How Cells Sense Oxygen: The Role of 2-Oxoglutarate-Dependent Dioxygenases

Jonathan Gleadle*

Flinders Medical Centre and Flinders University, Bedford Park, SA 5042

**Corresponding author: jonathan.gleadle@health.sa.gov.au*

Gene Expression in Response to Hypoxia

Cells respond to lack of oxygen or hypoxia with a substantial alteration in gene expression. Studies of the transcriptional response to hypoxia by the erythropoietin gene, which underlies the physiological feedback loop controlling red blood cell production, led to the cloning of the transcription factor hypoxia inducible factor (HIF) (1). The widespread operation of the *cis*-acting hypoxia responsive element (HRE), to which HIF binds in many, if not all, mammalian cells, and the widespread expression of HIF led to the realisation that this transcriptional mechanism of response to oxygen could underpin the hypoxic regulation of many genes. Subsequent studies defined a broad range of genes as responding to hypoxia through HIF, including glycolytic enzymes such as phosphoglycerate kinase and angiogenic factors such as vascular endothelial growth factor (VEGF). More recently, microarray studies have demonstrated a broad HIF-dependent transcriptional response to hypoxia with induction and repression of many genes. It also became clear that hypoxia was a common feature in many cancers and that HIF was commonly activated. A focus on the mechanism underlying the function of the gene product VHL (von Hippel Lindau), whose mutation causes the hereditary cancer syndrome von Hippel Lindau disease and is commonly mutated in sporadic renal cancer, provided insight into the mechanism by which oxygen is sensed by cells (2).

In studying cells for regulation of HIF by hypoxia, it emerged that HIF-α protein expression was induced in cells by oxygen deprivation and became rapidly degraded by the ubiquitin-proteasomal system in response to re-exposure to oxygen. Studies of cells with a mutated VHL gene showed constitutive upregulation of HIF-α expression and loss of hypoxic induction (2). These changes were mirrored by constitutive upregulation of HIF target genes such as VEGF and glucose transporter 1, but could be reversed by re-introduction of a wildtype VHL gene. Further studies demonstrated direct binding of the HIF-α molecule to VHL when exposed to oxygen. This binding was found to require specific prolyl residues within an oxygen-dependent degradation domain of HIF-α. Mass spectroscopic studies revealed that under oxygenated conditions these specific prolyl residues were modified to hydroxyproline, which then enabled VHL binding and, by virtue of its action as an E3 ubiquitin ligase, led to proteasomal degradation. Under hypoxic conditions, hydroxyproline was not formed and a stable HIF molecule could exert its transcriptional effects (3,4) (see **Fig. 1**).

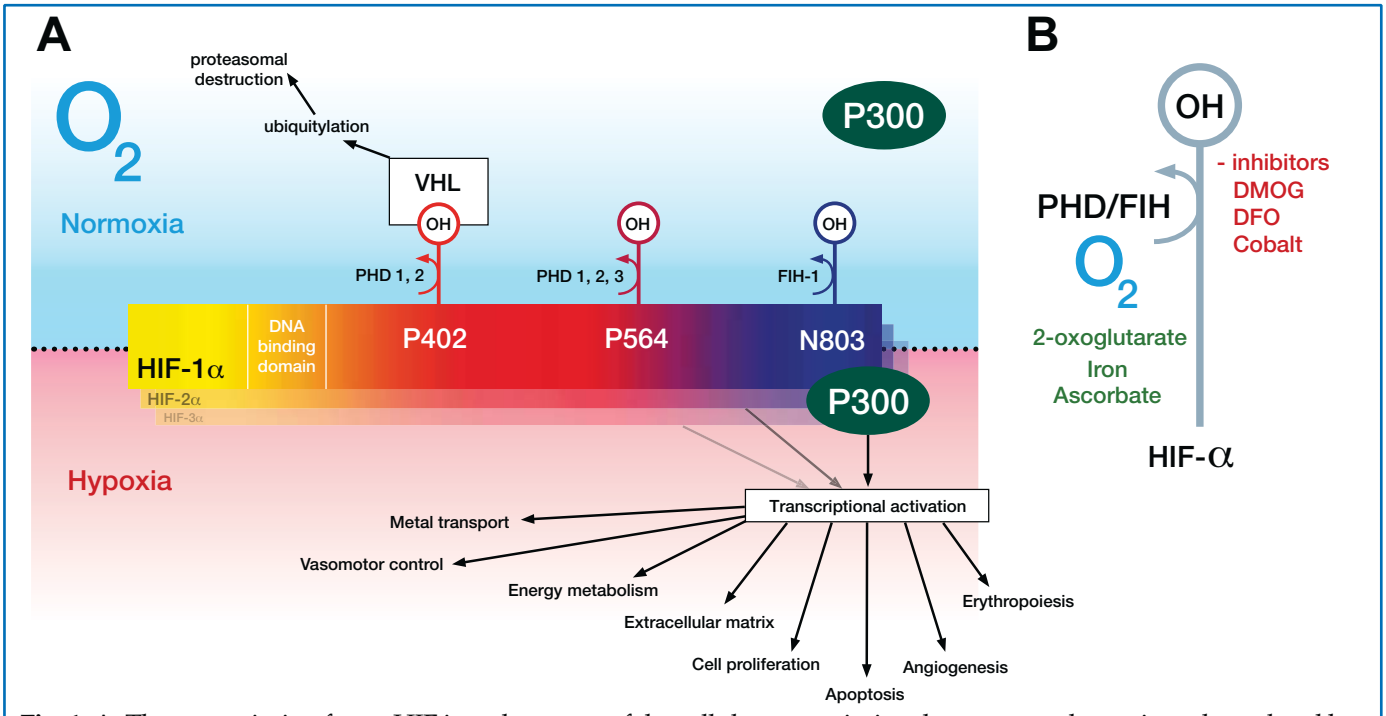

Fig. 1. A. The transcription factor HIF is at the centre of the cellular transcriptional response to hypoxia and regulated by the oxygen-dependent prolyl and asparaginyl hydroxylases.
B. The enzymatic reaction of the HIF hydroxylases, PHD and FIH, and their co-substrates (shown in green) and potential inhibitors DMOG (dimethyloxalylglycine), DFO (the iron chelator, desferrioxamine) and cobaltous ions (shown in red).

Regulation of HIF by Oxygen via Prolyl and Asparaginyl Hydroxylation

Biochemical precedence for enzymatic conversion of prolyl to hydroxyprolyl residues existed in collagen molecules, mediated by the action of collagen prolyl hydroxylases. Importantly, these enzymes, which are 2-oxoglutarate-dependent dioxygenases, require molecular oxygen for their enzymatic function, and this provided a potential direct link between the availability of oxygen and the regulation of HIF. Indeed, it was subsequently demonstrated that the oxygen atom that was added upon prolyl hydroxylation was derived from ambient molecular oxygen. Experiments in *Caenorhabditis elegans*, which possess a paralogous HIF-VHL system of response to hypoxia, showed that mutations of the known collagen prolyl hydroxylases did not affect the HIF response (5). However, in worms with mutations of the predicted prolyl hydroxylase *egl-9*, HIF was constitutively induced and unresponsive to hypoxia (5). A search for homologous mammalian enzymes led to the discovery of three similar molecules, PHD1, PHD2 and PHD3 (prolyl hydroxylase domain), which were capable of converting HIF to a VHL-binding form via the oxygen-dependent conversion of specific prolyl to hydroxyprolyl residues (5-7). Moreover, reactions conducted in a controlled oxygen environment revealed that the ability of the purified enzymes to hydroxylate HIF-1α was progressively diminished by exposing the enzyme to increasingly severe degrees of hypoxia (5), in keeping with functioning as an oxygen sensor. Subsequently, siRNA-mediated suppression of these genes has been shown to lead to HIF-α activation (8,9). Further support for their *in vivo* role has come from genetic studies of familial erythrocytosis, the selection of genetically advantageous traits in populations living at high altitude and observations in knockout mice.

The HIF-α molecules are also subject to specific asparaginyl hydroxylation by the related 2-oxoglutarate-dependent dioxygenase, factor inhibiting HIF (FIH) (10-12). This hydroxylation is also oxygen dependent and inhibits the ability of the HIF molecules to bind to the transcriptional co-activator p300, providing an additional mechanism for the inhibition of HIF action by oxygen. In keeping with this, the siRNA-mediated suppression of FIH does influence the expression of HIF target genes in cell culture (13), but in FIH knockout mice, effects on HIF were not so clearly discernible, with more prominent effects on metabolism and ventilation (14). This may indicate a generally more important role for the prolyl hydroxylase enzymes in regulating HIF, with FIH having a more subtle influence, but also raises the potential important role of other non-HIF substrates of FIH.

This understanding of the nature of the enzymes controlling HIF activity has also provided important pharmacological and biochemical insights into the control of HIF. The HIF hydroxylases are also subject to regulation by the provision of iron, a co-factor of the enzyme. Iron chelation by compounds such as desferrioxamine, or replacement by particular transition metal ions such as cobalt or nickel can inhibit enzyme activity, leading to HIF-α induction and increased expression of HIF-dependent genes. Very recently, the poly(rC)-binding proteins (PCBP) have been implicated in iron delivery to the HIF hydroxylases (15). The role of the HIF hydroxylases and other 2-oxoglutarate-dependent dioxygenases in mediating sensing of iron and 2-oxoglutarate is incompletely clarified. Interestingly, the enzymatic reaction of the 2-oxoglutarate-dependent dioxygenases mirrors respiration by consuming oxygen and generating carbon and links the sensor to the citric acid cycle. Furthermore, ascorbate (vitamin C) is required for activity of this family of enzymes by maintaining enzyme-bound iron in its active, reduced form (and accounts for the nutritional illness scurvy in which there is deficient collagen prolyl hydroxylation). The enzymatic requirements of the enzymes, notably their requirement for 2-oxoglutarate, has led to the development of pharmacological inhibitors (e.g., dimethyloxalylglycine) and insights into the mechanisms of HIF activation and oncogenesis that may arise in tumours with mutations of genes encoding members of the citric acid cycle (e.g., fumarate hydratase). There is increasing study of the use of HIF hydroxylase inhibitors in the treatment of ischaemic diseases and in increasing the production of proteins such as erythropoietin.

Role of Hypoxia Inducible Factor

The main effect of HIF activation by hypoxia is transcriptional activation of a large array of genes with roles in oxygen delivery, metabolism, cell cycle, extracellular matrix modification and apoptosis. Whilst much of this activation of gene expression seems to be mediated via direct HIF binding to hypoxically responsive DNA elements, the large number of genes that are repressed following hypoxic exposure appear to be regulated via indirect mechanisms (16). Chromatin immunoprecipitation experiments have suggested that these effects are mediated indirectly, potentially through other transcription factors, effects on mRNA stability and via the effects of microRNAs.

In addition to mRNA regulation, the regulation of expression of microRNAs such as miR-210 seems important in mediating responses to oxygen (17). Many of the responses to hypoxia can be regarded as facilitating adaptive or developmental responses with advantages to the cell or organism in surviving hypoxic exposure. It is important to recognise that in mammals there are three HIF-α isoforms with differing roles, regulation and distribution, with HIF-2α having a particular role in promoting malignant progression and HIF-3α functioning as a negative inhibitor of the HIF response.

Non-HIF Substrates of Prolyl and Asparaginyl Hydroxylation

Subsequent to the discovery of oxygen-dependent prolyl hydroxylation of HIF, several other substrates of the PHD enzymes have been reported (**Table 1**). For some of these purported substrates, secure *in vivo* mass spectrometry-based studies demonstrating specific prolyl hydroxylation is awaited, as is the extent to which variations in oxygen provision exert a functionally important effect. Of interest is the observation for some of these substrates that there is recognition of hydroxyprolyl residues by

Table 1. Substrates of the prolyl hydroxylases PHD1, 2 and 3 and their potential interaction with VHL.

Substrate	Enzyme	Interaction with VHL
HIF-1α	PHD1, PHD2, PHD3	+
HIF-2α	PHD1, PHD2, PHD3	+
RNA polymerase subunit Rpb1	PHD1, PHD2	+
β2-adrenergic receptor	PHD3	+
IkappaB kinase-beta	PHD1	?
Pyruvate kinase M2	PHD3	?
Myb binding protein p160	PHD1?	+
TRPA1 channel	PHD1, PHD2, PHD3	?

the VHL protein (**Table 1**). This raises the possibility of a more general function for VHL in the recognition of hydroxyprolyl residues in non-HIF proteins. However, further experimental validation and X-ray crystallographic structural studies of such interactions, such as have been provided for the HIF-VHL interaction (18), are required to appreciate the existence of and constraints on such binding. The relevance of these other potential VHL substrates to the oncogenic mechanism of VHL mutation is also unclear. Similarly, FIH-1 appears capable of acting as an asparaginyl hydroxylase on non-HIF substrates such as IkappaB and Notch, many of which are characterised by ankyrin repeat domains (19). However, what remains less clear is the functional consequences of asparaginyl hydroxylation on such proteins containing ankyrin repeat domains. Whilst functional consequences of the asparaginyl hydroxylation of HIF are linked to oxygen responses, the influence of FIH on the function of other protein substrates is unclear. It has been postulated that the high affinity of FIH for such substrates provides a means for sequestering FIH away from the HIF molecules. Whatever the explanation for this modification, its conservation in many eukaryotic organisms suggests an important role.

Role of Other 2-Oxoglutarate-dependent Dioxygenases in the Response to Hypoxia

A large number of 2-oxoglutarate-dependent dioxygenases exist with a variety of enzymatic functions in peptide modification, histone and DNA modification and repair (**Table 2**). They share with the PHDs and FIH an enzymatic requirement for molecular oxygen. The extent to which physiological or pathological variation in oxygen tension can thereby lead to alterations in the action of this family of enzymes requires further study. It is of interest that, as for several of the HIF hydroxylases, they themselves are subject to HIF-mediated transcriptional regulation by hypoxia. The ability to modify histones and nucleic acids

Table 2. Examples of 2-oxoglutarate dioxygenases with known substrates and enzymatic action.

Enzyme	Substrate	Modification
PHD1, 2, 3 (prolyl hydroxylase domain 1, 2, 3)	HIF	Prolyl 4*R*-hydroxylation
FIH-1 (factor inhibiting HIF)	HIF, ankyrin repeat domains	Asparaginyl 3*S*-hydroxylation
JMJD1A (Jumonji domain containing protein 1A)	Histone	Histone H3 (H3K9me2) demethylation
JMJD1B (Jumonji domain containing protein 1B)	Histone	Histone H3 (H3K9me3/2) demethylation
JMJD6 (Jumonji domain containing protein 6)	U2AF65 (U2 small nuclear ribonucleoprotein auxiliary RNA splicing factor 65 kD)	Lysyl-5-hydroxylation
JARID1B (Jumonji AT-rich interactive domain 1B)	Histone	Histone H3 lysine 4 (H3K4me3) demethylation
PHF8 (PHD finger protein 8)	Histone	Histone H4 lysine 20 (H4K20me) demethylation
ASPH (aspartate beta-hydroxylase)	EGF (epidermal growth factor)	Asparaginyl/aspartate 3*R*-hydroxylation
P4HA1 (prolyl 4-hydroxylase alpha 1)	Pro-collagen	Prolyl 4*R*-hydroxylation
PLOD1 (procollagen lysyl hydroxylase 1)	Pro-collagen	Lysyl 5*R*-hydroxylation
ABH1 (AlkB homologue 1)	DNA/RNA	1-Methyladenosine/ 3-methylcytosine demethylation
FTO (fat mass and obesity associated gene)	DNA RNA	3-Methylthymine demethylation N6-Methyladenosine demethylation

in an oxygen-dependent fashion provides a potential route for an influence of hypoxia in epigenetic modification. Important effects of hypoxia via such mechanisms on the extracellular matrix and on RNA splicing also seem likely.

It is unclear the extent to which the transcriptional responses of cells to hypoxia are mediated by HIF and the HIF hydroxylases. Under particular cell culture conditions, HIF hydroxylase inhibition with dimethyloxalylglycine leads to a very similar alteration in gene expression as hypoxia and many of these transcriptional changes can be affected by HIF-1α siRNA (20). This suggests a dominant role for the HIF signalling system under these conditions, but it remains possible that at other severities of hypoxia or in other cells, other transcriptional responses may contribute.

Conclusion

The definition of an oxygen-dependent enzymatic modification of transcription factors that regulate the transcriptional response to hypoxia has provided an important understanding of one central mechanism by which cells sense and respond to oxygen with alterations in gene transcription. The extent to which further cellular responses to oxygen may be mediated by other 2-oxoglutarate-dependent dioxygenases is being increasingly explored. Furthermore, it appears likely that these signalling mechanisms will have a broader role in other biochemical, cellular and physiological responses to oxygen.

References

1. Wang, G. L., and Semenza, G. L. (1993) *J. Biol. Chem.* **268**, 21513-21518
2. Maxwell, P. H., Wiesener, M. S., Chang, G.-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999) *Nature* **399**, 271-275
3. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) *Science* **292**, 468-472
4. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G. J. (2001) *Science* **292**, 464-468
5. Epstein, A. C., Gleadle, J. M., McNeill, L. A., *et al.* (2001) *Cell* **107**, 43-54
6. Ivan, M., Haberberger, T., Gervasi, D. C., Michelson, K. S., Gunzler, V., Kondo, K., Yang, H., Sorokina, I., Conaway, R. C., Conaway, J. W., and Kaelin, W. G. J. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 13459-13464
7. Bruick, R. K., and McKnight, S. L. (2001) *Science* **294**, 1337-1340
8. Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003) *EMBO J.* **22**, 4082-4090
9. Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) *J. Biol. Chem.* **279**, 38458-38465
10. Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruick, R. K. (2002) *Genes Dev.* **16**, 1466-1471
11. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) *Science* **295**, 858-861
12. Hewitson, K. S., McNeill, L. A., M.V., R., Tian, Y.-M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. (2002) *J. Biol. Chem.* **277**, 26351-26355
13. Stolze, I. P., Tian, Y. M., Appelhoff, R. J., Turley, H., Wykoff, C. C., Gleadle, J. M., and Ratcliffe, P. J. (2004) *J. Biol. Chem.* **279**, 42719-42725
14. Zhang, N., Fu, Z., Linke, S., Chicher, J., Gorman, J. J., Visk, D., Haddad, G. G., Poellinger, L., Peet, D. J., Powell, F., and Johnson, R. S. (2010) *Cell Metab.* **11**, 364-378
15. Nandal, A., Ruiz, J. C., Subramanian, P., Ghimire-Rijal, S., Sinnamon, R. A., Stemmler, T. L., Bruick, R. K., and Philpott, C. C. (2011) *Cell Metab.* **14**, 647-657
16. Mole, D. R., Blancher, C., Copley, R. R., Pollard, P. J., Gleadle, J. M., Ragoussis, J., and Ratcliffe, P. J. (2009) *J. Biol. Chem.* **284**, 16767-16775
17. Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., Davuluri, R., Liu, C. G., Croce, C. M., Negrini, M., Calin, G. A., and Ivan, M. (2007) *Mol. Cell. Biol.* **27**, 1859-1867
18. Hon, W. C., Wilson, M. I., Harlos, K., Claridge, T. D., Schofield, C. J., Pugh, C. W., Maxwell, P. H., Ratcliffe, P. J., Stuart, D. I., and Jones, E. Y. (2002) *Nature* **417**, 975-978
19. Coleman, M. L., McDonough, M. A., Hewitson, K. S., *et al.* (2007) *J. Biol. Chem.* **282**, 24027-24038
20. Elvidge, G. P., Glenny, L., Appelhoff, R. J., Ratcliffe, P. J., Ragoussis, J., and Gleadle, J. M. (2006) *J. Biol. Chem.* **281**, 15215-15226