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Review

Signalling hypoxia by HIF hydroxylases

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Abstract

Analysis of oxygen sensitive pathways that regulate the hypoxia inducible factor (HIF) transcriptional system has revealed a novel role for oxygenases in signalling hypoxia. The enzymes, which catalyse hydroxylation of specific prolyl and asparaginyl residues in the regulatory HIF-α subunits, belong to the superfamily of non-haem Fe(II)-dependent oxygenases that use the citric acid cycle intermediate 2-oxoglutarate (2OG) as a co-substrate. We review biochemical and physiological data that demonstrate a central role for these oxygenases in integrating multiple signals that coordinate cellular responses to hypoxia.

Keywords: Oxygenase; Signalling; Oxygen; Iron; Hypoxia inducible factor; Transcription

The matching of oxygen supply to metabolic demand across billions of cells is one of the principal physiological challenges facing animals. Work over the last 15 years has provided significant insights into the molecular processes underlying this complex task, and has revealed a central role for a set of dioxgenases belonging to the Fe(II) and 2OG (2-oxoglutarate)-dependent oxygenase superfamily in directing the activity of a major transcriptional regulator termed hypoxia inducible factor (HIF). These enzymes catalyse the post-translational hydroxylation of specific prolyl and asparaginyl residues, the oxidation state of which governs both the rate of degradation and transcriptional activity of HIF-α subunits. The sensitivity of HIF hydroxylase capacity to oxygen concentrations enables the regulation of a wide array of cellular and systemic responses to oxygen availability. Here we review recent information on the role of the HIF hydroxylases in signalling hypoxia.

Hypoxia inducible factor

The HIF system, and its role in angiogenesis, glucose/energy metabolism, tumour development, and ischaemic/hypoxic disease has attracted widespread interest; for review see [1,2].

HIF is an α/β-heterodimer of two proteins both of which contain basic-helix–loop–helix PAS domains, that bind to a core DNA sequence (G/ACGTG) in hypoxia response elements (HREs) coupled to target genes. Transcriptional regulation by oxygen is mediated by the α-subunits of which three forms (HIF-1α, HIF-2α, and HIF-3α) have been defined in humans, each encoded by a distinct gene locus. HIF-1α and HIF-2α are closely related and each possesses two conserved sites of prolyl hydroxylation within a central oxygen-dependent degradation domain (Pro402, Pro564 in human HIF-1α). In HIF-3α, this domain is partially conserved and to date a single site of prolyl hydroxylation (analogous to HIF-1α Pro564) has been identified. *Trans*-prolyl-4-hydroxylation at these positions enables interaction of HIF-α with the von Hippel-Lindau tumour suppressor (pVHL) by a hydrogen bonding network to the b-domain of pVHL. pVHL is the recogni-

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tion component of a ubiquitin E3 ligase that targets HIF-α subunits for proteasomal degradation. HIF-1α and HIF-2α also possess a highly conserved site of asparaginyl hydroxylation within a C-terminal transactivation domain (Asn 803 in human HIF-1α), that prevents interaction with the co-activator p300 that otherwise binds at this site to promote transcriptional activation. Hydroxylation at the pro-S position of the β-carbon of the asparaginyl residue is predicted to disrupt hydrophobic interactions between HIF-α and p300 (for review see [3,4]).

Prolyl hydroxylation in humans is catalysed by three oxygenases termed PHD1, PHD2, and PHD3 (PHD, prolyl hydroxylase domain enzymes), the catalytic domains of which are closely related. Asparaginyl hydroxylation is catalysed by a more distantly related oxygenase termed FIH (factor inhibiting HIF). Thus in the presence of sufficient oxygen the HIF system is inactivated by a dual process involving proteolytic destruction and inactivation of transcriptional activity (Fig. 1). Reduction of oxygenase activity in hypoxia inhibits these processes allowing HIF-α to accumulate, dimerize with HIF-β, recruit p300, and induce the expression of transcriptional targets (for review, see [3,4]).

Signalling hypoxia by HIF hydroxylation

Though the role of post-translational protein hydroxylation is well recognized in structural biology, particularly in the stabilization of collagen fibrils, the hydroxylation of HIF-α is the first identified example of this process being used in cell signalling. Recent analyses have indicated that like the protein hydroxylases involved in structural modification, the HIF hydroxylases are capable of high rates of turnover, with figures of approximately 50 mol/mol/min for recombinant PHD enzymes [5], and in excess of 100 mol/mol/min for FIH [6] (at saturating substrate concentrations), fitting well with values for the native enzymes [7]. These figures might at first sight suggest that hydroxylation capacity for a low abundance transcription factor would not be limiting. However, several lines of evidence indicate that hydroxylase activity is, in fact, limiting under circumstances in which HIF is induced, and that the HIF hydroxylases do play a key regulatory role. First, the co-substrate and co-factor requirements for oxygen and iron are in concordance with the classic properties of the HIF system of induction by hypoxia, cobaltous ions and iron chelators. Second, in almost all situations except blockade of the (downstream) pVHL ubiquitin/proteasome pathway, accumulation of HIF is in a non-hydroxylated form, implying that the hydroxylation step is limiting. Third, mutation of HIF-α polypeptides at the known sites of hydroxylation is associated with greatly enhanced stability and/or activity and reduced regulation by oxygen. Fourth, genetic or pharmacological inactivation of the HIF hydroxylases leads to a constitutive activation of the HIF pathway with little or even absent regulation by oxygen remaining. Fifth, modest changes in enzyme abundance, achieved, for instance, by siRNA suppression of a particular PHD isoform, or induction of the PHD encoding transcripts by hypoxia, alters the cellular capacity for HIF degradation in an appropriate direction. Thus, although there are clearly other regulatory inputs into the HIF system (such as regulation of HIF-α translation by a variety of protein phosphorylation pathways), oxygenase-catalysed HIF hydroxylation is a fundamental control mechanism. This discovery has raised many new questions as to how the biochemical properties of these oxygenases correlate with their regulatory role in the challenge of maintaining of oxygen homeostasis.

In common with the procollagen prolyl and lysyl hydroxylases [8], the HIF hydroxylases belong to the ubiquitous 2OG-dependent oxygenase superfamily, which

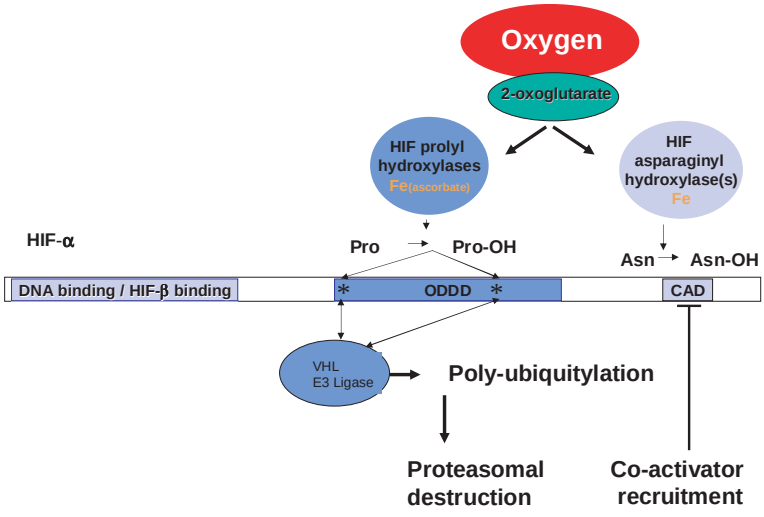

Fig. 1. Schematic illustrating regulation of HIF-α subunits by prolyl and asparaginyl hydroxylation. Prolyl hydroxylation at two sites (*) within a central oxygen-dependent degradation domain (ODDD) in HIF-α is catalysed by three, Fe(II), oxygen, and 2-oxoglutarate-dependent enzymes PHD1, PHD2, and PHD3. Asparaginyl hydroxylation is catalysed by an enzyme termed FIH at a single site within the HIF-α C-terminal activation domain (CAD).

require a single non-haem Fe(II) cofactor and use the citric acid cycle intermediate 2OG as a co-substrate [9–11]. For several members of this group kinetic, spectroscopic and crystallographic analyses suggest a common mechanism in which an enzyme–Fe(II) complex first binds 2OG, then its prime substrate. The binding of prime substrate displaces a water molecule that is co-ordinated to the Fe(II) and so triggers reaction with molecular oxygen. Oxidative decarboxylation of 2OG then occurs, producing succinate, CO_2, and a ferryl species (Fe^{IV}=O) at the iron centre (for review, see [11,3,10]). This highly reactive intermediate then oxidizes the prime substrate. In addition to hydroxylations, 2OG oxygenases from lower organisms catalyse a range of other oxidative reactions and there is evidence for variations of the typical mechanism. It is not yet clear to what extent the HIF hydroxylases are typical 2OG oxygenases, or whether their catalytic properties are specialized for an oxygen sensing function.

X-ray crystallographic analyses of all 2OG-dependent oxygenases studied to date (including the HIF asparaginyl hydroxylase, FIH) [3,9,10] have revealed a core of eight β-strands that are folded into a double stranded helix or 'jelly-roll' fold, the two sheets of which are typically extended by 2–3 other β-strands, and which is supported by conserved α-helices. The jelly roll core supports the three Fe(II) binding ligands which comprise a conserved two-histidine-one-carboxylate iron co-ordinating triad (HXD/EX_nH) on or close to the second and seventh β-strands [11,3,10]. In the HIF hydroxylases, the iron binding carboxylate is an aspartyl residue, as is most commonly observed. Inhibition by iron chelators, Co(II) ions, and the requirement for iron supplementation for full activity of the enzyme demonstrate that, unlike the P_{450} oxygenases, Fe(II) binding is reversible, accounting for the 'iron-dependent' characteristic of the hypoxic response. However, it is not yet clear how Fe(II) is delivered and maintained at the catalytic site in vivo.

Another co-factor requirement of some, but not all, 2OG-dependent oxygenases that appears to be shared by the PHDs, but not FIH, is ascorbate. In the case of procollagen prolyl hydroxylase, ascorbate oxidation (either directly to dehydroascorbate or via the semi dehydroascorbate radical) is required to complete cycles in which oxidation of 2OG to succinate is uncoupled from that of (procollagen) substrate oxidation, leaving an oxidized (Fe^{IV}=O) intermediate unable to return to the reduced form and complete the catalytic cycle [12]. Ascorbate may however have other actions including simple reduction of unwanted Fe(III) to the Fe(II) co-factor required by the oxygenase.

In addition to the requirement of the HIF hydroxylases for molecular oxygen, some or all of their other multiple co-substrate and co-factor requirements probably contribute to the flexibility inherent within this highly evolved system for signalling cellular hypoxia. Below we have summarized rapidly emerging data on these and other potential controls of HIF hydroxylase activity.

Regulation of the HIF hydroxylases

Molecular oxygen

Monitoring of the incorporation of ^{18}O into the sites of prolyl and asparaginyl hydroxylation by mass spectrometry has confirmed direct incorporation of oxygen into the HIF-α substrate with little or no (<5%) exchange with water [13,14]; almost complete (>95%) incorporation of a single dioxygen derived oxygen into the succinate coproduct occurs (Welford et al., unpublished data). Early studies using VHL capture by a short HIF-α polypeptide as a measure of hydroxylation demonstrated that PHD enzyme activity was indeed highly sensitive in vitro to hypoxia, being reduced progressively as ambient atmospheric oxygen was reduced from 20% to zero [15]. In keeping with this, more formal measurements of the apparent K_m for molecular oxygen using $^{14}CO_2$ release from labelled 2OG have yielded values in the range 230–250 μM for the the PHD enzymes [16] and 90 μM for FIH [6]. However, there are important caveats to both studies.

Although kinetic data for the procollagen prolyl hydroxylases have led to the proposal that oxygen binding precedes that of the polypeptide substrate [8], more recent mechanistic proposals for other 2OG oxygenases imply that it is most likely to be the combined enzyme–substrate complex that binds molecular oxygen [9–11]. In the case of FIH an enzyme · Fe(II) · 2OG · substrate has been observed by crystallography [3]. Mutational and deletional analysis of full-length HIF-α molecules has indicated that extensive domains are required for optimal efficiency of both oxygen-regulated proteolysis and oxygen-regulated transactivation. Thus, it is quite possible that apparent K_m values for oxygen obtained with short HIF-α peptide substrates do not reflect the kinetics of native HIF hydroxylation accurately, where the enzyme–substrate complex involves a much larger substrate molecule. Indeed, we have found that using a longer (ca 200 residue) HIF-1α polypeptide encompassing the Pro 564 residue, rather the minimal 19 residue hydroxylation site, the apparent K_m for oxygen is substantially lower (Ehrismann et al., unpublished work). However in considering whether the system will display oxygen regulated activity it is important to recognize that two criteria must be met. First, the threshold physiological pO_2 for a hypoxic response must be below saturating levels for the enzyme. Second, the overall process of hydroxylation must be rate-limiting in the system regulating HIF-α activity. Physiological tissue oxygen concentrations are notoriously difficult to measure because of steep oxygen gradients within tissues and, especially, cells. Existing measurements in the range 10–30 μM may well therefore be an overestimate and perhaps a major overestimate of oxygen concentrations within cells, where intracellular gradients exist (for reviews, see [17,18]) and oxygen may be complexed, e.g., with myoglobin in muscle cells. Indeed, measurements made close to respiring tissue culture monolayers have revealed surprisingly low values due to large standing gradients

developing in the unstirred culture medium (see below) [19]. Overall, therefore, it seems unlikely that there is special significance in the reportedly atypically high K_m values of the PHD enzymes for oxygen, since even very much lower values would still be high in relation to the physiological operating concentrations. In these circumstances, the critical requirement for oxygen sensitive function is simply that overall hydroxylation capacity is in a rate-limiting range for the system. We have outlined evidence above that this is the case and will expand on this below.

However, one further point deserves emphasis. If (as we have argued) intracellular oxygen concentrations in intact organisms are sufficiently low for the rate of hydroxylation to be oxygen dependent in any physiological condition, it is still possible that this rate is only limiting for the overall HIF degradation (or transactivation) pathway over a more limited range of oxygen concentrations (Fig. 2). It follows that any input that shifts the total hydroxylation capacity in relation to the total capacity of the pathway has the potential to alter the oxygen sensitive range. For example, decreases in enzyme abundance (see below) would be predicted to shift the regulatory range for oxygen upward, whereas increases in enzyme concentration should shift the regulatory range downward (and, if sufficiently large, render hydroxylation non-regulatory). Whether the system actually responds in this particular way remains unclear. However, it is certainly possible for different influences on the total hydroxylation capacity to effectively match the system to an oxygen sensitive range appropriate for the physiology of a particular cell type or tissue. This proposal rationalizes how the system can be tuned to operate over widely differing ranges of oxygen concentrations in different tissues within an intact organism.

Iron and divalent metal ions

The dependence of the HIF hydroxylases on Fe(II) and their inhibition by other transition metals [3] fits well with classical observation of the behaviour of HIF and HIF target genes in vivo, where induction by hypoxia is mimicked by iron chelators, and a series of metals including Co(II), Ni(II), and Mn(II) [20]. In keeping with this, when expressed in unpurified reticulocyte lysate, recombinant HIF hydroxylases are also sensitive to inhibition by iron chelators and cobaltous ions [15]. Recent data using purified recombinant PHD enzymes produced in insect cells have demonstrated very tight binding of Fe(II), with apparent K_m values of <0.1 μM, contrasting with a value of 0.5 μM for FIH and 2 μM for procollagen prolyl hydroxylase [5]. Moreover, desferrioxamine, Co(II), Ni(II), and Mn(II) were found to be weak or ineffective inhibitors of these PHD enzyme preparations, but more active against FIH, possibly reflecting the weaker Fe(II) binding of the latter. The discrepancy has led to the suggestion that the metals inhibit the PHD enzymes or stabilize HIF-α by other mechanisms in vivo. For instance, it has been proposed that Co(II) may stabilize HIF by binding a series of carboxylate residues lying close to Pro564 in HIF-1α [21], or that Co(II) and Ni(II) act by depleting intracellular ascorbate levels [22]. However, these mechanisms cannot explain the difference in activity of iron chelators on in vitro versus in vivo systems, leaving an intriguing discrepancy to

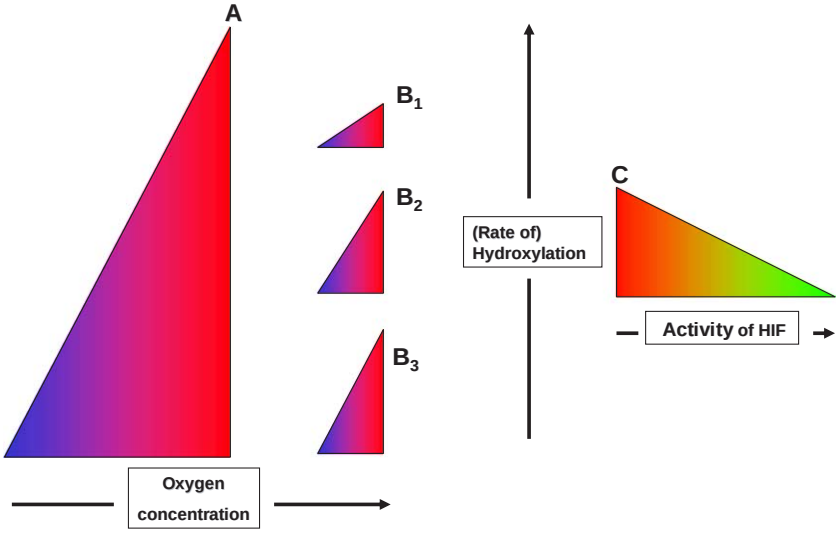

Fig. 2. Schematic illustrating the potential operation of 'range finding' inputs to match oxygen-dependent HIF hydroxylation to the rate-limiting range for the overall process of HIF activation. (A) The very wide range of potential rates of oxygen-dependent hydroxylation for enzyme with saturating kinetics for oxygen that equal or exceed physiological pO_2. (B1–B3) illustrate three potential ranges of hydroxylation that might be realized by physiological variation in pO_2 under different conditions. (C) The range of hydroxylation rates that would be limiting for the overall activation of the HIF-α. Oxygen-dependent regulation of the pathway requires the matching of (B,C). Oxygen-dependent hydroxylation in range (B2) would confer regulation by oxygen on the system whereas (B1) or (B3) would not. Processes that alter enzyme abundance or activity (even if not themselves directly oxygen sensitive, e.g., phosphorylation) could operate in a range finding function that matches (B,C).

be explained. Overall, it seems likely that the apparent lability of the PHD catalytic Fe(II) observed in vivo represents a more complex process than the unassisted exchange of the metal with a labile cellular pool, one possibility being the involvement of additional binding/transport proteins in the delivery and/or exchange processes.

An important question raised by these findings is whether changes in cellular iron availability play a physiological role in the regulation of the HIF hydroxylase system. Indirect evidence suggests that this may be the case. First, rapid proliferation of cells is associated both with depletion of the cellular iron pool [23]. Second, these conditions are commonly associated with the accumulation of non-hydroxylated HIF in apparently well-oxygenated cultured cells [24]. Third, addition of iron to such cultures reduces normoxic levels of HIF-α in a manner that is dependent on a functional hydroxylase/degradation system [25].

Ascorbate

The in vitro dependence of 2OG oxygenases on ascorbate varies significantly from none to being (near) essential for multiple turnovers. Studies of the PHD enzymes both in vitro and in vivo indicate a role for ascorbate in the HIF system. Kinetic analyses of recombinant HIF hydroxylases have given apparent K_m values of 140–180 μM for the PHDs a value in a similar range to those cited for procollagen prolyl hydroxylase [16]. Again these values are difficult to relate to physiological operating conditions in cells. However, several lines of evidence suggest that ascorbate provision may indeed be limiting for hydroxylase activity, at least under commonly employed tissue culture conditions. Thus, as with iron, addition of ascorbate has been shown to reduce the normoxic accumulation of HIF-α that is observed in certain rapidly growing tissue culture cells [25]. Such effects were not seen in cells treated with 2OG analogues that block HIF hydroxylase activity, and are not seen in VHL deficient cells, supporting the interpretation that they arise from the promotion of hydroxylase activity by ascorbate. In another recent study, ascorbate was shown to promote PHD activity and HIF hydroxylation in junD deficient cells with reduced anti-oxidant defence pathways, suggesting that ascorbate may mediate interaction between antioxidant and hypoxia pathways [26].

Normally ascorbate is obtained in the diet, or in the case of most species, synthesized from UDP-glucoronic acid in the liver by gulonolactone oxidase; in humans, this enzyme is mutated and not expressed. Cellular ascorbate is obtained from this source via the operation of the anion transporter Slc23a1 or recycled from the oxidized form of dehydroascorbate by the flavoprotein dehydroascorbate reductase [27]. Tissue culture cells, which lack a systemic source of the vitamin, may therefore be unphysiologically liable to ascorbate deficiency, and the circumstances under which ascorbate is limiting for HIF hydroxylases in intact organisms remain to be determined. Nevertheless, the potential role of ascorbate in the HIF system is of great interest, not least because the complex oxido-reduction chemistry of ascorbate suggests a number of mechanisms that might influence cellular levels of the active (reduced) form, and potentially account for effects of redox active substances on the HIF system.

Reactive oxygen radical species

Several studies have demonstrated that agents with predicted or measured effects on cellular levels of reactive oxygen species (ROS) have actions on HIF activity, leading to the hypothesis that ROS have a regulatory role in the HIF pathway (for review see [28]). The known susceptibility of 2OG-dependent oxygenases to oxidative damage, via active site hydroxylation [10], fragmentation (Welford et al., unpublished data), or inactivation via conversion of Fe(II) to Fe(III) suggests that such effects might be involved in HIF hydroxylase regulation. Evidence for this has recently been obtained from analysis of the effects of junD on the HIF system, as outlined above [26]. This study provided evidence that junD reduces ROS levels as part of a defence against oxidant stress. In junD deficient cells, reduced PHD2 activity was observed that could be rescued by ascorbate, together with EPR signals that were assigned to (oxidized) Fe(III) at the catalytic site of PHD2. One intriguing possibility is that antioxidant systems that scavenge ROS might have a tonic influence that enhances PHD activity. This would downregulate HIF resulting in a more 'oxic' as opposed to 'hypoxic' pattern of gene expression. Oxidant inactivation of PHDs, possibly by conversion of the active Fe(II) form to the inactivate Fe(III) form, could in theory account for observations that, under some circumstances, HIF-α can be stabilized by substances such as hydrogen peroxide [29].

It has also been postulated that ROS species produced by mitochondria are actually involved in signalling hypoxia [30]; it is proposed that ROS production at complex III of the electron chain is increased in hypoxia and that this signal is coupled to HIF stabilization. In support of this, the authors showed that inhibitors of mitochondrial electron transport, that reduce ROS production, reduce hypoxic stabilization of HIF-α. Though these findings have been controversial, an emerging picture is that mitochondrial inhibitors reduce the HIF-α stabilization that is observed in modest hypoxia but not that observed in more severe hypoxia, or in response to pharmacological blockade of the HIF hydroxylases [29,31,32]. This has suggested an action of mitochondrial inhibitors to increase PHD activity in moderate hypoxia. However, a further controversy has emerged in that it is not clear whether the increased PHD activity reflects a reduction in ROS signals that are proposed to inhibit PHD activity, or simply an elevation of cellular oxygen availability following reduction of oxygen consumption.

The existence of substantial oxygen gradients across the unstirred medium overlying respiring cell monolayers is

established [19]. Using both the hypoxia sensitive bioreduction marker pimonidazole [32] and a fluorescence based method [33] two groups have demonstrated that blocking oxygen consumption with mitochondrial inhibitors does, indeed, enhance intracellular oxygen availability. Moreover, in one study it was demonstrated that when cells were grown on highly oxygen permeable Teflon membranes that eliminate oxygen gradients, mitochondrial inhibitors no longer affected levels of HIF-α at any oxygen concentration [32]. On the other hand, groups arguing that reduced ROS rather than increased molecular oxygen is responsible for the reduction in HIF activation after mitochondrial inhibition have reported that the effect is specific to the site of inhibition in a way that is consistent with effects on the proposed site of ROS generation [30,34], whereas others have reported similar effects with all inhibitors [32,33]. To date, studies reporting different effects from different mitochondrial inhibitors have not measured effects on molecular oxygen availability at the monolayer.

Nitric oxide

There is evidence for regulation of the HIF by nitric oxide, and the HIF hydroxylases may represent a molecular interface between the two signalling systems. At sufficient concentrations nitric oxide can inhibit 2OG-dependent oxygenases by acting as an analogue of molecular oxygen [35]. Such an action might explain activation of the HIF system by nitric oxide donors under normoxic conditions [36], though it is unclear whether the necessary concentration of NO is reached, and even less certain that endogenous nitric oxide would act directly on HIF signalling in this way. Several observations indicate that the interaction is more complex. In particular, a second action of nitric oxide donors to reduce HIF activity in hypoxia has been demonstrated [37]. Whether these effects are mediated by actions on the HIF hydroxylases is unclear. However, one study has suggested that nitric oxide mediated inhibition of complex IV of the mitochondrial electron chain reduces oxygen consumption, effectively relieving cellular hypoxia as outlined above [33]. The issue is further complicated by the use in some studies of nitric oxide donors such as nitroprusside, which might have effects on cellular iron availability [38].

2OG, 2-oxoacids, and citric acid cycle intermediates

The requirement for the citric cycle intermediate 2OG as co-substrate for the HIF hydroxylases raises the question as to whether limiting availability may, in an appropriate cellular compartment, act as a further regulator of activity coupling metabolic to hypoxic signals. Reported values for the apparent K_m for 2OG are in the range 55–60 μM for the PHDs and 25 μM for FIH [6,16]. Again it is difficult to relate these figures to concentrations in the appropriate cellular compartment. Prediction of effects in vivo is further complicated by the potential role of other intermediates either in product inhibition (succinate) or as competitive inhibitors of 2OG.

Interestingly, reduction in HIF hydroxylase activity leading to upregulation of HIF-α or alternative (as yet unknown) targets of these enzymes is observed in hereditary cancer syndromes associated with defects at two points in the citric acid cycle. Thus, defects in succinate dehydrogenase (subunits B, C or D) are associated with hereditary paraglioma (a tumour that interestingly also occurs with increased incidence in subjects living at high altitude), whilst fumarate hydratase deficiency is associated with susceptibility to leiomyomata of the skin, uterine fibroids, and papillary renal cell carcinoma (for review, see [39]). Recent analyses have suggested that the defects cause accumulation of succinate or fumarate, respectively, and that (as has been described for 2OG-dependent oxygenases) these substances act as competitive inhibitors for 2OG [40–42]. The mode of oncogenesis and whether upregulation of the HIF pathway is itself responsible is unclear. Nevertheless, the findings raise the possibility that other perturbations of intermediary metabolism might modulate HIF hydroxylase activity. In this context, it is interesting that two other oxoacids (pyruvate and oxaloacetate) that have previously been reported to act as competitive inhibitors of some 2OG-dependent oxygenases [43] have recently been shown to induce the HIF system [44].

HIF substrate recognition and modification

In addition to hydroxylation, HIF-α polypeptides are subjected to a range of post-translational modifications with different studies identifying sites of phosphorylation, ubiquitylation, sumoylation, and (perhaps) acetylation. Such modifications could potentially enhance or reduce hydroxylation capacity if they occurred within recognition domains for the HIF hydroxylases or adjunct proteins. The extent of these domains and mechanisms of HIF-α substrate discrimination are to date unclear. Nevertheless, deletional analysis of intact HIF-α polypeptides and Gal-HIF-α fusion proteins indicates that large regions of the molecule are required for unimpaired regulation of HIF-α proteolysis and HIF-α transactivation, suggesting that the molecular determinants affecting hydroxylation capacity may be extensive and more complex than those defined to date in studies of short peptides.

Somewhat surprisingly, in vitro studies of substrate mutations on hydroxylase activity have indicated that very few residues outside the site of hydroxylation itself are critical for hydroxylation with even the Leu residues within a conserved LxxLAP motif at the sites of prolyl hydroxylation being tolerant of a range of substitutions [45,46]. This apparent lack of selectivity may in part reflect the use of high concentrations of short peptides in these assays or the omission from the assays of additional components that confer selectivity and which are normally required to promote hydroxylation at the very much lower concentrations of HIF-α polypeptides that exist under physiological conditions.

To date, the only X-ray structure for a hydroxylase bound to its (HIF-α) protein substrate is for FIH bound to the HIF-C-terminal activation domain [47]. This structure reveals that the enzyme-substrate interaction involves at least two sites; a tight turn that projects the side chain of the target asparagine residue towards the catalytic centre, and association of an α-helix lying distal to the site of hydroxylation in HIF-α with a hydrophobic surface of FIH. Whilst this structure readily explains the preference for Val802 or similar residues adjacent to the target site [48], it again does not explain the apparent requirement for more distant sequences [49]. Nevertheless, the proximity of a phosphorylation site at Thr796 prompted studies of this residue which have indicated that phosphorylation at this site does indeed interact to block hydroxylation, thus exemplifying a mechanism by which signalling pathways might be integrated in the regulation of HIF [48,50]. Though a similar interaction between acetylation at Lys532 in HIF-1α has been proposed to interact with hydroxylation or VHL recognition at Pro564 [51], the effects of modulating acetylation have not been reproduced in subsequent studies [52,53].

HIF hydroxylase associated proteins

That the HIF hydroxylases may exist as part of larger macromolecular complexes was suggested by the biochemical purification of PHD2 from a 320–440 kDa complex in rabbit reticulocyte lysate [54]. Several methods have been used in attempts to identify associated proteins. Thus,the chaperonin complex TriC was identified in immunoprecipitates of PHD3, but not other enzymes [55]. The functional significance of this is, however, still not clear, though it is interesting that TriC is also required for the production of a functional VHL protein [56]. In the converse approach, the PHDs were identified in immunoprecipitates of the ring-finger E3 ligases Siah1a/2. Though the Siah complex may target other PHDs, functional studies to date have focused on PHD3 and have revealed major effects on the proteolytic regulation of this protein [57]. Thus, in Siah1a/2 deficient cells PHD3 is constitutively upregulated and HIF is downregulated. In Siah2 deficient mice,the erythropoietic response to hypoxia is blunted, clearly demonstrating the physiological relevance of this additional control. Interestingly, Siah mRNA is itself upregulated by hypoxia potential providing another interface with oxygen availability.

Very recently two reports have identified other PHD-associated proteins that appear to have important actions through mechanisms which are as yet unclear. Using the yeast two-hybrid system a protein termed OS-9 was identified as a HIF-1α interacting protein that also interacts with PHD2 [58]. Functional studies have demonstrated an action to enhance total HIF prolyl hydroxylase activity and downregulate both HIF-1α protein levels and HIF target gene expression.

In another yeast two hybrid screen, the tumor suppressor ING4 was identified as a PHD2 interacting protein. Unexpectedly, functional studies have indicated that ING4 is not a PHD2 target, nor does it affect PHD2 activity or HIF-α stability [59]. Rather it appears to affect HIF target gene expression via an effect on transactivation. Whether this is mediated by the internal transactivation domain of HIF-α, which contains a site of PHD2-dependent prolyl hydroxylation, or reflects some more complex interaction with the C-terminal transactivation domain is unclear.

Regulation of hydroxylase abundance

The PHD enzymes are inducible proteins whose expression level is affected by a variety of stimuli. Thus, PHD1 is oestrogen inducible in breast cancer cell lines. In different cell types, PHD3 has been found to be inducible by p53, by nerve growth factor withdrawal, and by stimuli that induce smooth muscle differentiation (for review see [3]).

Interestingly, both PHD2 and PHD3 are themselves strongly inducible by hypoxia, whereas PHD1 levels are unchanged or even reduced in hypoxia [15,60]. The effects of this are clearly manifest in the response of HIF-α to re-oxygenation. Induction of PHD2 and PHD3 by hypoxia increases the total level of hydroxylase activity in cells [15] and results in enhanced rates of HIF degradation when cells are reoxygenated after longer (8 h) as opposed to shorter (1 h) periods of hypoxia [61]. Interestingly, both PHD2 and PHD3 are in fact transcriptional targets of HIF, effectively creating a feedback loop [62]. At present the physiological purpose of such a feedback is not clear. One possibility, however, is that it effectively shifts the dynamic range of oxygen sensitivity. As argued above, if the enzyme kinetics are such that the binding constant for molecular oxygen is high in relation to the physiological oxygen concentrations then the system will potentially be oxygen sensitive over the entire physiological range. However, an oxygen sensitive output on HIF-α will only be observed if total hydroxylase activity is limiting for the overall operation of the system and this may restrict that range. Thus higher levels of hydroxylase expression may act to extend the regulatory range to more severe hypoxia and vice versa (Fig. 2).

HIF hydroxylase mRNA and protein levels have been extensively studied in tissue culture cells and reveal that in normoxic cells PHD2 is substantially the most abundant enzyme [60]. In keeping with this, analysis of the effects of siRNA mediated suppression indicates that it is the most important enzyme in setting normoxic levels of HIF-α [63]. These studies have also revealed that inhibition of PHD2 alone is sufficient to upregulate HIF target gene expression in normoxia, despite continuing activity of FIH. Interestingly, the induction of PHD3 by hypoxia is substantially greater than that of PHD2. Thus in hypoxia, the balance of isoforms is altered and siRNA studies reveal that PHD3 plays a proportionally greater role in setting

hypoxic levels of HIF-α [60]. Other studies have compared the effects of changes in PHD2 levels with changes in FIH levels, on the expression of HIF target genes. Again these studies reveal that the relative importance of the HIF hydroxylases is dependent on the level of hypoxia, with PHD2 being more important in normoxia and modest hypoxia, and FIH being more important in severe hypoxia [64]. FIH levels do not change with hypoxia, so the difference presumably relates to an intrinsically greater capacity of FIH to operate in severe hypoxia.

HIF hydroxylase expression patterns also have the potential to alter the HIF response qualitatively. For instance, PHD3 appears to be more active on HIF-2α than on HIF-1α [60] and since the different HIF-α isoforms have distinct transcriptional targets [65], differential alteration of PHD activity could in theory alter the nature of the transcriptional output. Interestingly, mRNA expression patterns for the PHDs in the intact organism show marked tissue specificity. For instance, PHD1 is particularly abundant in the testis whereas PHD3 is strongly expressed in the heart [66]. However, the extent to which these patterns affect the operation of the HIF system in vivo has not yet been addressed.

In summary, the discovery of the HIF hydroxylases has revealed an unexpectedly direct connection between molecular oxygen and transcriptional responses to hypoxia. Relating the in vitro characteristics of these enzymes to their function in cells and intact organisms remains a challenge. However, accruing evidence indicates that they are able to integrate multiple signals that have the potential to deliver a flexible and controlled response to hypoxia, consistent with a major role in oxygen homeostasis. Important questions raised by these findings are whether pharmacological inhibition of the HIF hydroxylases will be an effective therapeutic strategy in ischaemic/hypoxic disease, and whether protein hydroxylation is involved in signalling in other hypoxia sensitive pathways.

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