Oxygen homeostasis and cancer: insights from a rare disease

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ABSTRACT – Many aspects of physiology and anatomy are precisely adjusted so that the right amount of oxygen reaches cells throughout the body. Hypoxia-inducible factor-1 (HIF-1) is activated by low oxygen tension in all mammalian cells and underpins many aspects of the impressive ability to match oxygen supply and demand. As examples, HIF-1 regulates:

- **local capillary architecture via angiogenic signalling**
- **red cell production via erythropoietin**
- **cellular metabolism via increased expression of glucose transporters and glycolytic enzymes.**

HIF-1 is also important in disease, for example in cancer where it is involved in angiogenesis. This review describes how HIF-1 is regulated by oxygen and the central role played by the von Hippel-Lindau tumour suppressor protein. The underlying oxygen sensor is provided by a family of enzymes which oxidise specific proline residues in HIFα subunits. Inhibiting these newly discovered enzymes provides a way of activating HIF-1 in the presence of oxygen – an exciting prospect for therapeutic intervention in ischaemic diseases.

KEY WORDS: hypoxia-inducible factor-1, oxygen, ubiquitin, von Hippel-Lindau

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Introduction

An important challenge for multicellular organisms is how to distribute oxygen reliably to every cell. Central to meeting this challenge in complicated organisms such as ourselves is the blood, its oxygenation in the lungs and its circulation in the vasculature. In normal tissues, the capillary vasculature and local metabolic requirements are precisely matched. In principle, this could perhaps be achieved by a blueprint design specifying every aspect of the vasculature, but in practice local oxygenation is used to regulate both cellular metabolism and vascular architecture. In a cancer, cells grow in an uncontrolled fashion. The vasculature, which comes from the normal host, does not supply enough oxygen. As a result, many regions of solid tumours are hypoxic, with important clinical correlates since the hypoxic regions are refractory to chemotherapy and radiotherapy. Even for patients treated with surgery, more tumour hypoxia correlates with poorer prognosis.

This article describes the HIF-1 oxygen response system which was originally recognised as regulating red blood cell production. The system appears to operate in all mammalian cell types, is a key mediator of a range of homeostatic responses, and underlies important aspects of cancer biology.

Control of red cell production

In response to anaemia or hypoxaemia, the fibroblasts in the kidney mount a rapid, high amplitude increase in erythropoietin (EPO) secretion[1]. How do they sense this need? Cyanide does not provoke the response, suggesting that sensing is not based on adenosine triphosphatase (ATP) depletion[2]. Exposure to cobalt or iron chelators mimics hypoxia, which suggests that the sensor might be a ferroprotein[3,4]. However, there are obvious limitations to this kind of approach in the complex setting of whole animals. A useful simplification came with the observation that a cultured hepatoma cell line produces EPO, increasing production dramatically when the level of oxygen in the tissue culture incubator is reduced[5].

This greatly simplified system has been used to work back from the EPO gene to an 'oxygen sensor'.

Glossary

Hypoxia-inducible factor-1 (HIF-1): a transcription factor found in cells exposed to low oxygen tensions. It consists of an α and a β subunit. HIF-1 binds to hypoxic response elements (HREs) and increases the expression of a range of target genes.

von Hippel-Lindau disease: an uncommon autosomal dominant condition. Affected individuals have a high risk of haemangioblastoma in the retina and central nervous system (CNS), renal cell carcinoma and phaeochromocytoma. The *VHL* gene, which is defective in these families, was cloned in 1993.

Prolyl hydroxylase domain (PHD) genes: these genes encode enzymes that oxidise specific proline residues in HIFα subunits to hydroxyproline. They use iron as a cofactor and 2-oxoglutarate (2-OG) and oxygen as co-substrates. The three PHD enzymes are related to the prolyl-4-hydroxylases that modify procollagen.

Key Points

- **Hypoxia-inducible factor-1 (HIF-1) is activated by hypoxia in all mammalian cells**
- **HIF-1 regulates a wide range of processes, including glycolysis, erythropoiesis and angiogenesis**
- **HIFα subunits are normally rapidly destroyed but are stable when the oxygen concentration is low**
- **Destruction of HIFα subunits involves enzymatic oxidation of specific proline residues and then capture by the von Hippel-Lindau gene product**
- **Inhibiting the enzyme reaction that regulates HIF-1 could be useful in diseases involving ischaemia**

In doing this, many tools have been important, including:

- cell culture systems and gene transfer techniques
- biochemical and physical analyses
- the model organism *Caenorhabditis elegans*, and
- information from genome sequencing projects.

These techniques are widely available and accessible, creating a real opportunity to get to the heart of many complex biological processes if the right questions are asked. A rare human genetic condition, von Hippel-Lindau disease, was decisive in dissecting the problem. As in many other cases, an invaluable contribution came ultimately from the resource of several billion genetically different humans and their phenotypic variation.

Definition of a hypoxia response element adjacent to the erythropoietin gene and isolation of hypoxia-inducible factor-1

A recombinant copy of the mouse or human EPO gene introduced into hepatoma cells was oxygen-responsive like the native gene. This oxygen-responsiveness depended on an enhancer element just downstream of the gene itself, termed an HRE[6]. The HRE binds a protein complex HIF-1 in extracts prepared from hypoxic cells but not from normoxic cells. Using a classical biochemical approach, the EPO HRE was used to purify HIF-1 which was found to contain two proteins, termed HIF-1α and HIF-1β[7]. Regulation by oxygen is through the HIF-1α subunit which is rapidly destroyed under normal oxygenation by proteasomal destruction. The central portion of the HIF-1α subunit is necessary for this oxygen-dependent degradation. At least two subregions can act as transferrable oxygen-dependent degradation domains (ODDD) , which means that they can be inserted into other proteins which then become unstable in the presence of oxygen[8]. A similar protein to HIF-1α, HIF-2α, has been identified; it also forms an HIF-1 complex with a β subunit, activates HREs and undergoes oxygen-dependent degradation[9].

Hypoxia-inducible factor-1 as a widespread oxygen response system

At an early stage, we reasoned that the oxygen-responsive system which regulates EPO through the HRE might well be used for other regulatory purposes. To test this hypothesis, cells which do not produce EPO were transfected with an HRE linked to a reporter gene. A wide range of mammalian cell lines regulated transcription of an HRE-linked reporter in response to changes in oxygen tension[10]. This had some interesting implications: first, it meant that the underlying oxygen sensing system could be studied in almost any cell type, which was subsequently shown to include simple multicellular organisms. Secondly, the presence of this oxygen sensing system in many cell types which were not involved in EPO production strongly suggested that it would have other biological functions. This indeed turns out to be the case. In the presence of low oxygen, HIF activation produces broad changes in gene expression, including increased expression of glycolytic enzymes and increased expression of angiogenic growth factors (Fig 1). Given this broad range of effects of HIF activation and the importance of oxygen homeostasis, it is not surprising that HIF activation has been centrally implicated in a range of physiological and pathological processes (Fig 2)[11].

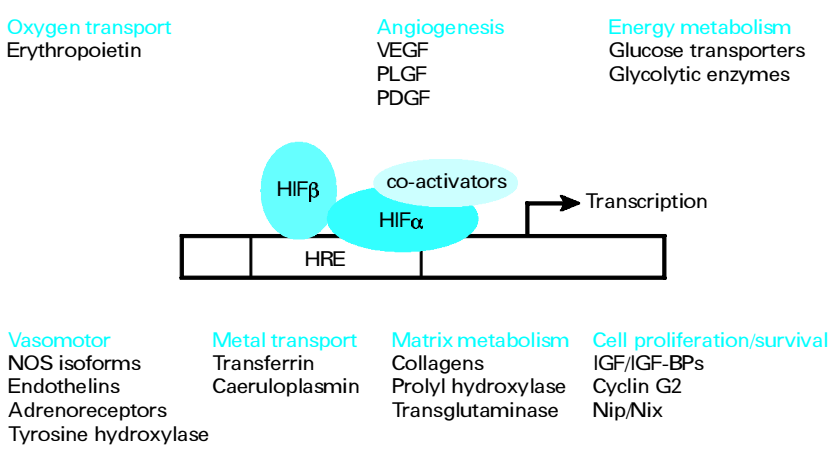

Fig 1. Examples of genes whose expression is increased when the hypoxia-inducible factor (HIF) transcription control system is activated
(HRE = hypoxia response element; IGF-BP = insulin-like growth factor-binding protein; Nip 3 = nineteen kilodalton interacting protein 3; Nix = Nip3-like protein x; NOS = nitric oxide synthase; PDGF = platelet-derived growth factor; PLGF = placenta growth factor; VEGF = vascular endothelial growth factor).

Regulation of glycolysis and angiogenesis by oxygen

Two adaptive responses mediated by HIF activation are particularly relevant to cancer: glycolysis and angiogenic signalling. The first steps in glucose utilisation lead to pyruvate production. Aerobic respiration centres around the generation of reducing equivalents (NADH and FADH) which are then used by the mitochondria to produce energy. Cells do not use the NADH in the absence of oxygen, which promotes lactate production. Lactate is then recycled by the liver. This effect of oxygen on lactate production was first described by Otto Warburg who won the 1931 Nobel Prize for Physiology for his work showing that cytochromes were necessary for respiration. He called it the Pasteur effect because of Pasteur's observation that yeasts stop producing carbon dioxide in the presence of oxygen. Although the glycolytic pathway generates ATP, this is much less efficient than aerobic respiration. Consequently, a greater metabolic flux from glucose to pyruvate is required in hypoxic conditions. An important contribution to meeting this requirement is HIF activation, which increases the expression of transporters and enzymes along the entire pathway[11].

It has also been known for many years that blood vessel growth is oxygen responsive. This has important clinical consequences: for example, if neonates are exposed to high levels of inspired oxygen, the network of retinal vessels is less extensive. On return to normal inspired oxygen, there is retinal ischaemia which stimulates new blood vessel growth. These exuberant blood vessels are fragile and leaky, resulting in the retinopathy of prematurity. The fact that HIF activation results in angiogenic growth factor production provides a mechanism for this plasticity, giving an insight into another homeostatic loop which matches oxygen supply and demand.

Interestingly, it has long been recognised that upregulation of glycolysis and angiogenesis are common features of cancer. The increased glycolysis of tumour slices compared with normal tissues led Warburg to suggest that the primary defect in cancer was damage to the normal mechanisms of aerobic respiration. Although this interpretation was incorrect, expression of glycolytic transporters and enzymes are commonly increased in cancer. Furthermore, it has emerged that the isoforms upregulated in cancer are the same subset that are HIF responsive. Angiogenic signalling was also previously recognised as a hallmark feature of solid tumours[12].

Hypoxic-inducible factor activation occurs in solid tumours and has effects on gene expression and growth

These findings raised the question as to what role HIF activation might play in cancer biology, and specifically whether HIF activation was an important contributor to the high levels of glycolysis and angiogenic signalling. To investigate this, we compared model tumours of murine hepatoma cells which lacked HIF-1β with other sublines which were HIF competent. Expression of the glucose transporter-3 and vascular endothelial growth factor (VEGF) was high around areas of necrosis in the wild-type tumours, but not in the HIF defective tumours, implying that this pattern is due to HIF activation[13]. The HIF defective tumours were also less well vascularised and grew more slowly.

One interesting aspect of HIF activation in tumours is that it results in a co-ordinate array of changes in gene expression, which might give some insight into the 'angiogenic paradox'. While angiogenesis is clearly in the interests of the tumour as a whole, it is hard to see how increased angiogenic signalling could offer an individual cell a competitive advantage over its neighbours since they will also benefit. In contrast, a genetic change which increases HIF activation might confer a survival advantage through another effect, with co-selection of increased angiogenic signalling – an obvious candidate would be enhanced glycolysis. In fact, as long as activating the HIF pathway has an overall positive effect on individual cell proliferation/survival, some downstream consequences could be neutral or negative in terms of 'reproductive fitness' and still be co-selected[14]. A good example is provided by the activation of some pro-apoptotic genes by HIF. Despite this, HIF activation is common in solid tumours, and inactivating the HIF pathway in experimental tumours has usually (but not always) slowed tumour growth.

Do mutations in the 'oxygen-sensing' pathway occur – and what can they tell us?

The discovery that the HIF system is both widespread and important in solid tumour behaviour added substantial impetus to efforts to understand the underlying oxygen sensing mechanism that regulates HIF. One route we took was to seek genetic changes which result in activation of the HIF pathway. Given the emerging biological insights, the reasoning was that such genetic

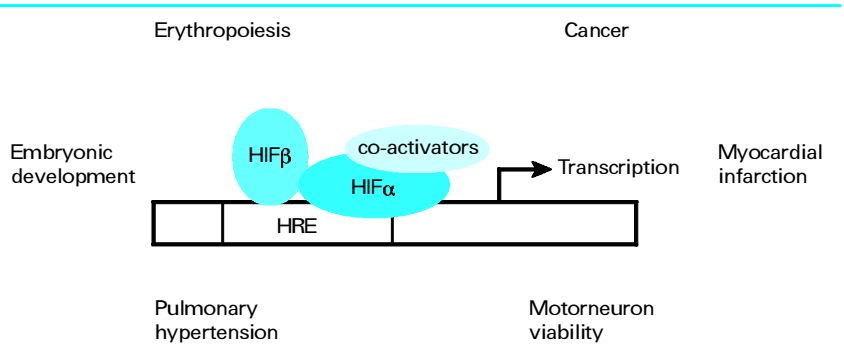

Fig 2. Physiological and pathological processes in which the hypoxia-inducible factor (HIF) pathway has been directly implicated (HRE = hypoxia response element).

changes might be associated with highly vascularised tumours. This is where the hereditary cancer, von Hippel-Lindau disease, enters the story in a decisive way. It turns out that cells lacking VHL function have full activation of the HIF pathway[15].

von Hippel-Lindau disease is relatively rare, with a frequency of about one in 36,000 live births. It is inherited in an autosomal dominant fashion, and the most important features are renal cell carcinoma, haemangioblastomas of the retina and CNS, and phaeochromocytoma. The underlying gene was identified by positional cloning in 1993[16]. Affected individuals inherit one defective allele in the germ line and the tumours have somatic inactivation of the second allele. Importantly, the great majority of non-familial clear cell renal carcinomas also show inactivation of both copies of the VHL gene[17]. VHL therefore conforms to Knudson's two-hit hypothesis[18] of tumour suppressor function.

In renal carcinoma cells with VHL defects, HIFα subunits were stable in the presence of oxygen and re-expression of VHL corrected this abnormality (Fig 3)[15]. This high level of HIFα subunits in normoxia increases transcription of target genes which include glucose transporters and VEGF.

The role of VHL in HIFα destruction fitted well with emerging evidence that VHL interacts with other cellular proteins, forming a complex with striking sequence and structural similarities to the SCF class of ubiquitin ligases (for Skp1-Cdc/CUL2-F-box)[19]. It was rapidly confirmed by us and others that the VHL complex acts as a ubiquitin E3 ligase specific for HIFα subunits[20–22]. The beta domain of the VHL molecule interacts directly with the ODD domain of HIFα subunits[21], while the alpha domain of VHL is in contact with other components of the E3 ligase complex.

In considering the role of HIF in tumour biology, it is clearly relevant that a genetic event which causes HIF stabilisation can cause cancer. However, since VHL has other reported functions, it does not prove that HIF stabilisation causes renal cancer. For example, VHL is required for proper assembly of a fibronectin matrix[23]. Two lines of investigation are pertinent to whether HIF activation and cancer are causally linked:

1 *Do missense VHL mutations which are associated with VHL disease or sporadic renal carcinoma alter its ability to regulate HIF?* Missense mutations in VHL which are associated with haemangioblastoma or renal carcinoma do interfere with its ability to capture or ubiquitylate HIFα subunits. In contrast, VHL mutations associated with familial phaeochromocytoma without other clinical manifestations (type 2C VHL disease) do not alter HIF regulation[24,25]. These observations from clinical mutations are consistent with a role for HIF dysregulation in haemangioblastoma and renal cell carcinoma but not in phaeochromocytoma.

2 *When does HIF activation occur temporally after VHL loss?* It seems that HIF activation occurs early (probably immediately) following VHL loss-of-function in renal tubular cells (S Mandriota, P Maxwell, *et al*; unpublished observations). This is again consistent with – but not proof of – a causative role for HIF activation in tumorigenesis.

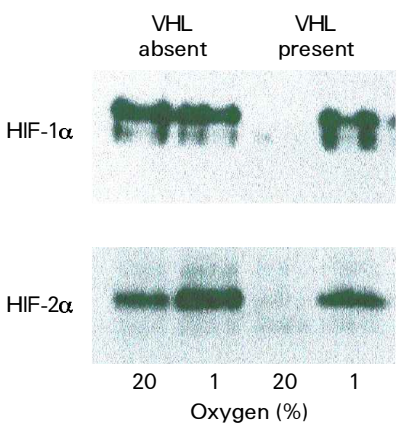

Fig 3. Immunoblot showing the effect of VHL status and oxygen tension on the abundance of hypoxia-inducible factor-1 (HIF-1) α proteins. In RCC4 cells, which lack VHL, HIFα proteins are readily detected under standard oxygenation (20%) with little increase in hypoxia (lanes 1 and 2). Re-expression of VHL suppresses the level of HIFα subunits in normoxia, restoring the normal hypoxic response (lanes 3 and 4).

From the interaction with von Hippel-Lindau protein to an oxygen-regulated modification of hypoxia-inducible factor α

In terms of understanding how the HIF system is regulated by oxygen, important insights came from studying how the HIFα subunits interact with VHL protein (pVHL) (Fig 4).

Using co-immunoprecipitation and supershift assays, HIFα subunits were shown to be physically associated with VHL protein. It is significant that this interaction was not observed in extracts either from cells treated with iron chelators or cobaltous ions or from hypoxic cells provided that oxygen was excluded from cells during lysis and extract preparation.

To simplify the system, the requirements for the interaction were analysed using recombinant proteins. A minimal sequence from the HIFα ODDD did not interact efficiently with VHL protein unless modified by mammalian cell extract, iron and oxygen. The modification process was temperature sensitive and required a high molecular weight component of the cell extract which was heat inactivatable. Taken together, these observations strongly suggest that the modification process is an enzymatic reaction[26].

Mutation of individual residues within the HIF-1α sequence demonstrated a critical requirement for the conserved prolyl residue 564. Furthermore, mass spectroscopic analysis of modified HIF-1α showed a mass shift of 16 Da, consistent with a single oxygen atom being added to this residue to form hydroxyproline. To test this further, a synthetic polypeptide was made in which this proline residue was substituted by 4-hydroxyproline; this was captured efficiently by VHL protein.

Enzymatic hydroxylation of proline is an important step in the processing of procollagen in which it is necessary to permit

proper assembly of mature collagen. In this case, the enzymes involved are 2-OG dependent dioxygenases. Importantly, these enzymes have a loosely co-ordinated ferrous ion at the catalytic centre and require oxygen as a co-substrate. Similar characteristics of the HIF prolyl hydroxylase would mean that the modification was oxygen-sensitive and that the effect of hypoxia would be mimicked by iron chelators. To test the idea that a similar prolyl hydroxylase acts on HIF, the effect of 2-OG analogues was examined and it was found that they antagonised the reaction[26].

One interesting aspect of these findings is that enzymatic oxidation of a polypeptide provides a new signal acting as a cue for recognition by a ubiquitin ligase and proteolytic destruction. This provided an attractive mechanism for an oxygen sensor, but also raised an obvious question as to the identity of the enzyme(s) involved. The route we took to identify the enzyme used the nematode, *Caenorhabditis elegans*.

Conservation of the hypoxia-inducible factor-1 von Hippel-Lindau system in *C. elegans*

First, the gene in *C. elegans* whose predicted protein product showed most similarity to HIF-1α was identified, and antibodies were raised against fragments of this protein product. Using these antibodies, we showed that the protein was expressed at a high level in worms exposed to hypoxia or a permeant iron chelator but was barely detectable in worms growing under standard conditions. A gene homologous to the human VHL gene was also identified and the effect investigated of inactivating this gene on HIF-1 protein expression. Strikingly, *vhl* defective worms showed constitutively high levels of HIF-1 expression[27].

Thus the VHL-HIF system is conserved in the nematode. This is intriguing in itself since there are less than a thousand cells in this relatively primitive multicellular organism, each cell being supplied with oxygen by diffusion alone. The VHL-HIF system therefore evolved before complex oxygen distribution systems. In contrast to the embryonic lethality of inactivating HIF or VHL in mice, effects in the worm are minor. It therefore seems likely that this system was originally involved in detecting oxygen as an important external variable. Later in evolution, the signal was exploited as a regulator of the internal architecture.

Identification of hypoxia-inducible factor prolyl hydroxylases in *C. elegans* and man

The entire genomic sequence of *C. elegans* is available. Using sequence and structural information concerning known 2-OG dependent oxygenases, candidate genes were identified for the HIF prolyl hydroxylase. These were then screened by determining the level of HIF-1 protein in worms with genetic mutations. Mutations in the *egl-9* gene caused high levels of HIF-1 protein. It was then confirmed that the gene product acted as a prolyl hydroxylase in experiments in which EGL-9 and HIF-1 proteins were expressed together in bacteria.

Homologues of *C. elegans* EGL-9 were then sought in humans and mice. Three closely related genes were identified, which

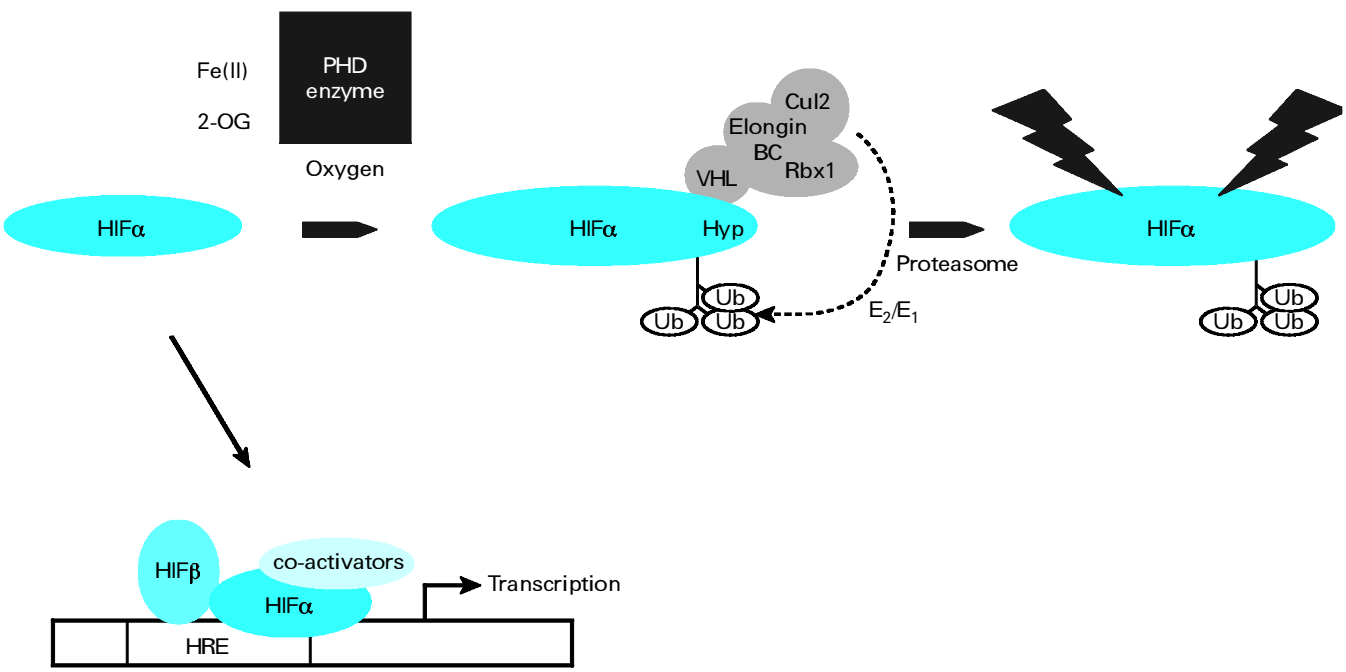

Fig 4. Summary of hypoxia-inducible factor (HIF) regulation. In hypoxia, HIFα subunits are stabilised and the HIF pathway activated. In the presence of oxygen (upper part of figure), specific prolyl residues in HIFα are hydroxylated by prolyl hydroxylase domain (PHD) enzymes. The von Hippel-Lindau protein (pVHL) captures modified HIFα protein, leading to its ubiquitylation and destruction (Fe (II) = divalent iron; HRE = hypoxia response element; Hyp = hydroxyproline; OG = oxoglutarate; Ub = ubiquitin).

were named PHD1, 2 and 3. Each of these gene products was shown in *in vitro* assays to modify HIFα subunits, enabling capture by VHL protein[27].

Hypoxia-inducible factor prolyl hydroxylases as oxygen sensors

The rate of HIF modification by PHD enzymes is exquisitely sensitive to the level of oxygen in *in vitro* assays (Fig 5). Furthermore, HIF hydroxylation is sensitive to iron chelators and cobaltous ions. Thus, HIF hydroxylation by PHD enzymes accords precisely with the characteristics of an oxygen-sensor underlying the HIF system.

The findings indicate substantial possibilities for complexity in the HIF response. First, the availability of intracellular iron and 2-OG could influence the rate of the reaction, and consequently modulate the hypoxic response. In fact, the interactions between Krebs cycle intermediates, intracellular iron and HIF signalling may be quite extensive. Teleogically, it is plausible that this results in HIF activation when energy demand is increased in non-hypoxic circumstances such as cell proliferation. This might be attractive since it could, for example, increase vascular supply before tissues become hypoxic.

Secondly, since the prolyl hydroxylation rate is not at equilibrium the amount of PHD enzyme is likely to alter the response to changes in oxygen. It is therefore likely to be important that the levels of expression of at least PHD3 are variable between tissues and/or cell types. They have been reported to be influenced by both pro-apoptotic and growth-promoting stimuli[28,29]. At least in some cells, hypoxia itself increases expression of PHD2 and PHD3, which would be expected to downregulate the HIF response. This accords well with a recent report that the rate of HIF destruction on re-oxygenation is increased on prolonged hypoxic exposure[30]. Complexity is also added by the recognition that there is a second prolyl hydroxylation site in the N-terminal portion of the ODDD of HIF-1α and HIF-2α[31]. This offers an explanation for the incomplete effects of deleting part of the extensive ODDD. Importantly, the different PHD enzymes appear to exhibit differential preferences for the two sites[27].

Overall, the scope for complexity in the HIF response would seem capable of adapting it for many different purposes in higher multicellular organisms.

It seems appropriate to highlight two particular questions:

1. As mentioned above, HIF is also regulated by hypoxia-induced nuclear translocation and transactivator recruitment. How are these regulated? In each case, iron chelation or cobaltous ions mimic hypoxia, suggesting that these might also involve PHD enzymes or other 2-OG dependent dioxygenases. Furthermore, since VHL defective cells show a complete, rather than a partial HIF response, it seems likely that pVHL is also involved in these aspects of HIF regulation.
2. An overlapping question is what other cellular processes may involve regulation of protein-protein interactions by enzyme–mediated hydroxylation reactions which depend on 2-OG and iron and are oxygen-sensitive?

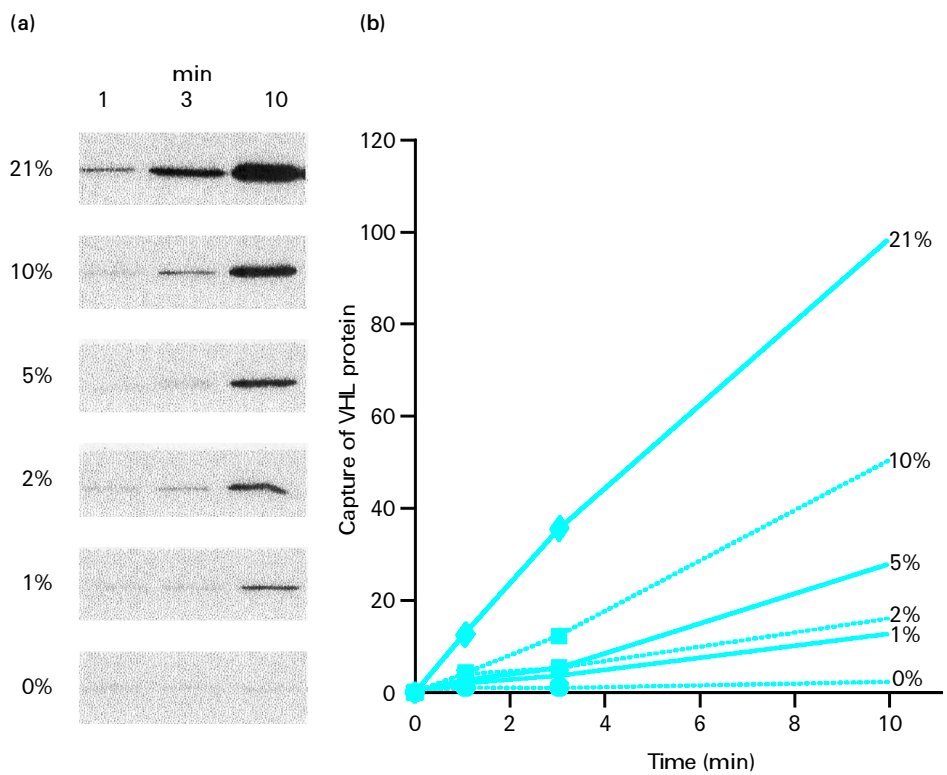

Fig 5. Experiment showing the oxygen-dependence of hypoxia-inducible factor (HIF) modification by prolyl hydroxylase domain (PHD) enzyme.
HIFα protein was incubated with cell lysate containing PHD enzyme at room temperature for the indicated times at different oxygen concentrations: (a) HIF modification detected by its ability to capture ^{35}S labelled VHL protein (left panel); (b) the data in graphic format. Data courtesy of Dr D Mole.

Potential therapeutic opportunity

Perhaps the most exciting aspect of improved understanding of HIF regulation is that it provides potential targets for therapeutic intervention. The HIF system is activated either by blocking the HIFα-pVHL interaction or by antagonising the PHD enzymes. We envisage that this could be used to great benefit in ischaemic settings, effectively co-opting a complete set of biological responses to improve local cellular adaptation and promote tissue perfusion and survival.

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