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Oxygen sensing, HIF-1α stabilization and potential therapeutic strategies

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The ability to sense oxygen is a fundamental biological phenomenon, and reduced oxygen supply (hypoxia) has profound effects on developmental and physiological processes in mammals. A specific response to hypoxia is the transcriptional upregulation of genes which help to restore oxygen homeostasis at the cellular, local, and systemic levels. These genes are involved in cell survival, glycolysis, angiogenesis, erythropoiesis, and iron metabolism (reviewed in [25, 33]). Recent reports showed that the oxygen-sensing mechanism requires prolyl hydroxylase activity. In this short review, we aimed to give an update on the latest developments in oxygen sensing and oxygen-regulated gene expression.

HIF-1 is a master regulator of oxygen homeostasis

The transcription factor hypoxia-inducible factor-1 (HIF-1) has emerged as a key regulator of oxygen homeostasis. It consists of HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator) subunits, both belonging to the PAS (Per-ARNT/AhR-Sim) subfamily of basic helix-loop-helix transcription factors [32]. HIF-1 is widely, perhaps universally, expressed in the tissue of vertebrates, insects, worms, and most probably other species [2, 8, 27]. Activation of HIF-1 is critically dependent on hypoxia-induced stabilization of its α-subunit, while ARNT is abundantly expressed and not affected the by oxygen partial pressure. In contrast to ARNT, HIF-1α cannot be detected above a critical oxygen partial pressure when it is subjected to rapid ubiquitination and proteasomal degradation. Only hypoxic exposure or addition of hypoxia-mimicking reagents (e.g., cobalt chloride or iron chelators) leads to increased HIF-1α protein levels and hence to activation of HIF-1-dependent target genes.

When exposed to hypoxia, HIF-1α is stabilized instantaneously [15]. Interestingly, a recent report, analyzing HIF-1α levels in different mouse organs, showed that in brain, kidney, liver, heart, and skeletal muscle, HIF-1α can be detected in mice kept under normoxic conditions and increases its concentration in response to systemic hypoxia [28]. Of note, the partial pressure of oxygen that leads to this increase of HIF-1α protein levels and the time frame of maximal HIF-1α expression is highly tissue dependent. These findings propose that HIF-1α may be required for the basal expression of genes that are necessary to provide the cellular energy requirements. This suggestion is supported by the observation that normoxic basal transcription levels of HIF target genes are diminished upon disruption of either the HIF-1α [13] or the HIF-1β [35] gene in embryonic stemcells.

Stabilization of HIF-1α under hypoxic conditions

Once stabilized, HIF-1α translocates to the nucleus guided by a nuclear localization signal present in the C-terminus [16]. This nuclear translocation is independent of ARNT, which constitutively resides in the nucleus [5]. Using cell lines that inducibly overexpress high HIF-1α levels under normoxic conditions, we recently showed that HIF-1α translocates into the nucleus under normoxic as well as hypoxic conditions with hardly any detectable immunoreactivity remaining in the cytoplasm [9]. This finding suggests that HIF-1α nuclear translocation occurs without the need of any further hypoxia-dependent signals. After translocation to the nucleus, HIF-1α het-

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Table 1 HIF-1 target genes to be directly regulated by HIF-1. Alphabetical list of all HIF-1 target genes identified to date. (adapted from [3])

adrenomedullin
aldolase A
α_{1B}-adrenergic receptor
carboanhydrase-9
ceruloplasmin
enolase 1
erythropoietin
glucose transporter 1
glyceraldehyde-3-phosphate dehydrogenase
heme oxygenase 1
inducible NO synthase
insulin-like growth factor binding protein
lactate dehydrogenase A
Nip3 proapoptotic protein
p35srj
phosphofructokinase L
phosphoglycerate kinase 1
plasminogen activator inhibitor-1
prolyl 4-hydroxylase α (I)
retrotransposon VL30
transferrin
transferrin receptor
vascular endothelial growth factor

Table 2 The HIF-1 consensus DNA-binding site contains CGTG as the conserved core sequence, that is usually preceded by an adenosine and followed by a cytosine residue. Adapted from [3]

T_{19}	A_{38}	**CGTG**	C_{31}	G_{16}	G_{19}	C_{16}
G_{16}	G_5		G_5	T_{13}	C_{12}	G_{12}
C_8	T_1		A_5	C_{11}	A_6	T_{11}
A_1			T_3	A_4	T_7	A_4

erodimerizes with ARNT, and the resulting HIF-1 complex binds to the conserved consensus sequence (R)CGTG within the hypoxia response element (HRE) present in oxygen-regulated target genes (Tables 1, 2).

Although HIF-1α is regulated mainly by the oxygen partial pressure, other factors modulate HIF-1α stability and its *trans*-activation activity. Post-translational modifications (mainly phosphorylation) of HIF-1α are essential for full transcriptional activation and stabilization of the HIF-1 complex. Extensive phosphorylation of HIF-1α by the MEK-1/p42/p44 MAPK pathway enhances the transcriptional activity of HIF-1. As such, addition of the MEK-1 inhibitor PD98059 does not alter either the hypoxic stabilization or DNA-binding ability of HIF-1α but it inhibits the *trans*-activation ability of HIF-1α, thereby reducing the transcriptional activity of HIF-1 target genes [11, 21]. The phosphatidylinositol 3′-kinase (PI3K)/PTEN/Akt signaling pathway is also involved in HIF-1α stabilization. Addition of the PI3-kinase inhibitor LY294002 inhibits HIF-1α induction and hence an upregulation of HIF-1 target genes [4, 34].

HIF-1α under normoxic conditions: the oxygen-sensing mechanism

Above a critical intracellular oxygen partial pressure, HIF-1α protein is maintained at low and often undetectable levels. Following its ubiquitination HIF-1α is proteasomally degraded [10, 17, 23]. It has been demonstrated that the von Hippel-Lindau tumor-suppressor protein (pVHL), a subunit of a multiprotein complex harbouring E3 ubiquitin ligase activity, is responsible for regulating cellular levels of HIF-1α [19] (Fig. 1). Deletion analysis revealed that pVHL directly interacts with the oxygen-dependent degradation (ODD) domain of HIF-1α [6, 20, 30]. Mutation of pVHL prevents it from binding to HIF-1α, causing constitutive expression of this transcription factor and its target genes.

The key enzymes controlling the oxygen-dependent step in this degradation cascade are specific HIFα-proline hydroxylases that require Fe(II) as a co-factor as well as dioxygen and 2-oxoglutarate as co-substrates [12, 14]. It has been demonstrated that HIF-1α is hydroxylated at the proline residues 402 and/or 564, two highly conserved amino acids within the ODD domain [18]. Under normoxic conditions, prolyl hydroxylation enables the specific interaction of pVHL with HIF-1α, whereas under hypoxic conditions, prolyl hydroxylation does not occur, preventing pVHL binding and the degradation of HIF-1α. Interestingly, both proline residues can function independently and are part of a common motif, LXXLAP, implicating increased combinatorial interactions that confer specificity to the destruction process of HIF-1α (Fig. 1).

In their seminal work, Epstein et al. [8] demonstrated the existence of a homologous HIF-VHL-prolyl hydroxylase system in the worm *Caenorhabditis elegans*. It was shown that the *C. elegans* HIF-1α homolog was strongly and rapidly induced during hypoxic exposure, but disappeared within minutes after reoxygenation. In contrast, mutant worms with a defective pVHL homolog showed no oxygen-dependent regulation of HIF-1α. In analogy to mammalian cells, HIF-1α-pVHL binding is dependent on specific hydroxylation of HIF-1α proline 621 within the conserved LXXLAP motif. Looking for putative 2-oxoglutarate-dependent dioxygenases, database searches led to the identification of the *C. elegans egl-9* gene. Different mutant worms containing defective *egl-9* alleles showed hypoxia-independent upregulation of HIF-1α and its target genes. The ability of EGL-9 to hydroxylate HIF-1α was shown by HPLC analysis, demonstrating the critical function of EGL-9 as a prolyl hydroxylase that targets HIF-1α to pVHL in *C. elegans*. Sequence and structural analysis predicted three *egl-9*-related genes in human and rodent genomes. The human protein products were termed “prolyl hydroxylase domain-containing” (PHD) 1, 2, and 3, respectively. Interestingly, PHDs were differentially efficient in promoting HIF-1α-pVHL interaction via the C-terminal (P564) compared to the N-terminal (P402) prolyl hydroxylation site. Whereas the pVHL interaction through P564 was promoted by all enzymes, it was less efficient when only P402 was present, and was only promoted by PHD1 and PHD2 [8].

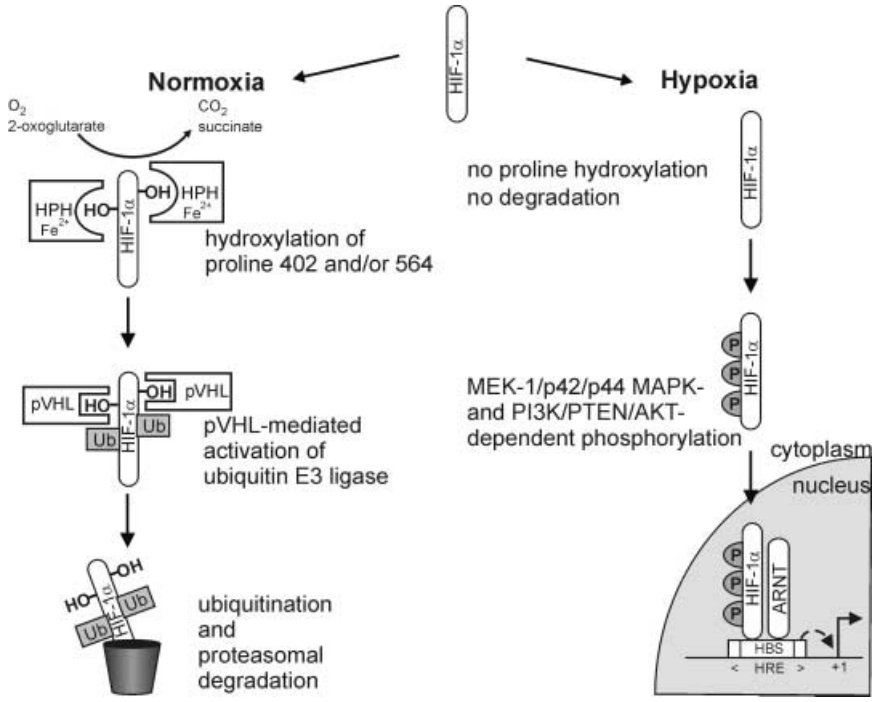

Fig. 1 The oxygen-sensing mechanism involves hypoxic stabilization of transcription factor hypoxia-inducible factor-1α (*HIF-1α*). Simplified schematic illustration of the HIF-1α activation and degradation pathway, respectively. Under normoxic conditions, HIF-1α is hydroxylated at proline 402 and/or 564, both representing highly conserved amino acids. Note that both proline residues are located within the oxygen-dependent degradation (*ODD*) domain (not drawn). Hydroxylated proline acts as a recognition site for pVHL-mediated ubiquitination that results in proteasomal degradation. Under hypoxic conditions, suppression of hydroxylation leads to stabilization and subsequent nuclear translocation of HIF-1α. Abbreviations: MAPK: mitogen-activated protein kinases; MEK-1: MAPK kinase-1; pVHL: von Hippel-Lindau tumor suppressor protein; HPH: HIFα prolyl hydroxylase; PI3K: phosphatidylinositol 3'-kinase

In accordance with the results described above, Bruick and McKnight [2] postulated the existence of three paralogous human HIF proline hydroxylases (HPH-1, 2, and 3, respectively) that are able to hydroxylate proline 564. Sequence analysis revealed that PHD1 corresponds to HPH-3, PHD2 to HPH-2, and PHD3 to HPH-1. In addition, they identified a single putative HIF proline hydroxylase gene (*dmHPH*) in *Drosophila melanogaster*. Suppression of dmHPH by RNA interference resulted in elevated expression of lactate dehydrogenase, a HIF target gene. These results indicate that HPHs are an essential component of the oxygen-sensing pathway [2].

Evaluation of HIF-1α as a target for gene therapy

Several reports have been published recently dealing with gene therapeutic approaches based on naked DNA or adenoviral gene transfer of HIF-1 target genes such as vascular endothelial growth factor (VEGF) or erythropoietin [22, 24, 31]. Transfer of VEGF, the most potent inducer of angiogenesis, has been successfully applied to treat ischemic diseases such as ischemic peripheral and diabetic neuropathy and coronary artery disease [22, 24]. Because HIF-1 mediates the transcriptional induction of VEGF and other angiogenic growth factors under hypoxic conditions [25, 33], HIF-1 itself is a target for gene therapy. Most likely, gene transfer of HIF-1α potentially would elicit a more complex and physiological induction of local angiogenesis, which might increase the proportion of successful therapeutic angiogenesis applications. To demonstrate the feasibility of HIF-1α gene transfer under normoxic conditions in vivo, we transplanted HeLa cells that inducibly overexpress HIF-1α under normoxic conditions into nude mice. Four weeks after injection, high levels of overexpressed exogenous HIF-1α were detected in mice kept under normoxic conditions [9]. Our results demonstrate the feasibility of HIF-1α gene transfer, though endogenous HIF-1α is unstable under normoxic conditions. The availability of cell lines stably overexpressing HIF-1α will aid the identification of critical co-factors required for full HIF-1 activation. The combination of these factors with HIF-1α might allow for the precise control of the extent of HIF-1-mediated therapeutic angiogenesis. These data are in accordance with a recently published report, in which transgenic mice overexpressed the constitutively active mutant HIF-1αΔODD [10], a HIF-1α polypeptide lacking the ODD domain, in basal keratinocytes and squamous epithelium. These mice developed dermal hypervascularity and showed an upregulation of VEGF and other hypoxia-dependent genes including glucose transporter-1 [7]. But unlike the microvasculature and increased vessel permeability found in transgenic mice overexpressing VEGF, no increase in vessel leakage was observed in HIF-1α transgenic mice. The fact that these vessels were fully

functional confirmed the potential benefit of HIF-1α compared to VEGF gene therapy.

In contrast to the potentially protective effects of HIF-1 for the treatment of ischemic diseases, HIF-1 plays a pathological role in tumor progression. Inactivation of tumor suppressor genes or activation of proto-oncogenes leads to an upregulation of HIF-1α protein expression and/or HIF-1 *trans*-activation activity [1]. As a consequence of these events, target genes with important roles in tumor progression (e.g., VEGF, glycolytic enzymes) are induced. In the last few years, several approaches have been exploited to treat hypoxic tumors, including the intratumoral gene transfer of an antisense HIF-1α plasmid [29]. Expression of HIF-1α antisense RNA within the tumor downregulated VEGF levels and tumor microvessel density, leading to a complete and permanent rejection of small tumors. Another strategy to exploit hypoxia in solid tumors was the development of an expression vector highly responsive to HIF-1 [26], which can be used to express therapeutic genes including enzymes that can metabolize nontoxic prodrugs into toxic drugs. The selective expression of these enzymes in the tumors will allow tumor-specific activation of these toxic drugs by poorly oxygenated regions of solid tumors.

Taken together, we expect that understanding the oxygen-sensing mechanism that involves HIF-1α modulation will open new doors to develop therapeutic strategies against a variety of diseases including tumor growth, ischemia/reperfusion injuries, and wound healing.

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