Progress on hypoxia-inducible factor-3: Its structure, gene regulation and biological function (Review)

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Abstract. Hypoxia inducible factors (HIFs) are transcription factors, which are commonly expressed in mammals, including humans. The HIFs consist of hypoxia-regulated α and oxygen-insensitive β subunits, and are key regulators of gene expression during hypoxia in normal and solid tumor tissues. Three members of the HIF family, HIF-1α, HIF-2α, and HIF-3α, are currently known. HIF-3α differs from HIF-1α and HIF-2α in protein structure and regulation of gene expression. For a long time, HIF-3α was considered as a negative mediator of HIF-regulated genes. HIF-3 has a transcriptional regulatory function, which negatively affects gene expression by competing with HIF-1α and HIF-2α in binding to transcriptional elements in target genes during hypoxia. Previously, certain target genes of HIF-3α have been identified, confirming the role of HIF-3α as a transcription factor. In this review, the protein structure, gene regulation and biological function of HIF-3 are discussed based on the literature.

Contents

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1. Introduction

Hypoxia-inducible factors (HIFs) are commonly expressed in humans and other mammals as transcriptional factors (1,2). They positively regulate the expression levels of >100 target genes, which encode protein products involved in the response to hypoxia (3-5). Tumor hypoxia was first described in the 1950s and currently there is increasing evidence to demonstrate that hypoxia is regulated by HIFs and is a common feature in several types of cancer (6-9). In 1992, Semenza *et al* (10) identified a nuclear factor, which binds to the 3'-flanking sequence of the human erythropoietin gene (EPO) and promotes the expression of EPO under anoxic conditions. This factor, termed HIF, is able to increase the number of erythrocytes and increase the efficiency of oxygen transportation (10). At present, three types of HIF have been identified, namely HIF-1 (10), HIF-2 (11) and HIF-3 (12). HIF-3α is the most recently identified member of the HIF family. Mouse HIF-3α (mHIF-3α) was initially identified by Gu *et al* in 1998 (12) and human HIF-3α (hHIF-3α) was identified in 2001 (13). HIF-3α has been investigated to a lesser degree compared with HIF-1 and HIF-2. HIF-1α and HIF-2α are often overexpressed in cancer tissue, leading to progression of aggressive tumors, tumor resistance to chemotherapy and radiation, and poor prognosis of the disease (14-17). The role of HIF-3α in tumors types remains to be elucidated, however, previous studies have indicated that HIF-3α may suppress the expression of genes, which are typically inducible by HIF-1α and HIF-2α in tumor cells (13,18). Therefore, HIF-3α has transcriptional regulatory functions and is a negative regulator of gene expression during hypoxia (13,18). Furthermore, HIF-3α is a true transcription factor since it actively stimulates the expression of a number of target genes (19). This review comprehensively discusses the current knowledge of the gene structure, regulation of expression and biological function of HIF-3.

2. Structure of human HIF-3

hHIF-3 is a heterodimer, which consists of hypoxia-regulated-α (HIF-α) and oxygen-insensitive β subunits, and is a member of the aryl-hydrocarbon receptor nuclear translator (ARNT) family (20). hHIF-3α is located at chromosome

19q13.13-13.2 (12), which differs from the locus of HIF-1α (14q21-24) and HIF-2α (2p16-21) (10,11). The first full-length hHIF-3α cDNA, now termed HIF-3α1, encodes a 668 amino acid protein with a relative molecular mass of 73 kDa (13). The N-terminus of HIF-3α is a basic-helix-loop-helix (bHLH) region, which is responsible for DNA binding (Fig. 1). Following the bHLH region is a Per/Arnt/Sim (PAS) region, which consists of ~300 aminophenoal residues. The PAS region contains PAS-A, PAS-B and two replication regions, which form dimmers with the bHLH region of HIF-1β (13). Following the PAS region is an oxygen-dependent degradation (ODD) domain. This oxygen regulatory region is involved in the degradation of HIF-3α (13). The C-terminus of HIF-3α is a transactivation domain (TAD). HIF-1α and HIF-2α have two TADs, which are located at the N and C-terminus, however, HIF-3α has only one TAD at the N-terminus (13). The TAD in HIF-3α shares 58% and 52% identity with the TADs in the N-terminus of HIF-1α and HIF-2α, respectively (12). Multiple splice variants of hHIF-3α, namely hHIF-3α1-10, have been reported (21,22). hHIF-3α has 19 exons spanning 43 kb in chromosome 19q13.2. Three unique exons, namely exons 1a, 1b and 1c, are likely to contain transcription initiation sites for the variants. Exon 2 encodes the bHLH domain and exons 3-9 contain the coding sequence for the PAS domain. HIF-3α2 consists of 632 amino acids and begins from exon 1a and ends at exon 13a, skipping exons 1b and 1c (22). hHIF-3α3 begins at exon 1b and ends at exon 17, skipping exons 1a, 1c, 15 and 16. hHIF-3α3 has 648 amino acids and contains ODD and LXXLL motifs, however lacks any recognizable DNA-binding sequences, including the bHLH or LZIP domains (22). hHIF-3α4 encodes a protein of 363 amino acids, which lacks NAD, CAD and ODD domains. Compared with other hHIF-3α variants, hHIF-3α4 contains no LXXLL or LZIP motifs (22). hHIF-3α5 and hHIF-3α6 start at exon 1b and lack exon 3. hHIF-3α5 contains a short exon 14c and ends at exon 15, and it encodes a protein containing partial PASa, PASb and PAC domains. hHIF-3α6, similar to hHIF-3α4, contains intron 7 and ends at intron 8, and it contains only a partial PASb domain at the C-terminus (22). Similar to the HIF-α subunit, HIF-1β contains bHLH, PAS and TAD domains (Fig. 1). However, HIF-1β lacks the ODD domain, therefore, it is constitutively expressed in all tissues under aerobic conditions (23-25).

3. Structure of mouse HIF-3α

The open reading frame of mHIF-3α spans 1.98 kb, containing 15 exons, and encodes a protein of 662 amino acids (12). mHIF-3α has also been reported to produce alternatively spliced variants, the mouse inhibitory PAS domain protein (IPAS) (26,27) and neonatal and embryonic PAS protein (NEPAS) (28). Mouse IPAS is a hypoxia-inducible short splice variant of mHIF-3α and shares three exons (2, 4 and 5) with HIF-3. Mouse IPAS lacks NTAD, CTAD and ODD domains (26) and is known to bind to HIF-1α, however not HIF-β (27). Similar to IPAS, NEPAS mRNA is derived from HIF-3α and contains the first exon (1a) of IPAS followed by the 2nd to 15th exon of HIF-3α (28). NEPAS encodes a polypeptide of 664 amino acids, containing the NTAD and ODD domains (28). Unlike IPAS, NEPAS is able to dimerize with HIF-β (28).

4. Expression and regulation of HIF-3α

The expression profiles of HIF-1α and HIF-2α have been well documented (29). However, the expression profiles of HIF-3α variants are only recently investigated. HIF-3α is expressed in human kidney (13) and lung epithelial cells (30). Northern blot analysis demonstrated that the mRNA expression of hHIF-3α is high in the heart, placenta and skeletal muscle, however, is low in the lung, liver and kidney (22). Immunofluorescence analysis demonstrated that HIF-3α is present in the cytoplasm and nucleus under normoxic conditions, and that exposure to hypoxia increases the nuclear fraction of HIF-3α (31). mHIF-3α is expressed in the adult thymus, lung, heart and kidney (12). In mice, IPAS is predominantly expressed in corneal epithelium and Purkinje cells of the cerebellum (26). NEPAS is expressed almost exclusively in the late embryonic and early postnatal stages, with the expression predominantly located in the lungs and heart (28). By contrast, IPAS is not detected during embryonic development (28).

The expression of HIF-3 is predominantly regulated at the transcriptional and post-transcriptional levels, which are described in the following sections.

Regulation of HIF-3α expression at the transcriptional level

Hypoxia. Hypoxia increases the mRNA expression levels of HIF-3α. Heidbreder *et al* (32) revealed that the mRNA expression levels of HIF-3α were significantly increased in the lung and other organs following a 2-h hypoxic exposure in rats. This was confirmed by two other studies (33,34). Zhang *et al* (35) demonstrated that hypoxia increases the mRNA and protein expression levels of HIF-3α in zebrafish.

HIF-1 and HIF-2. HIF-3α is a target gene of HIF-1 and modulates the expression of hypoxic genes (31). Tanaka *et al* (31) revealed that siRNA-mediated knockdown of HIF-1α in human renal cell carcinoma notably dampened the 2, 2'-dipyridy-stimulated induction of HIF-3α protein. In addition, immunohistochemical analysis revealed co-localization of HIF-1α and HIF-3α in cells (31). Pasanen *et al* (21) demonstrated that HIF-3α2 and HIF-3α4 are inducible during hypoxia and the inductions require HIF-1α. A similar previous study revealed that HIF-1α binds to the hypoxia response element (HRE) in the IPAS promoter and induces the expression of IPAS (36). However, the stabilized form of HIF-1α does not affect the mRNA expression levels of HIF-3α in zebrafish embryos (35) and 3T3-L1 cells (37). This contradictory result may be due to the different cell lines or animal models used in the experiments. In addition to HIF-1α, Hatanaka *et al* (37) observed that the promoter activity of HIF-3α is specifically activated by HIF-2α. HIF-2α specifically binds to the sequence between -251 and -228 in the mHIF-3α promoter, which is essential in response to the activation of HIF-2α (37). In human umbilical venous endothelial cells, the expression of HIF-3α is driven by HIF-1 and HIF-2 (18).

2-Deoxy-D-glucose (2-DG) and insulin. 2-DG and insulin are able to cause a widespread increase in the mRNA expression levels of HIF-3α. Following treatment with 2-DG in rats, the expression of HIF-3α was markedly increased in the lung, heart and kidney by 9.6-, 9.0- and 4.1-fold, respectively (38). Following treatment with insulin, the mRNA expression of HIF-3α is significantly increased in every major tissue and

Figure 1. Schematic overview of the domain structures of HIF-α isoforms. The majority of the isoforms contain the bHLH and PAS domains, which are required for dimerization and DNA binding. HIF-1α, HIF-2α, HIF-3α1, HIF-3α2 and HIF-3α3 also contain an ODDD, which contains the conserved proline(s). Only HIF-3α1 contains a LZIP domain. HIF, hypoxia-inducible factor; ODDD, oxygen-dependent degradation domain; bHLH, basic helix-loop-helix; LZIP, leucine zipper.

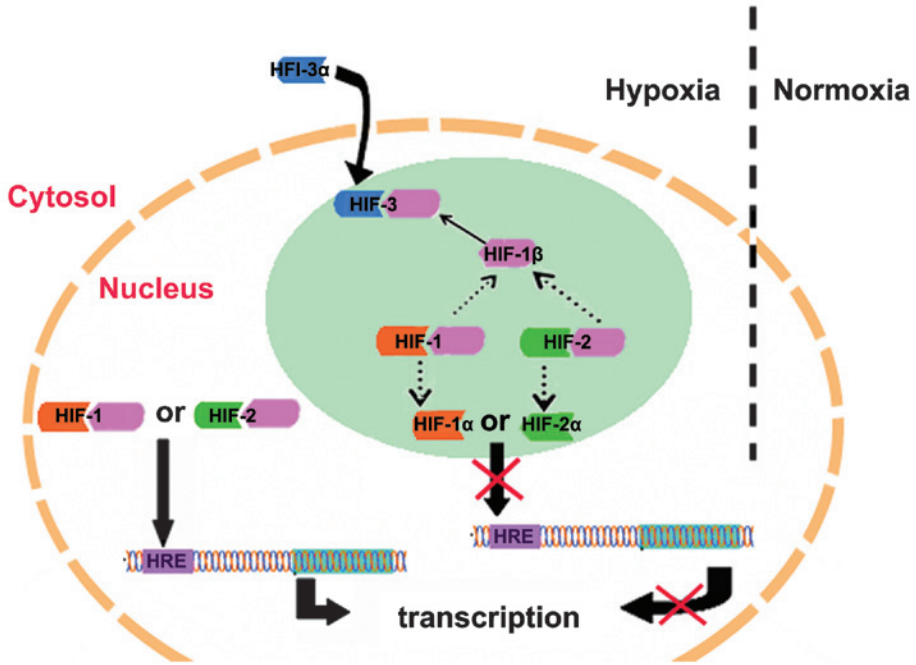

Figure 2. Mechanism of HIF-3α suppression of HIF-1 and HIF-2-mediated gene expression. HIF-3α competes with HIF-1α and HIF-2α in binding to the HIF-1β subunits, reduces the expression levels of HIF-1 and HIF-2, therefore repressing the upregulation of target gene expression stimulated by HIF-1 and HIF-2. HIF, hypoxia-inducible factor; HRE, hypoxia response element.

organ; however, the induction is not as high as that following treatment with 2-DG (38).

Regulation of HIF-3α expression at the post-transcriptional level

Hypoxia. The mRNA expression of HIF-3α is increased significantly in A549 cells exposed to a low oxygen environment for 2 h (30). On one hand, hypoxia markedly increases the protein synthesis of HIF-3α within 30 min exposure. Conversely, hypoxia reduces the degradation of HIF-3α protein and therefore, relatively increases its protein level (30).

Von Hippel-Lindau (VHL). VHL is a tumor suppressor gene involved in VHL syndrome and renal cell carcinoma (39-41). Maynard *et al* (22) demonstrated that hHIF-3α1-3 splice variants share a common ODD domain, which can be degraded by the pVHL ubiquitin-proteasome under normal oxygen partial pressure. The ability of VHL to degrade HIF3α is dependent on the proline 490 residue on HIF3α and this is increased in the presence of prolyl hydroxylase (PHD).

PHD. PHD is a cellular sensor for low-oxygen. Under normal oxygen partial pressure and in the presence of Fe^{2+} and acetone dicarboxylic acid, PHD catalyzes the hydroxylation of key amino acid residues in the HIF-α ODD domain (42,43). This is followed by VHL binding to HIF-α and inducing degradation via the ubiquitin-proteasome pathway (44,45). Chen *et al* (46) demonstrated that the *in vivo* protein level of HIF-3α under hypoxic conditions is negatively correlated with the protein expression levels of PHD2 and PHD3, whereas the

content of HIF-3α mRNA is positively correlated with the mRNA expression levels of PHD2 and PHD3. It is possible that PHD increases the protein degradation of HIF-3α, which is followed by negative feedback upregulation of the mRNA expression of HIF-3α in order to compensate for the loss of the HIF-3α protein.

Deferoxamine (DFX) and $CoCl_2$. DFX and $CoCl_2$ increase the protein expression levels of HIF-3α (30). DFX binds to iron and interrupts the hydroxylation of proline in the ODD domain of HIFs, by preventing the binding of the VHL ubiquitin-proteasome complex to HIF-3α and eventually leads to the accumulation of intracellular HIF-3α protein (30,47,48). Similarly, $CoCl_2$ reduces the degradation of HIF-3α by occupying its binding site for the VHL ubiquitin-proteasome complex, resulting in an increased protein expression of HIF-3α (30,49,50).

5. Biological functions of HIF-3

Following protein stabilization during hypoxia, HIF-1α and HIF-2α dimerize with HIF-β, bind to co-activators, including p300, and interact with the HRE of target genes (51,52). Compared with HIF-1α and HIF-2α, HIF-3α has dual functions: Inhibition of the activities of HIF-1α and HIF-2α, and regulation of its own target genes (19).

hHIF-3α1 has been demonstrated to suppress HIF-1 and HIF-2-mediated gene expression (13). Hara *et al* (13) transfected expression vectors of HIF-1α, HIF-2α or HIF-3α into COS-7 cells and demonstrated that HIF-1α and HIF-2α promote the transcription of HREs, whereas HIF-3α1 inhibits HRE transcription. A previous study revealed that HIF-3α1 inhibits the expression levels of HIF-1α and HIF-2α (13). It is suggested that HIF-3α competes with HIF-1α and HIF-2α in binding to HIF-1β subunits, reduces the levels of HIF-1 and HIF-2, and ultimately inhibits the upregulation of the target genes of HIF-1 and HIF-2 (Fig. 2) (13). Additionally, HIF-3α lacks a transcriptional activation domain, and its bHLH and PAS domains suppress the expression of target genes, which are typically inducible by HIF-1α and HIF-2α (13). Splice variant IPAS dimerizes with HIF-1α protein and disrupts the interaction between HIF-1α and the HRE of its target genes (36). Makino *et al* (36) demonstrated that there is a negative feedback mechanism between HIF-1α and IPAS. At first, HIF-1α binds to the HRE in the IPAS promoter and induces the expression of IPAS (36). Increased levels of IPAS dimerize with HIF-1α protein and inhibits further induction of IPAS (36). In hepatoma cells, ectopic expression of IPAS decreases the expression of vascular endothelial growth factor (VEGF), resulting in reduced tumor growth and decreased tumor vascular density *in vivo* (27). The splice variant, HIF-3α4, is different from IPAS in terms of its structure and gene regulation. For example, IPAS only binds to HIF-1α, whereas HIF-3α4 binds to HIF-1α and ARNT (53). The HIF-3α4/HIF-β complex binds to HREs, which inhibits the binding of the HIF-1α/HIF-β complex to the HRE (53). The HIF-3α4/HIF-β complex is not transcriptionally active, however, it significantly reduces HIF-1-mediated promoter activation by acting as a dominant negative regulator of HIF-1 (53). Similar to IPAS, ectopic expression of HIF-3α4 inhibits the endogenous expression of hypoxia-responsive genes, including glucose transporter-1 (GLUT-1), and knocking down the endogenous expression of HIF-3α4 using siRNA increases the transcription of HIF target genes (53). Besides HIF-1α and HIF-β, HIF-3α4 also binds to HIF-2α and inhibits HIF-2-mediated transactivation of HRE-driven genes (54). In addition, overexpression of HIF-3α4 in clear-cell renal cell carcinoma (CCRCC) cells reduces endogenous expression of HIF-2 target genes and inhibits the growth of CCRCC xenografts in severe combined immunodeficiency mice (54). These findings suggest that HIF-3α4 has a dominant negative role in suppressing CCRCC growth and has a potential therapeutic role in the treatment of CCRCC (54). A previous study revealed that overexpression of HIF-3α4 impairs angiogenesis, proliferation, and metabolism/oxidation of hypervascular meningioma (55). Therefore, HIF-3α4 is a potential molecular target for the treatment of meningioma (55).

It is reported that siRNA-mediated knockdown of HIF-3α induces the expression of certain HIF-1α-mediated genes and decreases the expression of ANGPTL4 in response to hypoxia (31). This indicates that HIF-3α also possesses transcriptional activity. All the hHIF-3α variants were demonstrated to be able to bind to HIF-β and overexpression of certain HIF-3α variants, together with HIF-β, induces the mRNA expression levels of several HIF-1 and HIF-2 target genes, including EPO (56,57), ANGPTL4 (58,59) and GLUT1 (60,61). However, the overexpression of HIF-3α variants reveals no significant stimulation of the expression of HRE-driven reporter genes (62), suggesting that the target genes induced by HIF-3α variants may contain specific response elements, which are not canonical HREs (31,62).

Zhang *et al* (19) revealed that HIF-3α exhibits significant transactivation activity in zebrafish. The authors performed transcriptomic analyses and identified a large number of HIF-3 target genes, which can be divided to three categories: i) Genes that are upregulated by HIF-3a only (e.g. sqrdl, mclb and zp3v2); ii) genes that are regulated by HIF-1α and HIF-3α with similar potencies (e.g. redd1 and mlp3c); and iii) genes that are regulated by HIF-1a and HIF-3a, however, with different potencies (e.g. igfbp1a) (19). Notably, the authors demonstrated that the transcriptional activity is conserved across species and hHIF-3a-9 isoforms stimulate similar target genes in different human cell types, including LC3C, REDD1 and SQRDL cells (19). These findings suggest that HIF-3 is an oxygen-dependent transcription factor, which activates a distinctive set of genes in response to hypoxia.

6. Association between HIF-3 and diseases

Tissue hypoxia is a pathological feature of several human diseases, including myocardial infarction, stroke and kidney disease (63-65). The expression of HIF-3α is often altered in these diseases and may contribute to their development (32,66). It has been reported that the mRNA expression of HIF-3a is increased as an early response to acute hypoxia and acute myocardial ischemia in humans and experimental animal models (32,66). Zolk *et al* (67) demonstrated that the mRNA expression level of HIF-1α is 51% lower in cardiac tissue from a patient with heart failure compared with that of a healthy control. By contrast, the expression of HIF-2α remains unchanged and the mRNA expression of HIF-3α is

72% higher in cardiac tissue from a patient with heart failure compared with healthy control (67).

In addition, HIF-3 exerts abnormal expression patterns in liver and kidney disease (68). Hypoxia-associated molecules are upregulated during cystic alteration into a heterogeneous appearance (68). In polycystic liver, VEGF is markedly and widely expressed in the cytoplasm of hepatocytes (68), and the expression of HIF-3α, however not HIF-1α, is observed in a few nuclei of hepatocytes adjacent to the biliary areas (68). By contrast, VEGF, HIF-1α and HIF-3α proteins are not present in the cytoplasm or nuclei of hepatocytes in the control livers (68). Therefore, it is hypothesized that the presence of HIF-3α in periportal hepatocytes is associated with the induction of VEGF (68). Fang *et al* (69) demonstrated that HIF-3α is one of the mediators, which contribute to the development of primary spontaneous pneumothorax.

7. Conclusion

Based on the current knowledge, HIF-3α has a dual role in response to hypoxia: It suppresses HIF-1 and HIF-2-mediated gene expression and induces the expression of their own target genes by binding to the HRE or specific response elements of varying lengths, which are distinct from the canonical HRE. The function of HIF-3α remains to be fully elucidated, however, it is an important factor for the fine-tuning of the hypoxic response in humans in physiological and pathological conditions (62,70). A previous study identified certain target genes of HIF-3α and confirmed its role as a transcription factor (19). Understanding the biological roles of HIF-3α is important for identifying a potential therapeutic target for the treatment of diseases.

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