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Review

HIF-1: the knowns and unknowns of hypoxia sensing✪

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Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that functions as a master regulator of cellular and systemic oxygen homeostasis. It consists of two constitutively produced subunits: HIF-1α and HIF-1β. Under normoxic conditions HIF-1α undergoes hydroxylation at specific prolyl residues which leads to an immediate ubiquitination and subsequent proteasomal degradation of the α subunit. Addi-

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Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix domain; BMAL, brain muscle ARNT-like protein; CBP, cAMP-responsive element-binding protein; DMOG, *N*-dimethyl-oxalylglycine; EPO, erythropoietin; FIH-1, factor inhibiting HIF-1; Flt-1, *fms*-like tyrosine kinase-1; FRAP, FKBP-rapamycin associated protein; HAS, HIF-1 ancillary sequence; HBS, HIF-1 binding site; HIF-1, hypoxia-inducible factor 1; HO-1, heme oxygenase-1; HRE, hypoxia response element; HUVEC, human umbilical vein endothelial cells; IPAS, inhibitory PAS protein; MAPK, mitogen-activated protein kinase; MDM2, murine double minute 2; NLS, nuclear localization signal; NOS2, inducible nitric oxide synthase; NPAS2, neuronal PAS domain protein 2; ODD, oxygen-dependent degradation domain; PAS, Per-AHR-ARNT-Sim homology domain; PHD, PH domain-containing protein; PI3K, posphatidylinositol-3 kinase; PKC, protein kinase C; pVHL, von Hippel-Lindau tumor suppressor protein; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TAD, transactivation domain; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

tionally, hydroxylation of an asparaginyl residue blocks the transcriptional activity of HIF-1 due to inhibition of its interaction with co-activators. In contrast, under hypoxic conditions, abolition of prolyl hydroxylation results in HIF-1α stabilization, whereas the lack of asparaginyl hydroxylation allows the transcriptional activity. Additionally, the transcriptional activity may be modulated by phosphorylation or redox modification of HIF-1. Despite its name, HIF-1 is induced not only in response to reduced oxygen availability but also by other stimulants, such as nitric oxide, various growth factors, or direct inhibitors of prolyl and asparaginyl hydroxylases. Therefore, it seems to be a crucial transcription factor elicited by a wide range of stresses such as impaired oxygenation, inflammation, energy deprivation, or intensive proliferation. However, the mechanisms of normoxic activation, as well as of oxygen sensing, are not yet fully known. Further understanding of the processes that control HIF-1 activity will be crucial for the development of new diagnostic and therapeutic strategies.

Oxygen homeostasis is strictly controlled in order to maintain intracellular levels of oxygen within tight limits dictated by the requirement for O_2 in many metabolic processes on the one hand, and its high toxicity on the other. The control occurs at the level of an entire organism as well as at the level of a single cell. It involves numerous mechanisms of regulation and adaptation to changes in O_2 tension. At the cellular level, decreased O_2 tension (hypoxia) leads to the activation of alternative metabolic pathways that do not require molecular oxygen. The switch from aerobic metabolism to anaerobic glycolysis is mediated by the induction of glycolytic enzymes and expression of glucose transporters. Additionally, the expression of various stress proteins responsible for cell death or survival is upregulated. Further adaptations which occur at the tissue and systemic levels lead to the increase in O_2 delivery. They include induction of erythropoiesis (red blood cell production), angiogenesis (new vessel formation), and hyperventilation. Among the various upregulated proteins there is erythropoietin (EPO), the main growth factor inducing maturation of erythrocytes, and vascular endothelial growth factor (VEGF), the major mediator of angiogenesis and vascular permeability. The hypoxia-dependent regulation of these and other proteins (Table 1) occurs at the transcriptional level and is mediated by hypoxia-inducible transcription factor (HIF-1).

Although the importance of HIF-1 in the induction of the complex response to hypoxia is widely appreciated, the molecular mechanisms of HIF-1 activation remain unclear. This review aims to summarize current knowledge on HIF-1 regulation under hypoxic and normoxic conditions.

HIF-1 TARGET GENES

The consensus DNA sequence for HIF-1 binding, 5′-(A/G)CGTG-3′, is common for many genes upregulated by low oxygen tension (Semenza *et al.,* 1996). The list of known genes activated by HIF-1 grows continuously. It includes genes whose protein products act both to maintain O_2 homeostasis and to adapt to changes in the oxygen concentration (Table 1). The first group comprises of genes involved in the development and functioning of the vascular system. They either promote angiogenesis (such as VEGF and its receptor Flt-1) or modulate vascular tone (such as inducible nitric oxide synthase, heme oxygenase-1, endothelin 1, adrenomedullin, and α_{1B}-adrenergic receptor). The second group is represented by genes whose protein products induce erythropoiesis. Besides erythropoietin, HIF-1 upregulates ceruloplasmin, transferrin, and transferrin receptor which facilitate the supply of iron to the erythroid tissues. The third group consists of genes whose products are involved in energy metabolism.

Their cooperation leads to increased glucose uptake and a switch to glycolysis as the main source of energy. The fourth group comprises genes whose products are responsible for cell proliferation and viability. Besides these four main sets of HIF-1-induced genes, there are others also important in response to various stresses (all references in Table 1).

HIF-1 STRUCTURE

HIF-1 is a heterodimer of two bHLH-PAS proteins

Hypoxia-inducible factor-1 is a heterodimer composed of the α and β subunits. Both of them contain two characteristic domains: the

Table 1. Genes upregulated by HIF-1.

Process		Gene product	References
Control of vascular system	Angiogenesis	Vascular endothelial growth factor	Forsythe *et al.*, 1996
		VEGF receptor 1	Gerber *et al.*, 1997
		Plasminogen activator inhibitor 1	Kietzmann *et al.*, 1999
		Transforming growth factor β3	Caniggia *et al.*, 2000
	Vasomotor control	Nitric oxide synthase 2 (inducible nitric oxide synthase)	Melillo *et al.*, 1995 Palmer *et al.*, 1998
		Endothelin-1	Hu *et al.*, 1998
		α_{1B}-adrenergic receptor	Eckhart *et al.*, 1997
		Adrenomedullin	Cormier-Regard *et al.*, 1998
		Heme oxygenase 1	Lee *et al.*, 1997
Maturation of red blood cells	Erythropoiesis	Erythropoietin	Jiang *et al.*, 1996
	Iron transport	Transferrin	Rolfs *et al.*, 1997
		Transferrin receptor	Lok & Ponka, 1999 Tacchini *et al.*, 1999
		Ceruloplasmin	Mukhopadhyay *et al.*, 2000
Energy metabolism	Glycolysis	Lactate dehydrogenase A	Firth *et al.*, 1994
		Phosphoglycerate kinase 1	Semenza *et al.*, 1994, 1996
		Aldolase A and C	Iyer *et al.*, 1998
		Phosphofructokinase L	
		Pyruvate kinase M	
		Enolase 1	
		Hexokinase 1 and 2	
	Glucose transport	Glucose transporter 1	Gleadle & Ratcliffe, 1997
		Glucose transporter 3	O'Rourke *et al.*, 1996 Iyer *et al.*, 1998
	Multifunctional enzyme	Glyceraldehyde-3-phosphate dehydrogenase	Lu *et al.*, 2002 Iyer *et al.*, 1998
Cell proliferation and viability	Arrest of cell cycle	p21	Carmeliet *et al.*, 1998
	Apoptosis	Bcl2/EIB 19kDa-interacting protein 3 (BNIP3)	Bruick, 2000
		Nip3-like protein X	Sowter *et al.*, 2001
	Growth factors	Insulin-like growth factor 2	Feldser *et al.*, 1999
		Insulin-like growth factor binding protein 1, 2 and 3	
Other	pH regulation	Carbonic anhydrase 9	Wykoff *et al.*, 2000
	Nucleotide metabolism	Adenylate kinase 3	O'Rourke *et al.*, 1996
	Matrix metabolism	Collagen prolyl-4-hydroxylase α1	Takahashi *et al.*, 2000
	Catecholamine synthesis	Tyrosine hydroxylase	Norris & Millhorn, 1995
	Feedback regulation	p35srj	Bhattacharya *et al.*, 1999

basic helix-loop-helix (bHLH) domain and the PAS (Per-AHR-ARNT-Sim) domain. The PAS domain was termed as an acronym for the first known members of the family: *Drosophila* period (Per) and single-minded (Sim) proteins and mammalian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins (Wang *et al.,* 1995a). It contains two internal homology units, the A and B repeats, and is involved in interaction between proteins. PAS-proteins have been found in many species, including prokaryotes, which indicates their high evolutionary stability (Wang *et al.,* 1995a).

The bHLH domain, which is common for a large number of transcription factors, is required for both protein dimerization and DNA binding. In the case of HIF-1, the highest efficiency of heterodimerization is obtained only if the bHLH and PAS domains are intact (Jiang *et al.,* 1996a). Moreover, the efficiency of dimerization and DNA binding of *in vitro*-translated HIF-1α and HIF-1β is weaker than those isolated from a cell, suggesting that one or both subunits may undergo posttranslational modifications (Jiang *et al.,* 1996a).

HIF-1α

HIF-1α is an 826-amino acid (120-kDa) protein (Fig. 1). In the N-terminal part it contains the basic domain (aa 17–30), the helix-loop-helix domain (aa 31–71), and the PAS domain (aa 85–298) with PAS-A (aa 85–158) and PAS-B (aa 228–298) repeats (Wang *et al.,* 1995a). Two transactivation domains, N-terminal and C-terminal TADs (also termed NAD and CAD), are localized in the C-terminal half of HIF-1α (aa 531–575 and 786–826, respectively; Pugh *et al.,* 1997). Moreover, the C-terminal part contains a domain responsible for degradation of HIF-1α under normoxic conditions (Huang *et al.,* 1998). This oxygen-dependent degradation domain (ODD at aa 401–603) contains two PEST-like motifs: sequences rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (aa 499–518 and 581–600) common for many proteins with a short half-life (less than 2 h) (Rechsteiner & Rogers, 1996). The HIF-1α half-life under normoxic conditions is less than 10 min and the protein is hardly detectable (Chun *et al.,* 2002). Additionally, HIF-1α contains two nuclear localization signals: N-NLS (aa 17–74) and C-NLS (aa 718–721). The C-terminal NLS is crucial in the nuclear import of HIF-1α, whereas the N-terminal one seems to be less important (Kallio *et al.,* 1998). Furthermore, alternative splice variants of HIF-1α and β have been observed (Wang *et al.,* 1995a; Gothie *et al.,* 2000) and both subunits contain multiple consensus sites for protein phosphorylation (Wang *et al.,* 1995b).

HIF-1β

HIF-1β was previously identified as aryl hydrocarbon nuclear receptor translocator (ARNT), which heterodimerizes with aryl hy-

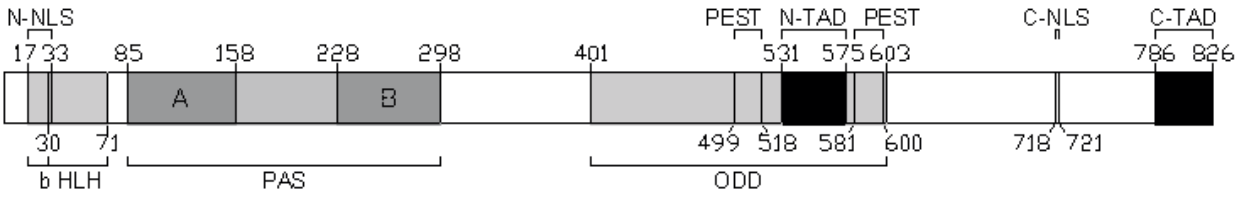

Figure 1. Structure of HIF-1α.

Numbers indicate the first and last amino-acid residues of each domain. The main domains are: basic helix-loop-helix domain (bHLH), Per-AHR-ARNT-Sim homology domains (PAS-A and PAS-B), oxygen-dependent degradation domain (ODD), and transactivation domains (N-TAD and C-TAD). Also position of PEST-like motifs and nuclear localization signals (NLS) is shown. Further description in the text.

drocarbon receptor (AHR) to form the functional dioxin receptor. It has two isoforms (774- and 789-aa that constitutes 92 or 94 kDa, respectively) that differ by the presence of the sequence encoded by a 45-bp alternative exon (Wang *et al.,* 1995a).

Additional HIFs

Two other members of the HIF-1α family have been identified: HIF-2α, also known as endothelial PAS protein (EPAS1), HIF-like factor (HLF), HIF-related factor (HRF), or a member of PAS domain family 2 (MOP2); and HIF-3α. Both of them heterodimerize with one of the members of the ARNT family: ARNT (HIF-1β), ARNT2, or ARNT3 (BMAL/MOP3) (all references in Semenza, 2000b; Wenger, 2002). The structure, regulation and function of all HIFs seem to be similar. However, the expression of HIF-2α, HIF-3α, ARNT2, and ARNT3 is tissue specific, which suggests that they may play more specialized roles (Semenza, 2000b).

OXYGEN-DEPENDENT HIF-1 REGULATION

Different levels of HIF-1 regulation

The regulation of HIF-1 activity concerns mostly the α subunit and occurs at multiple levels such as protein stabilization, post-translational modifications, nuclear translocation, dimerization, transcriptional activation, and interaction with other proteins. Moreover, changes in mRNA expression (Wang *et al.,* 1995a; Wiener *et al.,* 1996; Yu *et al.,* 1998) and alternative splicing (Gothie *et al.,* 2000) of both subunits have been observed. Under normoxic conditions, however, when HIF-1α and HIF-1β are constitutively transcribed and translated, the abrogation of HIF-1 activity results mainly from constitutive HIF-1α degradation (Fig. 2).

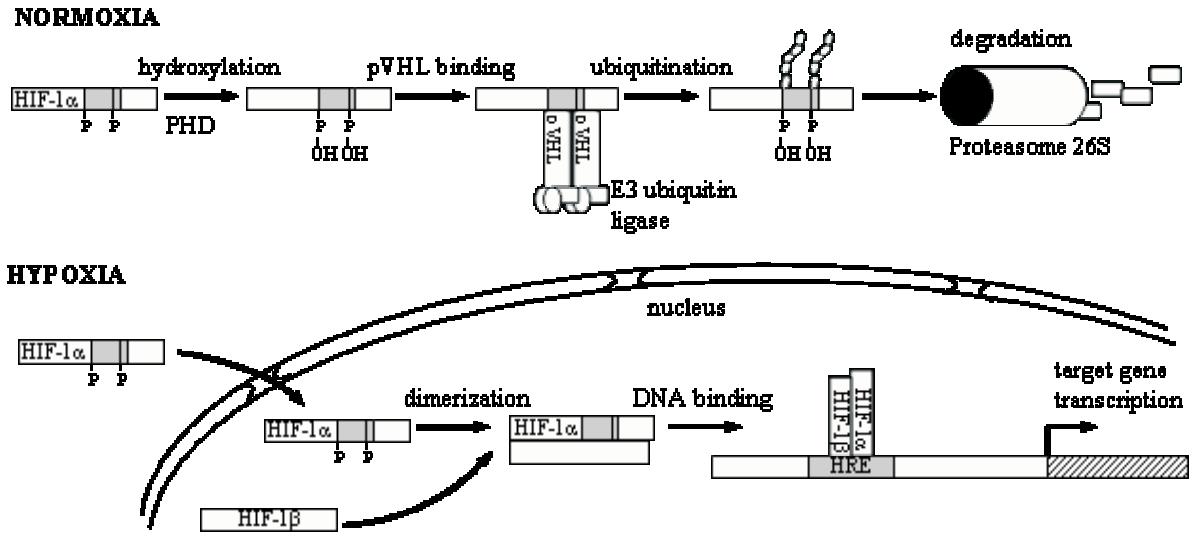

Figure 2. Regulation of HIF-1α protein stability.

Under normoxic conditions HIF-1-prolyl hydroxylases (PHDs) hydroxylate Pro 402 and 564 (P) within the ODD domain. After prolyl hydroxylation von Hippel-Lindau tumor suppressor proteins (pVHLs) bind to the ODD domain and recruit other proteins of E3 ubiquitin ligase complex. HIF-1α is subsequently ubiquitinated and degraded by the 26S proteasome. Under hypoxic conditions HIF-1-prolyl hydroxylases are inactive which prevents binding of pVHL. Therefore, HIF-1α escapes ubiquitination and proteasomal degradation, and can be transported to the nucleus where, after dimerization with HIF-1β, it stimulates target genes transcription.

HIF-1α degradation

Ubiquitination

Under normoxic conditions HIF-1α is subjected to ubiquitination and degradation by the 26S proteasome proteolysis (Kallio *et al.,* 1999). In HIF-1α ubiquitination the major role is played by the von Hippel-Lindau tumor suppressor protein (pVHL), whose β-domain interacts directly with the ODD domain of HIF-1α (Bonicalzi *et al.,* 2001). The inactivation of pVHL is associated with the von Hippel-Lindau disease, which is a hereditary cancer syndrome characterized by the development of highly vascularized tumors with constitutive HIF-1 expression (Ivan *et al.,* 2001). pVHL acts as the substrate recognition component of the E3 ubiquitin ligase protein complex and after binding to HIF-1α it recruits elongins B and C, cullin 2, and Rbx1 (reviewed in Semenza, 2001).

The interaction between pVHL and HIF-1α is oxygen-dependent: pVHL associates with ODD under normoxic but not under hypoxic conditions, thus the degradation does not occur during hypoxia (Maxwell *et al.*, 1999). The interaction of pVHL with HIF-1 depends on a posttranslational modification of HIF-1α, which was identified as an oxygen- and iron-dependent prolyl hydroxylation (Ivan *et al.,* 2001). The binding of pVHL to the ODD domain, and therefore the ubiquitination of its N- and C-terminal part (aa 390–417 and 549–582, respectively), must be preceded by the hydroxylation of proline residues (Pro 402 in the N-terminal part and Pro 564 in the C-terminal part of ODD, Fig. 2). These residues are embedded in the conserved amino acid motif, LXXLAP, in which Leu 562 strongly facilitates the hydroxylation of Pro 564 (Ivan *et al.,* 2001).

HIF-1 prolyl hydroxylases

The hydroxylation of the proline residues in the ODD domain of HIF-1α is catalyzed by HIF-1 prolyl 4-hydroxylases, members of the Fe(II)- and 2-oxoglutarate-dependent dioxygenase superfamily. Their activity, however, is distinct from the activity of procollagen prolyl hydroxylases, which belong to the same superfamily (Bruick & McKnight, 2001; Jaakkola *et al.,* 2001). The prolyl hydroxylase responsible for HIF-1α hydroxylation was first identified as a product of *egl-9* gene after searching *Caenorhabditis elegans* genome database (Epstein *et al.,* 2001). Egl-9 mutant worms, similarly to *vhl-1* mutants, constitutively expressed HIF-1α. Later three EGL-9 homologues in mammals were identified and designated PHD (PH domain containing protein) 1, 2, and 3. They are also termed HPHs (HIF-1 prolyl hydroxylases) 3, 2, and 1, respectively. They differ in intracellular localization: PHD1 was detected exclusively in the nucleus, PHD2 – in the cytoplasm, whereas PHD3 in both nucleus and cytoplasm (Metzen *et al.,* 2003a). Additionally, the existence of a fourth PHD has been described (Oehme *et al.,* 2002). Each of them hydroxylates Pro-564 in HIF-1α, whereas only PHD1 and PHD2 hydroxylate Pro-402. Molecular oxygen is a substrate of these enzymes, while carbon dioxide and succinate are by-products. During hypoxia, or in case of a lack of Fe(II) or 2-oxoglutarate, prolyl hydroxylases cease to function, thus HIF-1α does not undergo degradation (reviewed in Semenza, 2001).

HIF-1 activation

To obtain full transcriptional activity HIF-1 must bind to the DNA target sequence and recruit transcriptional cofactors. This phase also undergoes hypoxia-dependent regulation. The role of HIF-1α transactivation domains (TADs) is to recruit the transcriptional coactivator complexes to the promoters of HIF-1 target genes. The central integrating coactivator p300/CBP interacts through its CH1 (cysteine-histidine-rich) domain with HIF-1α TADs (Gu *et al.,* 2001) and recruits the accessory coactivators like histone

acetylotransferases SRC-1, TIF-2, and redox factor Ref-1 (Ema *et al.,* 1999).

The molecular mechanism of activation of the TAD domains was recently discovered. Under normoxic conditions the highly conserved asparagine residue within the C-TAD domain (Asn 803) is hydroxylated (Lando *et al.,* 2002a) which results in the silencing of transactivation domains (Fig. 3). The enzyme that hydroxylates the Asn residues is also a member of the Fe(II)- and 2-oxoglutarate-dependant superfamily of dioxygenases, thus it is blocked by the inhibitors of 2-oxoglutarate-dependent dioxygenases such as dimethyloxalylglycine (DMOG) and Fe(II) chelators. Treatment of cells with DMOG or iron chelators as well as replacing the critical Asn residue with Ala result in activation of C-TAD even at normoxia (Lando *et al.,* 2002a). Therefore, p300/CBP interacts with C-TAD only when the Asn is nonhydroxylated which enables the assembly of transcriptional coactivator complex, whereas the hydroxylation of Asn during normoxia silences C-TAD domain by preventing its interaction with p300/CBP. The asparaginyl hydroxylase that modifies HIF-1α was recently identified as the previously known factor inhibiting HIF-1 (FIH-1) (Lando *et al.,* 2002b). The final evidence that both residues, Pro within the ODD and Asn within the C-TAD, are necessary for the full activation of HIF-1 was provided by an experiment in which either critical Pro, or Asn, or both were replaced by Ala (Lando *et al.,* 2002a). Replacement of Pro within the ODD domain resulted in a stable protein even under normoxia which, however, exhibited low transcriptional activity at normoxia and high transcriptional activity at hypoxia. Replacement of the critical Asn within the C-TAD domain does not influence protein activity in comparison to wild-types proteins, because, despite the active C-TAD domain, the protein is unstable at normoxia. Finally, the double amino acid substitution resulted in nearly full activity of the protein at normoxia (Lando *et al.,* 2002a).

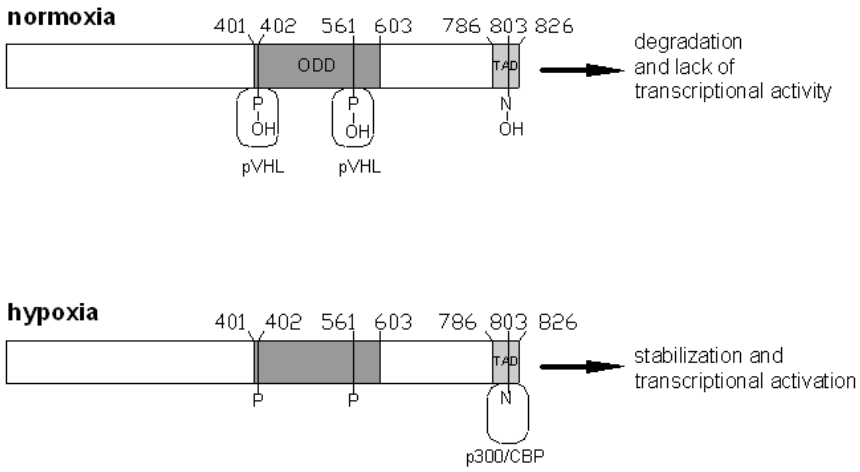

Figure 3. The role of prolyl and asparaginyl hydroxylation in the stabilization and activation of HIF-1α.

Under normoxic conditions specific prolyl (P) residues within the oxygen-dependent degradation domain (ODD) and asparaginyl (N) residues within the COOH-terminal transactivation domain (C-TAD) are hydroxylated by respective hydroxylases. The hydroxylation of prolines enables binding of pVHLs and thus constitutes a signal for proteasomal degradation. The hydroxylation of asparaginyl (N) residues blocks binding of transcriptional coactivator (p300/CBP) and subsequently inhibits transcriptional activity of HIF-1. In contrast, under hypoxic conditions HIF-1α escapes proteasomal degradation because of abolition of prolyl hydroxylation and may interact with p300/CBP due to inhibition of asparaginyl hydroxylation.

Other levels of HIF-1 regulation

Nuclear localization of HIF-1α

The dimerization of the HIF-1 α and β subunits occurs in the nucleus and is necessary for the DNA binding and subsequent activation of transcription (Kallio *et al.,* 1997). In contrast to HIF-1β which is present in the nucleus regardless of oxygen levels, nuclear translocation of HIF-1α is correlated with HIF-1 activity. Consequently, it was suggested that this translocation could be upregulated by hypoxia (Kallio *et al.,* 1998). However, when HIF-1α is overexpressed the translocation occurs even under normoxic conditions. Therefore, this process seems to be hypoxia-independent and the nuclear fraction of HIF-1α may simply reflect the overall level of this protein in the cell (Hofer *et al.,* 2001).

HIF-1 DNA binding

After the stabilization of the α subunit, nuclear translocation, and dimerization, HIF-1 binds to its consensus binding site (HBS, HIF-1 binding site) within the hypoxia response element (HRE) (Semenza *et al.,* 1996). The core sequence of HBS is (A/G)CGTG. HRE may be located within either promoter or enhancer regions (5′-flanking, 3′-flanking, or intervening) of target genes (Fig. 4, references in Table 1). Generally, HBS is the minimal sequence necessary for HIF-1 binding. However, the structure of HRE, methylation of the cytosine residue within HBS, or presence of additional transcription factors may influence HIF-1-induced response. Additionally, in the majority of hypoxia-induced genes HRE contains HIF-1 ancillary sequence (HAS), which is located 8–9 nt down- or upstream of HBS and is necessary for HIF-1-mediated transcription activation (Kimura *et al.,* 2001). HAS is an imperfect inverted repeat of HBS, thus the secondary structure of HRE seems be crucial for its activatory function (Fig. 5). It was also shown that HAS recruits protein complexes distinct from HIF-1 (Kimura *et al.,* 2001).

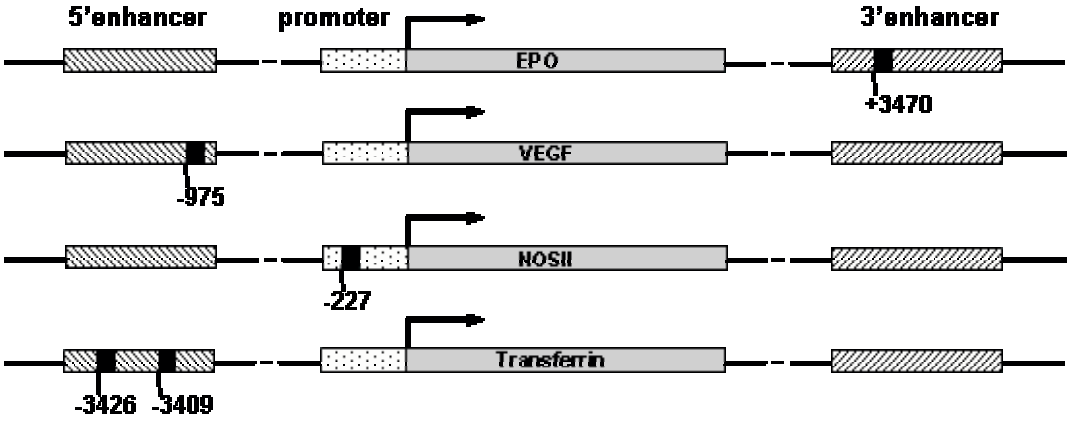

Figure 4. The localization of hypoxia response element (HRE) in various hypoxia-induced genes.

HRE (black rectangle) may be located within either 5′-flanking (VEGF and transferrin) or 3′-flanking (EPO) enhancer regions, or within the promoter (NOSII) of target genes.

Furthermore, efficient gene activation frequently requires recruiting of more than one HIF-1 or binding of additional transcriptional factors, which are not hypoxia-dependent. Two or three adjacent HBSs were found in some genes encoding glycolytic enzymes, glucose transporter 1, and transferrin. There is also a binding site for the ATF-1/CREB-1 factor (activating transcription factor-1/cAMP-response element-binding protein-1) in the

HRE of lactate dehydrogenase A gene, for AP-1 (activator protein-1) binding factor in *VEGF* gene, and for the HNF-4 (orphan receptor hepatic nuclear factor-4) in the erythropoietin gene (references in Wenger, 2002). All conditions (Frick *et al.,* 2003) or in response to hypoxia (unpublished data). In contrast, in human microvascular endothelial cells (Józkowicz *et al.*, 2004) and smooth muscle cells (Dulak *et al.*, 2002) hypoxia potently

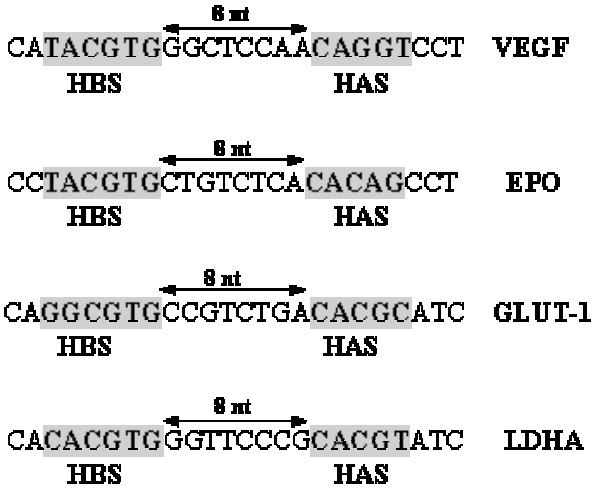

Figure 5. Human hypoxia response element (HRE) in VEGF, EPO, glucose transporter-1 (GLUT-1), and lactate dehydrogenase A (LDHA) genes (Kimura *et al.*, 2001).

HRE, besides the HIF-1 binding site (HBS), contains the HIF-1 ancillary sequence (HAS), which is located 8–9 nt down- or upstream of HBS and constitutes an imperfect inverted repeat of HBS.

these transcription factors modulate HIF-1 response. Such a requirement for additional transcription factors may (i) amplify hypoxic response in particular conditions, (ii) vary responses of distinct tissues to the hypoxia, and (iii) enable diverse induction of distinct target genes.

Another possible level of regulation may be the CpG methylation of the cytosine residue within HBS. Although, in the majority of HIF-1 target genes it remains unmethylated as it is located in the methylation-free CpG islands, the HBS of the erythropoietin gene might undergo methylation, which inhibits HIF-1 binding (Wenger *et al.,* 1998). To prevent methylation, HBS is at normoxia occupied by other DNA binding factors (reviewed in Wenger, 2002). It might also be hypothesized that methylation of HBS may constitute a mechanism of cell-specific regulation of hypoxia-induced gene expression. Interestingly, human macrovascular endothelial cells do not release VEGF either under normoxic modulates VEGF production. These data suggest that the response to hypoxia is cell type specific.

Molecular mechanism of oxygen sensing

Although the mechanisms of HIF-1α stabilization and activation are already known, the signaling pathways that lead to the inhibition of prolyl and asparaginyl hydroxylases remain unclear. The requirement of molecular oxygen as a substrate for these enzymes could explain the loss of HIF-1 degradation under hypoxic conditions. This assumption, however, appears to be oversimplified. It has been shown that collagen prolyl hydroxylases are active in hypoxic cells and that maximal HIF-1α stabilization occurs under 0.5% O_2 concentration rather than under anoxia, which would not be possible if prolyl hydroxylases were direct oxygen sensors (Jiang *et al.,* 1996b). Moreover, earlier studies provided evidence that other signaling

pathways, such as ROS-, protein phosphorylation-, and nitrosylation-dependent pathways are involved in oxygen sensing. Therefore, many models of intracellular oxygen sensing have been considered.

A hemoprotein as an oxygen sensor

The initial hypothesis suggested that the role of oxygen sensor is mediated by an unknown protein containing heme as a prosthetic group. The putative role of heme, bound either to HIF-1 PAS domains or to a distinct sensor hemoprotein, was suggested because of the fact that HIF-1 activation is induced not only by the lack of molecular oxygen but also by Fe(II) chelators, such as desferrioxamine (Wang *et al.,* 1993), and also by some transient metals that could replace Fe(II) in heme (Huang *et al.,* 1997). However, currently these observations seem to confirm the iron-dependence of prolyl and asparaginyl hydroxylases rather than hemoproteins acting as the oxygen sensor.

ROS-dependent signaling pathways

Two opposing models postulate signalization by changes in the cellular levels of reactive oxygen species (ROS) (reviewed in Semenza, 2000c; Michiels *et al.,* 2002). The first of them assumes that ROS are continuously produced by an unknown NADPH oxidase that reduces O_2 to superoxide anion ($O_2^{\bullet -}$) which is subsequently converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). According to this model the reduction of molecular oxygen concentration would be followed by the reduction of ROS levels, and therefore a decrease in ROS generation would be a direct or indirect signal for HIF-1 activation (Fig. 6). The experiments confirming this hypothesis showed: (i) decreased ROS production under hypoxic conditions, (ii) suppression of HIF-1 target genes by exogenous H_2O_2 in hypoxia, (iii) the presence of a non-mitochondrial cytochrome *b*-containing NADPH oxidase that could respond to oxygen levels (references in Michiels *et al.,* 2002), and (iv) induction of HIF-1 target genes in the presence of exogenous catalase or antioxidants (Salceda & Caro, 1997). However, some observations were inconsistent with this hypothesis. A nonspecific inhibitor of NADPH oxidases (diphenylene iodonium, DPI) blocked HIF-1 activation in response to hypoxia (Gleadle *et al.,* 1995) and HIF-1 activity was sustained in cells deficient in a subunit of NADPH oxidase (Archer *et al.,* 1999). Moreover, some experiments indicated that the production of ROS was increased rather than decreased during hypoxia (Chandel *et al.,* 1998).

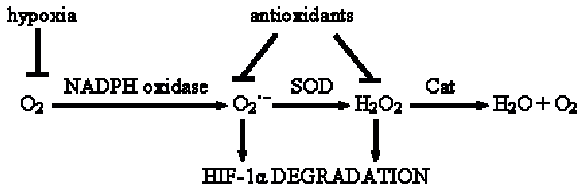

Figure 6. Model I of ROS-dependent hypoxia signal transduction.

According to this model hypoxia results in decreased reactive oxygen species (ROS) generation, which leads to HIF-1α stabilization. The same effect is observed in the presence of antioxidants and catalase (Cat).

The second model concerning ROS-dependent pathway is diametrically opposed to the previous one as it assumes that hypoxia results in an increased generation of ROS by mitochondria, which constitutes the signal to HIF-1α stabilization (Fig. 7). According to this model under hypoxic conditions the consumption of oxygen at cytochrome *c* oxidase (mitochondrial complex IV) is lower and electrons accumulate at preceding complexes. Such an accumulation leads to increased generation of ROS at complex III. Compatibly with this theory inhibitors of complexes I and III blocked the induction of HIF-1 activity at hypoxia (due to inhibition of ROS produc-

tion), whereas inhibitors of complex IV were able to induce HIF-1-dependent transcription at normoxia (due to induction of ROS generation) (Chandel *et al.,* 1998). Another evidence that mitochondrial complex III acts as an oxygen sensor was shown in ρ^0 Hep3B cells, lacking the functional respiratory chain, in which the induction of HIF-1 did not occur under hypoxic conditions (Chandel *et al.,* 1998). Moreover, the substrate of complex II (succinate) restored hypoxic response in cells chloride or desferrioxamine instead of hypoxia (Chandel *et al.,* 1998). The explanation of the contradictory results of Chandel *et al.* (1998), and Vaux *et al.* (2001) may lie in different mechanisms of oxygen sensing in the case of anoxia and hypoxia. Prolyl and asparaginyl hydroxylases, which use molecular oxygen as a substrate, act as direct oxygen sensors in the total absence of oxygen (Schroedl *et al.,* 2002). Similarly, desferrioxamine and cobalt chloride are direct in-

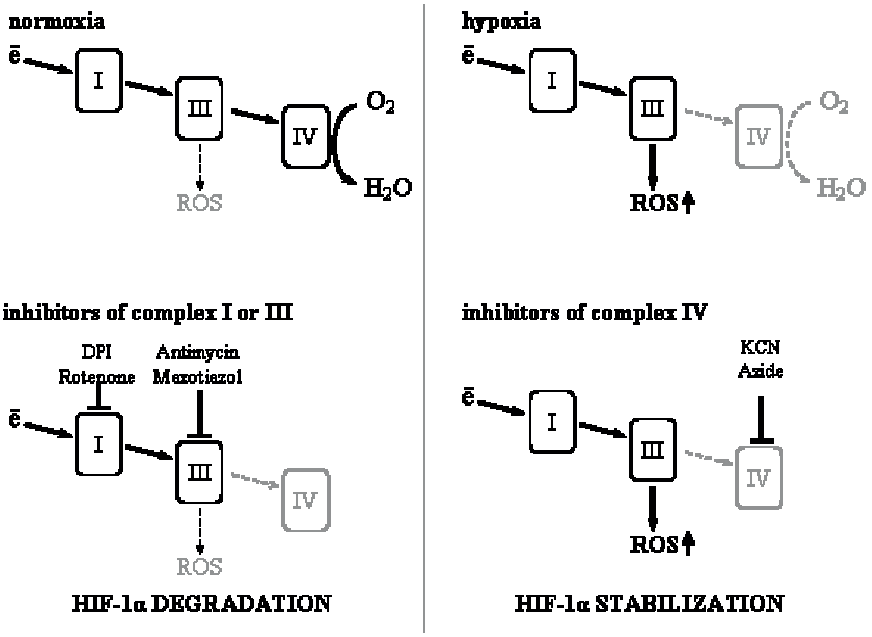

Figure 7. Model II of ROS-dependent hypoxia signal transduction.

According to this model hypoxia results in an increased generation of reactive oxygen species (ROS) due to attenuation of cytochrom *c* oxidase (mitochondrial complex IV) activity. The generation of ROS is mediated by complex III at which, in the absence of functional cytochrome *c* oxidase (due to hypoxia or in the presence of complex IV inhibitors), electrons are accumulated. High levels of ROS induce HIF-1α stabilization, whereas low levels lead to HIF-1α degradation. Low levels of ROS are maintained either when the electron transport chain is fully functional (normoxia) or when electrons do not reach complex III (in the presence of inhibitors of complex I and II, or III).

with a defect in complex I (Agani *et al.,* 2000). Consistent with this theory are also our results showing that overexpression of SOD, leading to increased production of H_2O_2, induces HIF-1-dependent VEGF expression (Grzenkowicz-Wydra *et al.*, 2004).

However, these findings were not confirmed in other experiments on ρ^0 cell lines cultured under severe hypoxic conditions (0.1% O_2) (Vaux *et al.,* 2001) or after exposure to cobalt hibitors of the hydroxylases. Thus, at anoxia or after treatment with desferrioxamine or cobalt chloride, stabilization of HIF-1α occurs independently of ROS generation and the presence of functional electron transport chain is not necessary. This hypothesis explains why inhibitors of complex I prevent hypoxia-induced but not desferrioxamine- or anoxia-induced accumulation of HIF-1α (Schroedl *et al.,* 2002). In contrast, in the

hypoxia-mediated HIF-1α stabilization additional intracellular signaling, including phosphorylation- and ROS-dependent pathways, is required (Schroedl *et al.,* 2002). The preservation of hypoxia-induced stabilization of HIF-1α in cells with a defect in electron transport chain (Vaux *et al.,* 2001) may have resulted either from the presence of marginal complex I and III activity, sufficient to generate ROS, or from anoxic rather than hypoxic conditions (0.1% O_2) used in the experiments. It is possible that signals induced by hypoxia, as well as signals from some cytokines, vasoactive hormones, and nitric oxide (see below) lead to the inhibition of proline hydroxylation (Fig. 8).

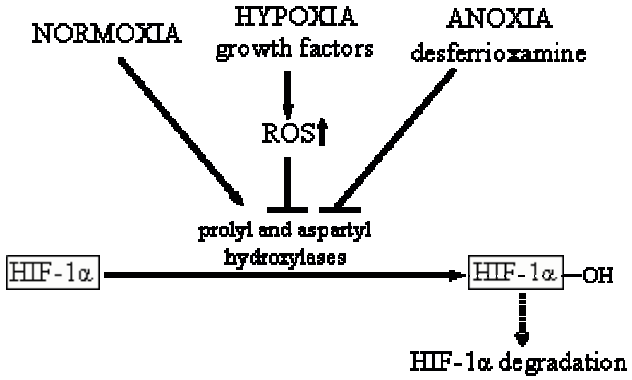

Figure 8. Prolyl and asparginyl hydroxylases function as direct (anoxia) or indirect (hypoxia) oxygen sensors.

Phosphorylation cascades

Initial studies indicated that the activation of HIF-1 involves protein phosphorylation (Wang *et al.,* 1995b). This process might be mediated by several different protein kinase pathways. First, the p42/p44 (Erk2/Erk1) mitogen-activated protein kinases (MAPKs) are able to phosphorylate HIF-1α (Richard *et al.,* 1999). This modification, however, enhances HIF-1 activity but is not involved in HIF-1α stabilization. Thus, this is rather the way in which growth factors modify HIF-1 function as many of them act *via* the receptor tyrosine kinase→Ras/Raf→MEK (mitogen-activated kinase kinase)→MAPK pathway (reviewed in Wenger, 2002). The second pathway able to upregulate HIF-1 activity involves the receptor tyrosine kinase→PI3K (posphatidylinositol-3 kinase)→ prolyl-4-hydroxylases (protein kinase B)→FRAP (FKBP-rapamycin associated protein) pathway. Some studies showed that inhibition of the PI3K or AKT kinases impairs HIF-1-dependent gene expression (references in Wenger, 2002). Moreover, loss of PTEN (phosphatase and tensin homolog deleted on chromosome ten) activity, which is a tumor suppressor protein and a negative regulator of PI3K, results in increased HIF-1α expression (Zundel *et al.,* 2000). Finally, FRAP can stimulate HIF-1α expression even under normoxic conditions (Zhong *et al.,* 2000). Additionally, the involvement of ROS-dependent p38 MAP kinase pathway has been suggested because inhibition of HIF-1 activity by p38 kinase blockers was observed (Gorlach *et al.,* 2001).

Other signaling pathways

Other suggested signaling pathways concern redox-dependent regulation and protein N-nitrosylation. For example, it was shown that reduction of cysteine within C-TAD enhances HIF-1 trans-activation by enabling the interaction with CBP. This reduction is provided by the system: thioredoxin/the redox factor Ref-1 (Ema *et al.,* 1999). Co-expression of Ref-1 and thioredoxin enhanced the transactivation by C-TAD, but not by

N-TAD, in a hypoxia-dependent manner. Additionally, upon hypoxic conditions, thioredoxin was found to be translocated to the nucleus where it can interact with Ref-1 (Ema *et al.,* 1999).

HIF-1 negative regulation

Several negative feedback regulatory pathways that could limit the response to hypoxia have been proposed (reviewed in Wenger, 2002). The downregulation of HIF-1α might occur on the level of transcription, translation, protein stabilization, and/or protein activation. Responsible for the latter could be Cited2 (also named p35srj), a member of the CBP/p300-interacting transactivators with a glutamic acid and aspartic acid-rich tail. Cited2 competes with HIF-1 in binding to the cysteine-histidine-rich (CH1) region of p300 and CBP (Leung *et al.,* 1999). Notably, Cited2 expression is induced by HIF-1 (Bhattacharya *et al.,* 1999).

Recently it was shown that the downregulation occurs also at the level of HIF-1α stabilization as HIF-1 induces synthesis of prolyl hydroxylases which enable rapid HIF-1α hydroxylation and degradation during reoxygenation (D'Angelo *et al.,* 2003). Also some anti-inflammatory factors could act through the inhibition of the HIF-1 pathway. We recently showed that under hypoxic conditions prostaglandin-J_2 attenuates VEGF expression by inhibition of HIF-1 activity (Józkowicz *et al.,* 2004). Furthermore, heme oxygenase-1, which is a stress inducible enzyme that degrades heme to carbon monoxide, iron ions, and biliverdin, could enhance activity of prolyl and asparaginyl hydroxylases by an increased release of iron. Biliverdin and its derivate, bilirubin, scavengers of peroxyl radicals, could also modulate HIF-1α stability due to the attenuation of reactive oxygen species generation. On the other hand, it was observed that HO-1 overexpression enhances VEGF synthesis (reviewed in Dulak *et al.,* 2004).

Interaction between HIF-1 and p53

There are many similarities between the two transcription factors: HIF-1α and the tumor suppressor p53. Under hypoxic conditions both proteins accumulate and gain their transcriptional activity which requires recruitment of p300 as a co-activator. Moreover, under normoxic conditions p53, similarly to HIF-1α, is ubiquitinated by E3 ligase (MDM2 for p53) and subsequently degraded by 26S proteasome (Giaccia *et al.,* 1998). It was observed that the loss of p53 activity results in an increased accumulation of HIF-1α under hypoxic conditions (Ravi *et al.,* 2000). This relationship was explained by the observation that p53 binds to HIF-1α and recruits the MDM2 ubiquitin ligase which preferably targets HIF-1α for degradation. Thus, in the case of prolonged anoxic conditions, p53 can act as a negative regulator of HIF-1 in two ways. First, it inhibits HIF-1 activity by competitive binding of p300 (Schmid *et al.,* 2003), and second, the interaction between p53 and HIF-1α results in HIF-1α degradation (Ravi *et al.,* 2000). Such cooperation explains the angiogenic switch that occurs during tumorigenesis in case of p53 mutation.

HIF-1 natural inhibitors

The natural inhibitors of HIF-1 identified so far are mainly splice variants of the α subunit and thus are able to act as HIF antagonists. The first of them, inhibitory PAS protein (IPAS) is a HIF-α without the transactivation domain. High levels of IPAS were indicated in the corneal epithelium of the eye where HIF-dependent angiogenesis is significantly impaired (Makino *et al.,* 2001). Other potential HIF-1 antagonists are: the zinc-inducible isoform lacking exon 12 (HIF-1αZ) and the dominant-negative HIF-1α isoform lacking exons 11 and 12. In addition, an antisense RNA specific for the 3' untranslated region of HIF-1α was identified (references in Wenger, 2002).

NON-HYPOXIC ACTIVATION OF HIF-1 AND MODULATORS OF HIF-1 ACTIVITY

Interestingly, an increasing number of studies indicate that HIF-1α stabilization might occur in a hypoxia-independent manner. The inducers of HIF-1 activity that act independently of O_2 levels may be divided into several groups. The first group consist of direct inhibitors of prolyl and asparaginyl hydroxylases such as iron chelators, iron replacing molecules, and analogs of 2-oxoglutarate. The second group is represented by various hormones and growth factors. Also hallmarks of inflammation such as proinflammatory cytokines, nitric oxide, increased temperature, or mechanical stress, may induce HIF-1 activity.

Direct inhibitors of prolyl hydroxylases

Transition metal ions, such as cobalt and nickel, induce HIF-1α stabilization under normoxic conditions. Initially this effect was thought to confirm the hypothesis of a heme protein acting as a putative oxygen sensor (Huang *et al.,* 1997). Recently, however, it was suggested that transition metals could inhibit prolyl hydroxylases by substituting the ferrous ion coordinated by PHDs (Epstein *et al.,* 2001). Another well-known activator of HIF-1, desferrioxamine (Wang *et al.,* 1993), could also inactivate PHDs by removal of iron from their catalytic domains. Furthermore, compounds competing with 2-oxoglutarate such as *N*-dimethyl-oxalylglycine (DMOG), a 2-oxoglutarate analog, are also able to inhibit PHDs activity (Jaakkola *et al.,* 2001).

Vasoactive hormones and cytokines

The induction of VEGF expression in vascular smooth muscle cells (VSMC) by vasoactive hormones like angiotensin II and thrombin is mediated through the activation of HIF-1 (Richard *et al.,* 2000). Two separate pathways are responsible for this hypoxia-independent induction of HIF-1 in VSCM. The first leads to an increase in HIF-1 gene transcription, whereas the second results in an increase in translation of HIF-1 mRNA. The central role in both pathways belongs to the diacylglycerol-sensitive protein kinase C (PKC). It was also shown that the increase in HIF-1 translation by angiotensin II is mediated by ROS-dependent activation of the phosphatidylinositol-3 kinase (PI3K) pathway (Page *et al.,* 2002). Together these two pathways increase HIF-1α in VSMC to levels that surpass hypoxic induction.

Also some growth factors such as insulin, insulin-like growth factor 1 and 2, epidermal growth factor, fibroblast growth factor 2, platelet-derived growth factor, transforming growth factor β_1, and inflammatory cytokines such as interleukin-1, or tumor necrosis factor α, (all references in Wenger, 2002) can evoke HIF-1 activation under normoxic conditions. Most of them act through the tyrosine kinase receptor→PI3K→AKT→FRAP pathway. This growth factor-dependent HIF-1 activation is important in tumorigenesis as the overexpression of some growth factors may cause HIF-1-mediated induction of intratumoral angiogenesis.

Nitric oxide

HIF-1 induces nitric oxide production through the enhancement of inducible nitric oxide synthase transcription (Table 1). Conversely, NO affects the accumulation and activity of HIF-1. Initial studies focusing on the NO influence on HIF-1 activity showed that under hypoxic conditions, or after treatment with $CoCl_2$, NO inhibits HIF-1α stabilization and transcriptional activation (Liu *et al.*, 1998; Sogawa *et al.*, 1998; Huang *et al.*, 1999). On the other hand, it was indicated that under normoxic conditions diverse NO donors (with the exception of sodium nitroprusside, SNP) or endogenously produced NO (by inducible or endothelial nitric oxide synthase,

NOS2 and NOS3, respectively) causes HIF-1α stabilization and activation of its target genes (Kimura *et al.*, 2000; Dulak *et al.,* 2000; Sandau *et al.*, 2001a; 2001b; Józkowicz *et al.*, 2001; Dulak & Józkowicz, 2003b). Normoxic NO-induced upregulation of HIF-1 occurred through the PI3K/Akt pathway (Sandau *et al.*, 2001b; Brüne *et al.*, 2001; Natarajan *et al.*, 2003) and was independent of soluble guanylyl cyclase (sGC) activity (Brüne *et al.*, 2001). The type of the NO donor and its concentration used in experiments seem to be crucial for the results obtained. The inhibitory effect of SNP may be evoked by toxic by-products of its decomposition such as cyanides and iron ions which probably surpass the effect of NO (Dulak *et al.,* 2000; Józkowicz *et al.*, 2001).

NO and PHDs

Recently it was reported that the NO-mediated activation of HIF-1 at normoxia is caused by attenuation of prolyl hydroxylation (Metzen *et al.*, 2003b) and, therefore, PHDs might constitute direct or indirect targets of NO. An interaction between NO and iron could constitute a way of affecting prolyl hydroxylation as NO directly binds to the ferrous ion in heme or non-heme iron-containing proteins. Prolyl hydroxylases contain Fe(II) in their catalytic domains, thus NO could compete with molecular oxygen for the catalytic site and subsequently inhibit enzyme function (Metzen *et al.*, 2003b). This hypothesis, however, does not explain why NO inhibits hypoxia-induced HIF-1 accumulation. Helpful in searching for the answer could be the fact that NO down-regulates HIF-1α in hypoxia by activation of PHDs in cytoplasmic extracts (Wang *et al.*, 2002), although this effect has not been confirmed by *in vitro* protein interaction assays (Metzen *et al.*, 2003b). Therefore, activation of PHDs by NO might be caused by cytoplasmic components such as protein phosphorylation cascades or/and ROS-dependent signaling pathways. Recently, however, it was reported that NO donor, NOC18, induces HIF-1α synthesis at normoxia, whereas neither hydroxylation nor stabilization of HIF-1α is influenced. Instead, it was shown that this effect depends on the PI3 and MAP kinases pathways (Kasuno *et al.,* 2004).

NO and ROS

On the other hand, NO as a free radical reacts rapidly with ROS, particularly in a diffusion-controlled fashion with superoxide anion to create peroxynitrate, one of the most reactive compounds in nature. If hypoxia activates HIF-1 through the increase in mitochondrial ROS generation, NO would inhibit HIF-1 activation by attenuation of ROS levels. To confirm this hypothesis it was shown that NO blocks HIF-1α stabilization in the presence of superoxide anion donors or H_2O_2 (Sandau *et al.*, 2001b). Thus ROS and NO might be able to induce HIF-1 activity but, when present together, they may react to form compounds such as peroxynitrite that lacks this property and might cause HIF-1α degradation.

Carbon monoxide

Initially it was reported that carbon monoxide (CO) inhibits hypoxia-induced HIF-1α expression, HIF-1 DNA binding, and HIF-1 transcriptional activity (Huang *et al.,* 1999). Such a correlation would be consistent with the hypothesis of a heme protein acting as an oxygen sensor. However the concentrations of CO used in those experiments were very high (up to 80%) and therefore non-physiological. On the contrary, it is possible that CO, as an inhibitor of mitochondrial complex IV, could lead to an increase in ROS generation and, therefore, induce HIF-1α accumulation. We have shown that low (1% or lower) concentrations of CO strongly induce VEGF expression in vascular smooth muscle cells (Dulak *et al.,* 2002) and in endothelial cells (Józkowicz *et al.,* 2003; reviewed in Dulak & Józkowicz,

2003a). Another interesting hypothesis was proposed after the discovery that mammalian neuronal PAS domain protein 2 (NPAS2), a bHLH-PAS transcription factor involved in regulation of circardian cycle, is regulated not only by the NADPH/NADP ratio but also by carbon monoxide (Dioum *et al.,* 2002). Incorporation of CO into hemes which are bound to the PAS domains of NPAS2 inhibits NPAS2-BMAL1 heterodimerization and, subsequently, its transcriptional activity. The NPAS2 binding sequence, CACGTG (Rutter *et al.*, 2001), may be recognized by HIF-1, thus reciprocal relationship between these two transcription factors is possible and requires further studies.

Mechanical and thermal stress

It has been reported that HIF-1α is present in the nuclei of cardiac myocytes in non-hypoxic myocardium (Kim *et al.,* 2002). This upregulation of HIF-1α occurs as a result of a wall stretch and is mediated by the PI3K→AKT→FRAP pathway. A similar effect was observed in aortic VSMC where hypertension induced HIF-1α accumulation (Kuwahara *et al.,* 2002). Therefore, HIF-1 seems to be responsible not only for the adaptation to hypoxia but also for the adaptation to mechanical stress.

It was also indicated that HIF-1α accumulation might be evoked by exposure to increased temperature. This effect seems to be mediated by direct protein stabilization that involves the heat shock protein HSP90 and is hypoxia-independent (Katschinski *et al.,* 2002).

CONCLUSIONS

Undoubtedly, HIF-1 plays the leading role in the induction of complex response to various stresses and thus contributes to the maintenance of cellular and systemic homeostasis. This function gives HIF-1 a major importance in development, physiology, and pathophysiology. The central role in the regulation of HIF-1 activity belongs to prolyl and asparaginyl hydroxylases, which function cooperatively as direct or indirect oxygen sensors. The question is why HIF-1 activity is controlled by two independent processes. The answer might be that multiple levels of the regulation enable graded responses to subtle changes in O_2 concentration and ensure tight control of the hypoxic response pathway (Bruick & McKnight, 2002).

The analysis of HIF-1 expression pattern and the modulation of HIF-1 activity may bring new diagnostic and therapeutic approaches in many diseases such as cancer and vascular disorders (reviewed in Semenza 2000a). It is possible that inhibition of HIF-1 could suppress intratumoral angiogenesis and thus attenuate cancer growth. Additionally, it was observed that *Hif1a*$^{+/-}$ mice had significantly weaker responses to chronic hypoxia such as reduced polycythemia, right ventricular hypertrophy, and pulmonary hypertension (Yu *et al.,* 1999). Thus in case of chronic lung disease local downregulation of HIF-1α could be useful in treating or preventing these disorders. In contrast, in the case of myocardial ischemia or other ischemic disorders induction of HIF-1 expression could prevent ischemic tissues from permanent damages. HIF-1 upregulation in pro-angiogenic therapies could facilitate the formation of fully matured vascularity.

Therefore, the understanding of the molecular mechanisms of HIF-1 activation will be crucial for the development of new drugs acting either as agonists or as antagonists of the hypoxia response pathway. Possible targets of future therapies could be not only HIF-1α itself but also the proteins mediating its stabilization such as prolyl and asparaginyl hydroxylases.

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