissue tends to remain relatively constant. Since individual cells must retain the ability to sense and respond to changes in oxygen availability, an implication of this observation is that at a cellular level the particular oxygen level required to trigger an active response would be expected to vary.

Hypoxia-inducible factors (HIF)

One paradigm for the investigation of the oxygen-sensing mechanisms has been control of erythropoietin gene expression. Erythropoietin is a glycoprotein hormone which, by acting as a survival factor for red cell precursors, is dominantly important in controlling erythropoiesis and thus the ability of higher organisms to transport oxygen effectively in the blood [7]. A substantial body of evidence accumulated to indicate that erythropoietin levels were determined by the rate of erythropoietin gene transcription and responsive directly to changes in oxygen level, rather than indirectly via compromise of metabolism [8, 9]. In addition to up-regulation by hypoxia, erythropoietin production can also be enhanced by iron chelation and treatment with some, but not all, transition metals [10–12]. Erythropoietin is produced predominantly by specialised cells within the kidney, although liver, brain and testis cells may also contribute [13–19]. The discovery that oxygen-sensitive changes in erythropoietin gene transcription could be encapsulated in particular human hepatoma-derived tissue culture lines [20] led ultimately to the discovery of the hypoxia-inducible factor (HIF) family of transcriptional activators [21, 22].

The route that led to this discovery was initially the identification of an oxygen-responsive transcriptional enhancer lying 3′ to the gene [hypoxia response element (HRE)] [23–25], followed by affinity purification of the associated DNA binding proteins, protein sequencing and subsequent PCR with degenerate oligonucleotides to identify cDNAs and thus the gene [21]. The importance of this system was emphasised by the early discovery that HREs were widely operative, even in cells that did not make erythropoietin, implying that the sensing mechanisms were widely expressed and would be controlling processes other than erythropoiesis [26]. These predictions have subsequently been vindicated.

Hypoxia-inducible factor-1 (HIF) is an alpha/beta heterodimeric protein complex that binds to the HRE consensus, RCGTG, and regulates the expression of many genes in response to changes in oxygen availability. In addition to controlling erythropoiesis HIF-responsive genes influence processes including angiogenesis, vascular tone, metal transport, glycolysis, mitochondrial function, cell growth and survival, emphasising HIF's central involvement in oxygen homeostasis. HIF has earned the epithet of being the 'master regulator' of hypoxically regulated transcriptional systems [27–32]. However, it is clear that a variety of other transcriptional systems, including NFκB, ATFs and p53, are responsive to ambient oxygen levels [33–37]. This raises the question of whether these other effects are indirectly driven by HIF or arise directly, perhaps via shared, overlapping or independent oxygen-sensing mechanisms [38]. Array analysis in the presence and absence of siRNA against HIF shows that in MCF7 breast cancer cells the majority of transcripts that were significantly up-regulated by hypoxia were HIF-dependent, but a significant minority (105/246) were not [39]. This analysis probably flatters the importance of HIF since the conditions used (16-h exposure to 1% oxygen above the culture medium) are known to be particularly good at activating HREs. Under different stimuli other systems will predominate. Examples include signalling via the

NFkappaB pathway in preference to HIF activation during intermittent hypoxia and re-oxygenation [37] and ATF signalling triggered by absolute anoxia [40, 41], although the independence of this effect from the unfolded protein stress response is debated.

The HIF alpha and beta proteins contain basic helix–loop–helix (bHLH) and PAS (Per; AHR; ARNT; SIM) domains and are part of a large family of proteins with these features [42]. Intriguingly, the PAS domain has primitive origins and is a component of several proteins involved in circadian rhythms, perhaps indicating links between the circadian light–dark cycle via variations in photosynthetic rate to oxygen availability and thus the response to oxygen levels [42, 43]. The HIF alpha chains confer oxygen regulation on the complex as a whole. The HIF beta chains are constitutively expressed and had previously been identified as the aryl hydrocarbon receptor nuclear translocator (ARNT), contributing in combination with the aryl hydrocarbon receptor (AHR) to the transcriptional response to xenobiotic chemicals, such as dioxin [44–47]. Under stimulatory conditions HIF-alpha chains enter the nucleus and heterodimerise with HIF-beta. Active complexes bind DNA and where appropriate recruit CBP/p300 transcriptional co-activators to the transcriptional complex, thereby augmenting their inherent *trans*-activating abilities and influencing transcription of downstream genes [48]. The details of these processes and their regulation are discussed below.

Multiple HIF-alpha chains

Evolutionary precursors of the HIF system are found in *Caenorhabditis elegans* and *Drosophila melanogaster*, where the HIF-alpha chain is termed hif-1 [49] and Similar [50, 51] and the HIF-beta chain aha-1 [49] and Tango [52], respectively. Following the initial identification of HIF-1 several different genes encoding HIF-alpha and -beta subunits, and differentially spliced versions of these transcripts, have been found in mammalian systems. Three alpha chains, now commonly referred to as HIF-1 alpha [21], HIF-2 alpha [53–55] and HIF-3 alpha [56–60] and three beta chains, ARNT1, 2 and 3 [44, 61–64] are encoded at distinct genetic loci.

These components have different gene expression patterns. HIF-1alpha is widely expressed in normal tissues. HIF-2alpha was initially defined as an endothelial cell-specific isoform [53], though it is now clear that its expression is more widespread [65]. ARNT1 is widely expressed [66], whereas ARNT2 is highly expressed in the central nervous system and kidney [67], and ARNT3 in brain and muscle [68]. The expression pattern of HIF-3 alpha has been less fully studied [56].

Knock-out studies have thrown light on the function of these components, and this work is now being complemented by gene array studies and molecular dissection of the differential responses. HIF-1 alpha, HIF-2 alpha and ARNT have non-redundant functions and are all essential for normal embryogenesis. Targeted inactivation of HIF-1 alpha and HIF-2 alpha genes produces significantly different phenotypes. HIF-1 alpha null mice die around day 10.5 of embryogenesis with defective cardiac morphogenesis, vascularisation and neural tube closure [69, 70]. HIF-2 alpha inactivation has been reported to cause diverse phenotypes in the context of different genetic backgrounds, with death occurring in utero with defective catecholamine production [71] or vascularisation [72] or a few days or weeks after birth due to respiratory distress [73] or mitochondrial dysfunction [74]. ARNT1 negative mice also suffer embryonic death by day 10.5 associated with placental, vascular and haematopoietic defects [75]. ARNT2 null embryos die perinatally and exhibit impaired hypothalamic development [76]. Whilst differences in phenotype between HIF-1 and -2 alpha null animals may arise because of differences in their temporal and spatial expression, it is now evident that they also activate overlapping but distinct sets of genes, even in a single cell type.

Despite similar activity in promoting transcription of reporter genes linked to minimal multimerized HREs, endogenous target genes are often differentially activated by either HIF-1 alpha or HIF-2 alpha in individual cell types. In MCF7, a breast cancer cell line, these effects have been systematically studied using RNA arrays to assess transcription in a single cell type after RNA interference-mediated knock-down of individual HIF-alpha isoforms [39]. In other contexts, examples of specific genes with clear-cut preferential activation by one or other HIF isoform include activation of the Tie-2 promoter and the erythropoietin gene by HIF-2 alpha, but not HIF-1 alpha [53] and activation of glycolytic genes and carbonic anhydrase IX by HIF-1 alpha, but not HIF-2 alpha, in cell lines of kidney origin [77–79].

Differential gene activation inevitably leads to altered cellular phenotypes, although precise effects are likely to depend on the cellular background. Forced expression studies have revealed that HIF-2 alpha, but not HIF-1 alpha, is sufficient to promote renal clear cell tumourigenesis [79–82]. Analysis of different types of lesions in the kidneys of von Hippel-Lindau (VHL) patients has revealed that more advanced neoplastic lesions show proportionally greater expression of the HIF-2 alpha isoform, again suggesting that the HIF-2 alpha-induced phenotype has a selective advantage in this context [83]. Similarly, a recent study of mouse ES cell teratoma xenografts demonstrated enhanced tumour growth of cells bearing a HIF-2 alpha ‘knock-in’ allele at the HIF-1 alpha locus [84]. In

contrast, it has been reported that genetic ablation of HIF-1 alpha, but not HIF-2 alpha, in murine embryonic stem cells produces insensitivity to hypoxia-induced apoptosis [85].

Taken together these findings indicate that, despite many similarities, HIF-1 alpha and HIF-2 alpha isoforms have contrasting roles. Interest has centred around the mechanisms responsible for these effects. It has been shown that a post-DNA binding mechanism is responsible [86]. It has been suggested that differential gene regulation may result from the relative activities of the N-terminal and C-terminal transactivation domains of the HIF-1 alpha and HIF-2 alpha [87]. Titratable binding partners that suppress the activity of one or the other HIF isoform have been reported in different contexts [77], and more recently differences between HIF-1 and HIF-2 alpha in interactions with c-myc signalling have been shown [88].

Further complexity may arise from effects of HIF-3 alpha, a protein that exhibits a high degree of sequence similarity with HIF-1 and HIF-2 alpha over the bHLH and PAS regions but a low degree of sequence similarity across the C-terminus [89]. Transfection assays revealed that HIF-3 alpha could suppress basal and hypoxia-induced transactivation of a reporter gene linked to a promoter containing previously defined HIF-1 binding sites and antagonised hypoxia-inducible gene expression mediated by HIF-1 or HIF-2 alpha [90]. HIF-3 alpha also dimerises with ARNT1 and recognises the same DNA sequences as, and may compete for binding with, heterodimers containing HIF-1 and HIF-2 alpha [89]. However, alternative splicing of the HIF-3 alpha transcript produces a protein, termed inhibitory PAS domain protein (IPAS), that forms transcriptionally inactive heterodimers with HIF-1 alpha [59, 60] that could potentially bias the transcriptional response towards HIF-2 alpha-dependent genes.

Regulation of HIF-alpha chain activity—amino acid hydroxylation

HIF activation in hypoxia requires many processes including HIF-alpha chain synthesis, stabilisation, nuclear import, dimerisation, DNA binding and co-activator recruitment. Whilst HIF-alpha chain synthesis may be maintained under the cellular stress of hypoxia by the presence of an IRES sequence in the 5′-UTR, obviating the need for cap-directed translational initiation [91], and nuclear accumulation may be governed by hypoxia-sensitive mechanisms [92], it is widely agreed that the dominant regulation of HIF-alpha chain activity arises from the combination of protein stabilisation and enhanced co-activator recruitment in hypoxia (Fig. 1).

Under normoxic conditions HIF-alpha chains are rapidly degraded by the proteasome [93] following

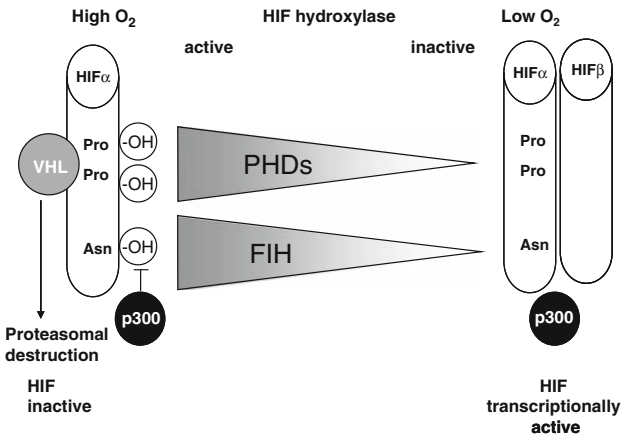

Fig. 1 Scheme showing the regulation of HIF alpha chain activity by HIF hydroxylases. When oxygen levels are high the HIF hydroxylases are active. Prolyl hydroxylases modify specific prolyl residues within the oxygen-dependent degradation domain of HIF alpha chains, allowing enhanced recognition by the pVHL ubiquitin ligase, ubiquitylation of the HIF alpha chain and subsequent proteasomal destruction. Factor-inhibiting HIF (FIH), the HIF asparaginyl hydroxylase, hydroxylates a C-terminal asparaginyl residue. This blocks recruitment of the transcriptional co-activators p300/CBP. As oxygen levels fall the HIF hydroxylases become progressively less active. In the absence of prolyl hydroxylation HIF alpha chains are stable and can heterodimerise with HIF beta chains to form a transcriptional complex that binds to hypoxia response elements (*HREs*). In the absence of asparaginyl hydroxylation p300/CBP can be recruited to this transcriptional complex, enhancing its activity and leading to increased transcription of downstream genes

promiscuous ubiquitylation at several sites [94, 95] by an E3 ligase in which the von Hippel-Lindau tumour suppressor protein (pVHL) acts as the substrate recognition component [96]. Mutations of pVHL found in renal clear cell cancer disable this process, leading to accumulation of high levels of HIF alpha proteins and contributing to the phenotype of these tumours [96–99]. Other mutations, associated with phaeochromocytoma (type 2C) [97, 100] and the rare recessive mutation found in Chuvash polycythaemia (R200W) [101], appear to distort HIF signalling without completely ablating HIF ubiquitylation and consequent degradation. Recognition of normoxic HIF-alpha proteins by pVHL arises from the enzymatic hydroxylation of either of two critical prolyl residues, residing within the N-terminal oxygen-dependent degradation domain (NODD) and the C-terminal oxygen-dependent degradation domain (CODD) of HIF-1 and HIF-2 alpha chains (HIF-3 alpha only contains a single prolyl residue) [102–106]. The presence of a hydroxyl group excludes a water molecule from the pVHL:HIF-alpha interface and allows the formation of two additional hydrogen bonds between HIF-alpha and pVHL, thereby stabilising the interaction and enhancing ubiquitylation [107]. Co-activator recruitment is also blocked by oxygen-dependent enzymatic hydroxylation of an asparaginyl residue in the

carboxy-terminal activation domain (CAD) of HIF-1 and HIF-2 alpha chains [108–110].

This amino acid hydroxylation is a function of a family of 2-oxoglutarate and iron(II)-dependent dioxygenases, the HIF hydroxylases. In each case part of the HIF-alpha chain enters the active site of the relevant hydroxylase in an extended conformation, bringing the residue to be hydroxylated into close proximity with the iron atom, which is essential for catalysis [111, 112]. During hypoxia, iron chelation or treatment with transition metal ions such as cobalt(II), HIF hydroxylase activity is reduced, allowing HIF to escape from both destruction and blockade of co-activator recruitment, thereby up-regulating genes involved in cellular and systemic hypoxia responses such as angiogenesis, apoptosis, erythropoiesis, glycolysis, inflammation, matrix metabolism, metal transport, mitochondrial function, proliferation, control of vascular tone and ventilation [113] and micro RNAs [114].

The mammalian genome encodes three HIF prolyl hydroxylases, named PHDs 1-3 [103], whereas in *Drosophila melanogaster* and *Caenorhabditis elegans* there are single proteins with this function named Fatiga and EGL9, respectively [103, 115]. A mammalian endoplasmic reticulum prolyl hydroxylase has also been reported to influence HIF activity [116], and mammals have a single HIF asparaginyl hydroxylase, factor-inhibiting HIF (FIH) [108–110]. Neither the endoplasmic reticulum HIF prolyl hydroxylase activity nor the asparaginyl hydroxylase activity is present in *Drosophila melanogaster* or *Caenorhabditis elegans*.

Function, regulation and mutations of the HIF hydroxylases

The HIF hydroxylases are part of a family of enzymes that all use dioxygen to hydroxylate their prime substrate and simultaneously convert 2-oxoglutarate into succinate with the release of carbon dioxide. Structurally their active site contains eight beta strands folded into a beta barrel jelly roll motif that positions oxoglutarate and iron, generally liganded to two histidine residues and a carboxylate group (HXD….H) [111, 112]. Somewhat different estimates of the K_m of these enzymes have been published using a variety of partially physiological assays. However, in all cases the K_m is thought to be significantly higher than the ambient oxygen level in cells, making enzyme activity exquisitely dependent on oxygen concentration, and allowing these enzymes to act as true oxygen sensors. The K_m of FIH, which functions as a dimer [117], has been measured to be less than that of the prolyl-hydroxylases, theoretically allowing FIH to regulate the transcriptional activity of any HIF-alpha chains that survive the degradative pathway [118–120]. However, practically it has been shown that HIF transcriptional targets can be up-regulated by inhibition of either PHD2 or FIH even when the other enzyme is fully active [121].

These enzymes catalyse the forward reaction but not the reverse reaction, and thus the net reaction rate is increased with the enzyme level (rather than this simply determining the speed with which equilibrium is achieved). In general in normoxic conditions PHD2 is the most abundant HIF prolyl-hydroxylase, and in keeping with this it has been reported to be the dominantly active enzyme [122]. However, it has been clearly established that when their abundance is increased PHD1 and PHD3 both also contribute to the control of HIF level and thus activity [123]. PHD1 production can be induced by oestrogens [124], and in some species at least, two variants with different translational initiations are produced, although the functional significance of this is not known [124]. PHD2 and PHD3 levels are both themselves up-regulated in response to hypoxia [103]. PHD2 and PHD3 regulation is achieved both by transcriptional and protein stability effects. The rate of degradation of PHD2 and PHD3 is mediated at least in part by ubiquitin ligases of the SIAH family [125, 126], which have more recently also been implicated in the regulation of FIH [127]. Additionally, proteolytic regulation of PHD2 through interaction with FKBP38, a peptidyl prolyl *cis/trans* isomerase, has been reported [128].

Enzyme activity can be regulated by a variety of factors including the availability of the prime substrate, the critical co-factor, iron, co-substrate, oxoglutarate, and the removal of the products, succinate (and possibly carbon dioxide). Modulation of iron(II) levels, either directly by supplementation or chelation, or indirectly using ascorbate or other redox agents, clearly affects enzyme activity [129]. Similarly, succinate has been shown to inhibit enzyme activity, and other Krebs' cycle intermediates also affect the system [130]. To date no convincing post-translational modifications have been shown to modify the specific activity of these enzymes, although evidence for changes in specific activity has been put forward [131].

The relative amounts of the different PHD enzymes present in a particular cell type at a particular time may also contribute to the transcriptional bias between HIF-1 alpha and HIF-2 alpha responsive genes. Despite highly conserved sites of prolyl hydroxylation between HIF-1 and HIF-2 alpha proteins, the effects of PHD enzymes on the levels of these proteins are not equivalent, with PHD2 contributing more to the regulation of HIF-1 alpha than HIF-2 alpha, while PHD1 and PHD3 contribute somewhat more to the regulation of HIF-2 alpha than HIF-1 alpha. Differential action of the PHD proteins on the NODD and

CODD of HIF-1 alpha and HIF-2 alpha may contribute to this bias [123].

The tissue distribution of the different HIF hydroxylase isoforms is gradually becoming clear. At the RNA level under basal conditions PHD1 is peculiarly abundant in the testis, PHD2 and FIH are ubiquitous, and PHD3 is highly expressed in heart and smooth muscle [132, 133]. Analysis of protein levels has lagged behind due to the relatively slow development of antibodies capable of specifically recognising endogenous levels of these proteins by immunoblotting and immunohistochemistry, and levels of protein responsible for functional effects are still close to the limits of detection, particularly for rodent PHD1. Protein levels generally match those of mRNAs in those tissue studied to date. Over-expression and physiological studies have shown that PHD1 is dominantly nuclear, PHD2 and FIH are mainly present in the cytoplasm, and PHD3 is present in both nucleus and cytoplasm [133, 134]. The significance of these different intracellular localisation patterns is unproven, although it is tempting to speculate that whilst cytoplasmic enzymes keep the system under check in normoxic conditions, the hypoxically induced intra-nuclear PHD3 enzyme may be particularly important for inactivating HIF, and perhaps particularly HIF-2 alpha, following re-oxygenation, although this would logically also require nuclear ubiquitylation and proteasomal destruction.

Functionally relevant mutations of PHD2 have now been reported in patients with primary polycythaemia [112, 135, 136], and it is likely that further mutations will also be found as other populations of people are surveyed. Additionally, given the genetic instability of cancer cell lines and the importance of the HIF system for tumour growth it seems inevitable that cancer cells will select HIF hydroxylase variants.

Knock-out mouse models provide clues about unique functions of individual HIF hydroxylases and may thus provide pointers to the existence, and possible nature, of alternative substrates. Studies of constitutive gene inactivation have shown that PHD2 is required for embryonic development. PHD2−/− embryos suffer defects in placentation and die in utero at dpc 12.5–14.5, but PHD2+/− animals survive to term and appear to develop relatively normally [137]. Somatic inactivation of PHD2 results in erythrocytosis and congestive cardiac failure [138]. PHD1−/− and PHD3−/− animals survive to term. PHD1−/− animals appear relatively normal in unstressed conditions, but their tissues have an enhanced ability to survive ischaemic insults [139]. PHD3−/− animals have a hyperplastic, but hypofunctional sympatho-adrenal axis, leading to relative hypotension in mid-life, but they develop cardiac failure in later life [140]. Further studies using tissue-specific conditional gene inactivation are required to assess the interplay between these phenotypes. FIH−/− mice have only recently been produced; preliminary reports suggest they are viable but of reduced size (R. Johnson; personal communication).

Other substrates for the HIF hydroxylases

Several laboratories are searching for other HIF hydroxylase substrates to discover novel oxygen-sensitive pathways or equally pathways regulated by iron or oxoglutarate availability. Other substrates for PHDs have been suggested to include the large sub-unit of RNA polymerase II [141, 142], IKK beta [143], ATF4 [38] and beta(2)-adrenergic receptors [144]. FIH hydroxylates many ankyrin repeat domains (ARD), and evidence exists to show this happens under endogenous circumstances [145–148]. FIH substrates appear to conform to a consensus motif that is present in most of the 300 human ARD-containing proteins, implying that FIH-mediated ARD hydroxylation is common. It is currently unclear what the functional consequences of FIH are on ARD biology since reported effects of FIH on the activity of ARD containing proteins are either subtle [145, 146] or may not be attributable to hydroxylation [147, 148]. However, it has been suggested that ARD proteins act as competitive inhibitors of FIH-dependent HIF alpha CAD hydroxylation: the ARD of Notch1 binds to FIH more tightly than HIF alpha CAD, and is a better substrate [146]. Overexpression of Notch1 ARD inhibits HIF1 alpha CAD hydroxylation, resulting in enhanced HIF1 alpha transcriptional activity (see "Attenuation of HIF activity in sustained hypoxia" below). The role of FIH in regulating other ARD pathways is unknown, but the notion that FIH-mediated ARD hydroxylation has a HIF-independent function is supported by bioinformatic analysis, which suggests that FIH and ARDs predate HIF-alphas (C. Loenarz; personal communication).

Other influences on HIF activity

The HIF system has pleiotropic actions and must be under subtle control. Whilst the actions of the HIF hydroxylases are vital, particularly for the hypoxic regulation of these transcriptional cascades, other mechanisms contribute to basal and induced activity.

HIF-alpha proteins are heavily phosphorylated [21], particularly in their oxygen-dependent degradation domains. In lower organisms such as nitrogen-fixing bacteria hypoxia-dependent phosphorylation activates transcription factors. A variety of kinase/phosphatase systems, including casein kinase II, AMP-activated protein

kinase (AMPK), p42/44 mitogen-activated kinase (MAPK) and PI3K, have been reported to affect HIF transcriptional activity. Some sites of phosphorylation exert relatively direct influences on the transcriptional activity of HIF-alpha proteins. For example, phosphorylation of HIF-1 alpha at Thr-796 by casein kinase II appears to enhance HIF transcriptional activity [149], and consistent with this observation a synthetic peptide phosphorylated at this position is a poor substrate for FIH in vitro [117]. Similarly, MAPK also affects activity of the C-terminal activation domain [150]. However, more indirect effects also occur. Examples include MAPK also suppressing CRMI-mediated nuclear export of HIF [151, 152] and AMPK phosphorylating tuberous sclerosis complex-2 (TSC2), thereby inhibiting the mammalian target of rapamycin (mTOR), which in turn, independently of hypoxia, affects HIF-alpha protein levels [153–155].

In addition to phosphorylation, hydroxylation and ubiquitylation HIF has been reported to be subject to acetylation [156] and SUMOylation [157], although more recent data refuted the presence of ARD1-driven acetylation [158–160] or the functional significance of SUMOylation [161].

Redox chemistry also plays a critical role in the transactivation of oxygen-responsive genes in unicellular organisms and modulates the activation of HIF-1, although it remains controversial as to whether these effects are direct, mediated via HIF hydroxylases or by entirely separate routes [162].

Nitric oxide, nitric oxide donors, scavengers and iNOS levels also influence the HIF system. The effects are complex, and some observations appear paradoxical [163, 164]. Proposed mechanisms include inhibition of HIF hydroxylases in normoxic conditions [165, 166], inducing HIF hydroxylase levels [167], possibly acting as partial HIF hydroxylase substrates in hypoxia and influencing intracellular oxygen levels available to the HIF hydroxylases by affecting mitochondrial function [168].

A wide variety of tumour suppressor and oncogenic proteins, including PTEN, p53, MDM2, Ha-Ras, v-Src and c-Myc, influence HIF activity, as do a variety of growth factors, including epidermal growth factor, insulin, insulin-like growth factors 1 and 2, angiotensin II, thrombin and PDGF.

Recent work has expanded the number of components of the HIF pathway. OS-9 (amplified in osteosarcoma-9) [169], inhibitor of growth family member 4 (ING4) [170], MAPK organizer 1 (Morg1) [171], TCP-1 ring complex (TRiC) [172] and NEMO (NF-KB essential modulator) [173] have been reported to interact with HIF and/or HIF hydroxylases. It is claimed that these interactions contribute to regulation of the system, although further work is required to clarify the general scope of these actions.

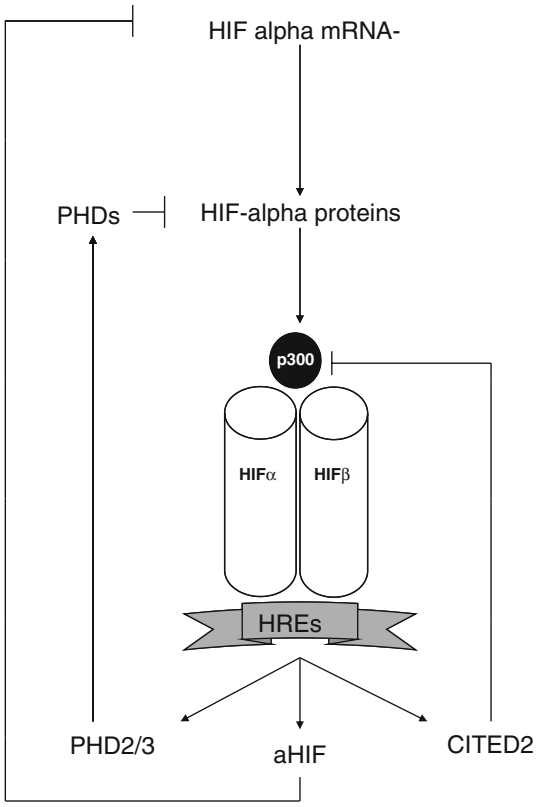

Fig. 2 Negative feedback systems affect HIF activity at multiple levels. Under hypoxic conditions HIF is active and leads to the production of a variety of transcripts. HIF-induced antisense HIF (*aHIF*) can specifically reduce HIF-1 alpha mRNA levels. HIF-induced up regulation of PHD-2 and PHD-3 production can increase the total PHD pool in the cell. The increased amount of prolyl hydroxylase protein does increase the amount of HIF alpha hydroxylation, and thus destruction, even though oxygen levels are low. Active HIF leads to production of CITED2, which can bind to p300 in its CH-1 domain, thereby blocking the ability of this co-activator to bind to the HIF transcriptional complex

Attenuation of HIF activity in sustained hypoxia

Like other biological systems the HIF system is subject to a variety of negative feedback mechanisms. These both attenuate HIF activity in sustained hypoxia and contribute to adjusting the system to sense relative hypoxia in tissues with different ambient oxygen levels.

HIF transcriptional targets include a variety of genes, the products of which, directly or indirectly, attenuate the HIF response. The first example identified was CITED2, previously known as p35-srj, which blocks co-activator recruitment [174]. A second mechanism is the induction of an antisense RNA to HIF-1 alpha (aHIF) driven by an HRE 3′ to the HIF-1 alpha gene. A third mechanism involves the hypoxic induction of HIF hydroxylase levels, modulated at the transcriptional level by HIF, but also affected by hypoxic influences on protein stability (Fig. 2). More indirect effects occur because HIF exerts major influences on

glycolysis, mitochondrial metabolism and iron transport, which in turn affect HIF hydroxylase activity by determining the availability of oxoglutarate and iron and the levels of downstream products (succinate, fumarate) [130, 175–177]. HIF-driven induction of nitric oxide synthase may also contribute to these effects. The involvement of HIF-induced microRNAs in further modulating the transcriptional outflow from this system is just being recognised [114]. Differential effects of these feedback mechanisms on the various components of the HIF system clearly have the potential to adjust the balance of HIF-1 versus HIF-2 activity. This is most clearly illustrated by effects of aHIF [178]; a more complex example is the preferential hydroxylation of HIF-2 alpha by PHD3, itself a preferential transcriptional target of HIF-2 alpha [86].

HIF-3 alpha induction also has the ability to negatively regulate HIF activity by heterodimerising with HIF alpha or HIF beta chains [59, 60, 89], but the generality of these effects has not been fully elucidated.

Clearly further adjustment of the system to sense relative hypoxia in tissues with different ambient oxygen levels can be mediated by the effects of phosphorylation, redox, nitric oxide, tumour suppressors and oncogenes discussed above. A new insight into tuning of this system has come from the identification of alternative substrates for the HIF hydroxylases. It has been shown that ARDs competitively inhibit FIH-mediated HIF-alpha CAD hydroxylation [146]. As these alternative HIF hydroxylase substrates have relatively long half-lives compared with HIF-alpha proteins, their hydroxylation status, and thus their ability to compete with HIF for hydroxylase activity, will depend on the longer term oxygenation of the cell (Fig. 3).

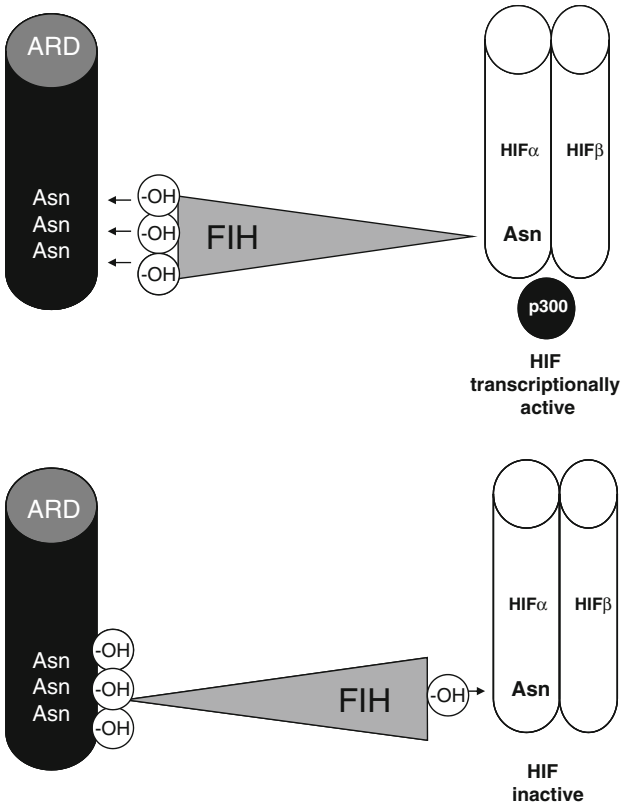

Fig. 3 Effects of ankyrin repeat domain hydroxylation on FIH and HIF activity. FIH will preferentially hydroxylate asparaginyl residues in ankyrin repeat domains (*ARD*) over those in HIF alpha chains. Under circumstances where ankyrin repeat domains have not been hydroxylated, the majority of FIH activity will be directed towards ankyrin hydroxylation, leaving the asparaginyl residue of HIF alpha chains unhydroxylated, allowing p300 recruitment and thus HIF transcriptional activity. Conversely, once ankyrin repeat domains have become fully hydroxylated FIH activity will be directed towards HIF alpha chains, blocking p300 recruitment and reducing the activity of the transcriptional complex

HIF hydroxylases and medicine

HIF has been implicated in disease processes, including cancer, ischaemic heart disease, pulmonary hypertension, mountain sickness, diabetic eye disease and arthritis [179, 180].

In human diseases some HIF effects are beneficial to patients, and medical benefit would be gained by augmenting these responses. For example, occlusive vascular disease is a major health problem in the western world, presenting as stroke, heart attack or peripheral vascular disease. Current therapies, including thrombolysis, percutaneous angioplasty or surgical revascularisation all aim to restore the blood supply to the tissue at risk. Promotion of angiogenesis in ischaemic tissues has been attempted using exogenous growth factor therapies. This has been of limited use, since it only drives certain aspects of the angiogenic response and has no direct cytoprotective effects. An alternative approach is to enhance the normal physiological response to ischaemia, driven at least in part by the HIF pathway. Several gene therapy approaches have adopted this strategy [181, 182]. Additionally, both desferrioxamine [183] and cobalt(II) chloride [184], known to activate the HIF system, have been shown to protect the rodent myocardium in ischaemia models. Therapeutic manipulation of the HIF pathway by inhibition of HIF hydroxylase activity is under active investigation. Many experiments reported to date have all used generic 2-oxoglutarate analogue based hydroxylase inhibitors, such as FG-0041 [185, 186], the plant extract L-mimosine [187], dimethyloxalylglycine [188], FG-2216 [189] or FG-4487 [190] to produce benefits in various ischaemic models. Other HIF responses are detrimental to the patient, for example, the growth of new vessels to supply an expanding cancer, and medical benefit would be gained by their inhibition. Benefits have been reported from both peptide-based and small molecule-induced

inhibition of co-activator recruitment in experimental models of cancer [191, 192].

Whether up-regulating or down-regulating the HIF pathway for therapeutic benefit, the presence of negative feedback loops and antagonists in the HIF pathway (discussed above) will all need to be considered in evaluating therapeutic approaches and optimising dosing schedules. Differential gene activation by different HIF alpha isoforms in a particular tissue also needs to be taken into account.

For HIF up-regulation differential effects of individual HIF hydroxylases on particular HIF alpha isoforms raise the possibility that therapeutic use of inhibitors of individual HIF hydroxylases could be tailored to bias the expression of hypoxia responsive genes. Indeed, analysis of the protective response of PHD-1 knockout mice to hindlimb [139] and hepatic ischaemia (M. Schneider; personal communication) not only provides further evidence in favour of HIF hydroxylase inhibition as a treatment for ischaemia diseases but also suggests that this hydroxylase isoform should be specifically inhibited for this purpose. Studies in renal cancers discussed above suggest that benefit may be derived from upsetting the balance of HIF-1 versus HIF-2 and that it is naïve to think simply in terms of overall up-regulation or down-regulation of the pathway [79, 88, 193, 194].

The wider 2-oxoglutarate and iron(II)-dependent dioxygenase (2OG oxygenase) family

Since most enzyme inhibitors will not be totally specific, knowledge of the consequences of down-regulating the activity of closely related enzymes is likely to be of benefit in anticipating potential toxicity and interpreting off target effects. The 2-oxoglutarate (2OG) and iron(II)-dependent dioxygenase (2OG oxygenase) families are widespread in biology [195], with approximately 60 members in humans. They catalyse a wide range of oxidative reactions, including hydroxylations, desaturations and ring closures [196]. They are involved in a variety of medicinally critical processes beyond oxygen-sensing, including DNA repair, chromatin remodelling, biosynthesis of antibiotics, the assembly of collagen and effects on lipid metabolism, diabetes and obesity [197–201]. Since enzyme activity can be regulated by a variety of factors, including enzyme level, the availability of the prime substrate and the critical co-factors and co-substrates, oxygen, iron and oxoglutarate and probably the removal of the products, succinate and carbon dioxide it is highly likely that these enzymes are involved in sensing, regulating and integrating many vital functions including oxygen supply and demand, iron balance, energy metabolism, ventilation and perhaps pH regulation.

The best characterised target of posttranslational hydroxylation by a 2OG oxygenase is the extracellular protein collagen [200]. The critical need for adequate co-factor availability for the proper function of this enzyme is illustrated by the effects of ascorbate deficiency, which lead to the disease scurvy. A second well-defined example of a disease associated with a 2OG oxygenase is Refsum's disease, a neurological syndrome characterised by adult-onset retinitis pigmentosa, anosmia, sensory neuropathy and phytanic acidaemia. Many cases are caused by mutations in peroxisomal oxygenase phytanoyl-CoA 2-hydroxylase (PAHX), a 2-oxoglutarate dependent dioxygenase that catalyses the initial β-oxidation step in the degradation of phytanic acid [202, 203].

There is now great interest in the roles of 2OG oxygenase enzymes in DNA repair and epigenetic control of gene expression. DNA is damaged by a variety of mutagenic alkylating agents. The ABH enzymes are human homologues of *E. coli* AlkB that repair 1-methyladenine and 3-methylcytosine [197]. Hydroxylation of the methyl group produces an unstable intermediate that decomposes to the repaired base plus formaldehyde. Inactivity of these enzymes in the hypoxic microenvironment of cancers may contribute to the general phenomenon of genetic instability of cancers, which makes them so hard to treat.

Histone methylation regulates gene expression by modulating transcription factor recruitment. Methyltransferases catalyse the methylation of H3 and H4 at specific residues, with the site of methylation dictating whether modification results in transcriptional activation or silencing. Recent studies suggest that 2OG oxygenases can reverse this process [204, 205]. Histone demethylases of the 2OG oxygenase family have been shown to regulate gene expression and the proliferation of tumour cells [206].

Other 2OG oxygenases also have important roles in metabolism. Gamma butyrobetain hydroxylase and trimethyl lysine hydroxylase are both involved in carnitine biosynthesis of particular relevance in muscles and cardiac disease [207–210]. The effects of oxoglutarate analogues on iron-regulatory protein 2 signalling also suggest an as yet unidentified member of this enzyme class is involved in regulating iron levels [211]. Excitingly, links between 2OG oxygenases and diabetes and obesity have recently been suggested. Genome-wide searches for type 2 diabetes-susceptibility genes have identified a common variant in the FTO (fat mass and obesity associated) gene that predisposes to diabetes through an effect on body mass index. Sequence analysis suggested that FTO is a Fe(II)- and 2-oxoglutarate-dependent oxygenase, and recombinant murine Fto catalyses the Fe(II)- and 2OG-dependent demethylation of 3-methylthymine in single-stranded DNA. In mice Fto messenger RNA is most abundant in the brain, particularly in hypothalamic nuclei governing energy

balance, and Fto mRNA levels in the arcuate nucleus are regulated by feeding and fasting [201]. Further studies are now required to determine the physiologically relevant FTO substrate and how nucleic acid methylation status is linked to increased fat mass.

Clearly this important and fascinating family of enzymes warrants much further investigation in its own right as well as because of its interplay with the HIF signalling system.

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