Published in final edited form as:

Clin Chim Acta. 2008 September ; 395(1-2): 6–13. doi:10.1016/j.cca.2008.05.002.

Regulation of gene expression by hypoxia: integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element

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Abstract

Cells experiencing lowered O₂ levels (hypoxia) undergo a variety of biological responses in order to adapt to these unfavorable conditions. The master switch, orchestrating the cellular response to low O₂ levels, is the transcription factor, termed hypoxia-inducible factor (HIF). The α subunits of HIF are regulated by 2-oxoglutarate-dependent oxygenases that, in the presence of O₂, hydroxylate specific prolyl and asparaginyl residues of HIF- α , inducing its proteasome-dependent degradation and repression of transcriptional activity, respectively. Hypoxia inhibits oxygenases, stabilized HIF- α translocates to the nucleus, dimerizes with HIF- β , recruits the coactivators p300/CBP, and induces expression of its transcriptional targets via binding to hypoxia-responsive elements (HREs). HREs are composite regulatory elements, comprising a conserved HIF-binding sequence and a highly variable flanking sequence that modulates the transcriptional response. In summary, the transcriptional response of a cell is the end product of two major functions. The first (trans-acting) is the level of activation of the HIF pathway that depends on regulation of stability and transcriptional activity of the HIF- α . The second (cis-acting) comprises the characteristics of endogenous HREs that are determined by the availability of transcription factors cooperating with HIF and/or individual HIF- α isoforms.

Keywords

Hypoxia; hypoxia-inducible factor; transcriptional regulation; hypoxia-responsive element

Introduction

Oxygen (O₂) is essential for the survival of all aerobic organisms. O₂ is required for aerobic metabolism that maintains intracellular energy balance. Aerobic energy metabolism is dependent on oxidative phosphorylation, in which the oxido-reduction energy of the mitochondrial electron transport is converted into the high-energy phosphate bond of ATP. In this process, O₂ serves as the final electron acceptor. Depending largely on the distance from the nearest functional blood vessel, cells in mammalian tissues typically experience O₂ concentrations in the 40-60 mmHg range [1]. Hypoxia, defined as a state of reduced O₂ level below normal values, occurs under various physiological (embryonic development, adaptation

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to high altitudes, wound healing) as well as pathological (ischemic diseases, cancer) conditions [1]. In order to cope with hypoxia, organisms undergo a variety of systemic and local changes to restore O₂ homeostasis and limit the effect of low O₂ [2]. Systemic adjustments include enhanced O₂ delivery by the bloodstream, whereas angiogenesis features prominently among the local adjustments [3]. At the cellular level, the most noticeable response to hypoxia is reduction in oxidative phosphorylation, accompanied by increased glycolysis to compensate for lower ATP production [4].

Although hypoxia generally inhibits mRNA synthesis, transcription of subsets of genes increases dramatically. At the molecular level, the master switch orchestrating the cellular response to low O₂ tension is generally considered to be the transcription factor hypoxia-inducible factor (HIF) [5,6]. The importance of the HIF pathway can be inferred from the fact that it is present in virtually all cell types and all higher eukaryotes [7]. HIF is a heterodimer that consists of one of the regulatable HIF- α subunits and the constitutively expressed HIF-1 β (also known as aryl hydrocarbon receptor nuclear translocator or ARNT). Both α and β subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain-containing transcription factors [8]. bHLH and PAS domains mediate DNA binding and dimerization; the other domains in the α subunits include a unique O₂-dependent degradation domain (ODDD) and two transactivation domains: the N-terminal activation domain (NAD) and C-terminal activation domain (CAD) [7] (Figure 1). Three structurally closely related α subunits (HIF-1 α , HIF-2 α , and HIF-3 α) have been identified to date [7]. In addition to these three isoforms, their splicing variants also have been described. HIF-1 α variants lacking exon 14 or exons 11 and 12 display severely compromised transcriptional activity [9,10]. Inhibitory PAS protein is a splice variant of HIF-3 α that preferentially dimerizes with HIF-1 α and thus precludes formation of active HIF-1 α /HIF-1 β heterodimers [11].

Hypoxia activates the HIF pathway by a sophisticated mechanism that regulates post-translational modifications of the α subunits. In this mechanism, two independent but co-regulated molecular switches can be recognized: the first switch controls the abundance of HIF- α whereas the second regulates its transcriptional activity. Upon activation by the hypoxic signal, HIF- α translocates to the nucleus, dimerizes with HIF- β [12], recruits p300/CBP, and induces the expression of its transcriptional targets via binding to hypoxia-responsive elements (HRE) [5,6] (Figure 2).

Regulation of HIF- α stability

The abundance of HIF- α can be regulated by O₂-dependent and O₂-independent mechanisms. On the one hand, the constitutive transcription and translation of the α subunits allow for almost instantaneous induction of HIF by hypoxia [13] but on the other hand require a mechanism for removal of HIF- α at other times. In the presence of O₂ the overall levels of α subunits are low due to the rapid degradation by a complex mechanism with several distinct steps (Figure 2). Enzymes that initiate degradation of HIF- α by hydroxylating either of the two prolines in the ODDD (P402 and P564 in HIF-1 α) are the prolyl hydroxylase domain proteins (PHDs). PHD1, PHD2, and PHD3 have closely related catalytic domains and belong to the superfamily of 2-oxoglutarate (2OG)-dependent oxygenases. In order to be active, PHDs require O₂, the citric acid cycle intermediate 2OG as a co-substrate, plus Fe(II) and ascorbate as cofactors [14,15]. A high rate of turnover of PHD enzymes might suggest that hydroxylation capacity for a low abundance transcription factor would not be limiting. However, there is sufficient evidence for the key regulatory role of PHD activity in HIF- α turnover and under conditions when HIF is induced PHD activity does become limiting (reviewed in [16]).

Hydroxylated prolines enable specific recognition of HIF- α by the von Hippel-Lindau (VHL) protein [17,18] which, in a complex with elongin B, elongin C, and Cul2 functions as an E3

ubiquitin ligase for HIF- α [19]. Hydroxylation of either of the two conserved prolines by PHDs is sufficient for recognition by VHL as mutation of either proline alone only partially stabilizes HIF- α , whereas mutation of both markedly increases its stability and activity [20]. VHL is a classical tumor suppressor that has no intrinsic catalytic activity, yet its critical role in regulation of the HIF system, along with the significance of this regulation in cancer progression, is now generally accepted (for review see [19]). VHL has two subdomains, a and b, binding directly to elongin C and HIF- α , respectively [21]. The loss of VHL function in classical VHL disease is either due to mutations that render VHL defective in respect to binding to elongin C or HIF- α , or epigenetic silencing of the wild type VHL allele, resulting invariably in inappropriate stabilization of HIF- α [19].

Binding of hydroxylated HIF- α by VHL is followed by rapid polyubiquitylation. The E2 ubiquitin-conjugating enzyme UbcH5 requires K532, K538, and K547 acceptors for the VHL-mediated ubiquitylation HIF-1 α . Ubiquitylation of the KKK/RRR HIF-1 α mutant was inhibited in a manner similar to the P402, P564 mutant [22], leading to its considerable stabilization. In the final step, polyubiquitylated HIF- α is translocated to and degraded in the 26S proteasome [23].

In addition to hydroxylation, other post-translational modifications also affect stability of HIF- α . For example, a recently identified protein termed RWD-containing sumoylation enhancer is induced by hypoxia and enhances the sumoylation of HIF-1 α , promoting its stabilization and transcriptional activity in hypoxia [24].

HIF- α levels can be also increased in an O₂-independent manner by various factors through the phosphatidylinositol-3-kinase (PI3K)-FKBP-rapamycin associated protein signaling pathway. The underlying mechanism appears to be the stimulation of HIF- α expression by a generalized increase in protein translation under normoxic conditions [25]. Thus, upon activation of the PI3K pathway by heregulin or insulin-like growth factor 1, increased translation of HIF- α will eventually saturate the O₂-dependent regulatory mechanisms and an apparently specific increase in HIF- α levels and HIF activity is observed [26]. Inactivation of the tumor suppressor PTEN, a negative regulator of the PI3K pathway frequently mutated in tumors, correlates with enhanced normoxic expression of HIF- α in tumors [27].

There is evidence that the tumor suppressor p53 protein negatively affects HIF function. When activated, the wild-type, but not mutant, p53 can accelerate the proteasome-dependent degradation of HIF-1 α , thus decreasing its levels in hypoxic cells [28]. In the context of cancer, these examples provide evidence that deregulation of oncogenic and/or tumor suppressor pathways in tumors activates the HIF pathway by increasing the abundance of HIF- α protein.

Regulation of transcriptional activity of HIF

To be transcriptionally active, the HIF complex has to assemble on the HRE in the regulatory regions of target genes. In contrast to HIF-1 β , which contains one activation domain, HIF- α has two activation domains that act synergistically: the centrally located NAD, overlapping with the ODDD, and the CAD, located at the C-terminus [7] (Figure 1). The CAD function is critically dependent on transcriptional coactivators CBP/p300 [29], and regulation of the HIF- α CAD – CBP/p300 interaction is the second molecular switch controlling transcriptional activity of HIF [30]. In normoxia, the factor inhibiting HIF-1 (FIH-1) binds to the C-terminal part of HIF- α and, via hydroxylation of N803 (N847 in HIF-2 α) in the conserved amino acid sequence YDCEVNV/AP [30], blocks interactions with p300/CBP [31]. The asparaginyl-hydroxylase FIH-1 is distantly related to PHDs and also requires O₂, Fe(II), and 2OG for activity [32]. Inhibition of hydroxylation of N803 in hypoxia allows the CAD to interact with the cysteine/histidine-rich domain 1 (CH1) of CBP/p300 [31].

In addition to controlling the accessibility of the HIF- α CAD by asparagine hydroxylation, the HIF- α CAD - CBP/p300 interaction can be regulated by competitive inhibition. Because the coactivators p300/CBP interact with a large number of transcription factors, their amounts could become limiting, e.g. CITED2 (previously p35srj/Mrg1) binds p300/CBP CH1 with high affinity and competitively inhibits other p300/CBP CH1-dependent transcription factors, including HIF [33]. Activated p53 also inhibits the HIF function by sequestering p300/CBP away from HIF-1 [34].

Coactivators SRC-1 and transcription intermediary factor 2 further enhance the transactivation potential of HIF-1 α and produce a synergistic effect with CBP/p300 [35]. MAP kinases have also been reported to modulate transcriptional activity of HIF but the molecular mechanism remains controversial. Both p42/44 and p38 MAP kinases can stimulate HIF- α activity without affecting its stability [36]. Although inhibitors of MEK1 and p38 block HIF-mediated gene expression, the significance of MAP kinases, with respect to influencing HIF function, is likely to be cell-type specific. For instance, it was reported that a minimal activation of the p42/44 MAPK pathway is required for the expression of the HIF-1 regulated hypoxia-marker carbonic anhydrase IX (CAIX), but activity of the p42/44 pathway does not generally correlate with levels of CAIX expression [37]. Moreover, despite the fact that both p42/44 and p38 MAP kinases phosphorylate HIF- α *in vitro*, the transactivation effect of these kinases *in vivo* is more likely to be exerted at the level of HIF coactivators [38].

Co-regulation of stability and transcriptional activity of HIF- α

The strategy of constitutive expression of a pool of HIF- α that will be almost instantaneously available in the rare times of need (e.g. hypoxia) is apparently wasteful. Moreover, the presence of functional HIF- α at other times is likely to be deleterious and the cells not only rapidly dispose of unwanted HIF- α in the proteasome but also make sure that HIF- α that escapes proteasomal degradation will be transcriptionally inactivated by FIH-1. This ingenious two-tier regulatory mechanism of controlling activity by regulating HIF- α stability (PHDs-VHL-proteasome) and its transcriptional activity (FIH-1) accounts for the exceptionally tight control of the HIF pathway. On the other hand, the existence of these two tiers also means that for proper induction of HIF activity both have to be inactivated. Because both PHDs and FIH-1 require O₂ for function, hypoxia, the physiological stimulator of the HIF pathway, simultaneously inhibits proline and asparagine hydroxylation. Hypoxia thus allows concomitant accumulation of HIF- α and its transcriptional activation, leading to a robust transcriptional response.

Although the HIF system originally evolved for the adaptation of organisms to hypoxia, it has become evident that both HIF- α protein abundance and its transactivation potential can be modulated under apparently normoxic conditions in response to various stimuli. A number of agents or genetic factors co-inhibiting PHDs and FIH-1 have been described (reviewed in [16,39]. The requirement for the same co-substrate (2OG) and co-factor Fe(II) [16] suggests that activities of PHDs and FIH-1 could be regulated by their availability. Indeed, iron chelation and divalent metal cations (Co(II), Ni(II), and Mn(II)) have hypoxia-mimicking effects [40]. Analogs of 2OG dimethyloxalylglycine and citric acid cycle intermediates (pyruvate and oxaloacetate) act as competitive inhibitors of PHDs and FIH-1 and induce the HIF system [41]. Interestingly, in the hereditary cancer syndromes, hereditary paraganglioma (HPGL), and hereditary leiomyomatosis and renal cell carcinoma (HLRCC), accumulation of succinate and fumarate, respectively, due to inactivating mutations of various subunits of succinate dehydrogenase [42] and fumarate hydratase [43], results in reduced PHD activity and upregulation of the HIF pathway. Nitric oxide also regulates the HIF system, presumably because it can act, at sufficiently high concentrations, as an analogue of molecular O₂ and inhibit 2OG-dependent oxygenases [44]. Activation of HIF by reactive oxygen species has

been explained in terms of inactivation of 2OG-dependent oxygenases by oxidative damage through hydroxylation of the active site, fragmentation, and conversion of catalytic Fe(II) to inactive Fe(III) [45]. However, the relation between oxidative stress and HIF function is apparently more complex than this, as it was reported that attenuation of oxidative stress by antioxidants can also stabilize HIF-1 α [46]. In all of the described cases, co-inhibition of PHDs and FIH-1 result in HIF- α accumulation in a transcriptionally active form.

In the classical VHL-associated hereditary cancer syndrome, affected individuals are heterozygous for a germline VHL mutation that predisposes to specific types of tumors: clear cell renal carcinomas, hemangiomas of the retina, or hemangioblastomas of the retina and central nervous system [19]. Following somatic inactivation of the second allele, disruption of hypoxic signaling and clinical manifestations are confined to tumors. Unable to target HIF- α for proteasomal degradation, cells in these tumors overproduce angiogenic factors and other HIF-activated transcripts, even under normoxic conditions [19]. Interestingly, erythrocytosis due to overproduction of erythropoietin (EPO), one of the HIF target genes (Table 1), is not a characteristic feature of the VHL disease. In contrast, erythrocytosis is observed in the Chuvash variant of familial polycythemia, a rare autosomal recessive condition linked to a C598T mutation in *VHL* that impairs but does not ablate HIF regulation [47]. Introduction of the wild-type VHL restores the O₂-dependent regulation of HIF- α and accordingly down-regulates the expression of hypoxia-inducible genes [19]. Furthermore, VHL behaves as a *bona fide* tumor suppressor in that restoration of wild type VHL expression results in inhibition of tumor growth *in vivo* [19]. The presence of defective VHL has no effect on PHD activity and HIF- α accumulates in a proline-hydroxylated form. Nevertheless, this form is fully functional because the loss of VHL function also adversely affects FIH-1 and compromises its capacity to inhibit the HIF- α CAD [48].

Although in most cases the stability and transcriptional activity of HIF- α are co-regulated, there are several notable exceptions. Measurements of the K_m of FIH-1 for O₂ revealed that it is less than half that of the PHD family members [49], suggesting that a hypoxic window could exist in which HIF-1 α would be stable due to the absence of prolyl hydroxylation and yet would be transcriptionally inactive due to hydroxylation of N803. In another example, proteasomal inhibitors, despite having a strong positive effect on HIF-1 α stability, not only do not activate HIF under normoxia but they considerably interfere with hypoxia-induced HIF-1 activity [50,51]. Although some theories have been put forward (reviewed in [52]), the mechanism by which proteasomal inhibitors inactivate HIF is not known at present.

In this section, we outlined the importance of co-regulation of HIF- α stability and its transcriptional activation, each regulated by different but related entities. In most cases, stability and transcriptional activity of HIF- α are co-regulated, leading to maximal activation of the HIF pathway. There are, however, situations in which regulation of stability and transcriptional activity are uncoupled, leading to accumulation of a transcriptionally inactive HIF- α . Theoretically, it should be also possible to de-repress transcriptional activity of HIF- α without significantly affecting its stability. The observation that even wild-type VHL-containing cells express variable levels of HIF-1 α in the basal state [53] provides the groundwork for this possibility. In this case, selective inhibition of FIH-1 would result in low to intermediate activation of the HIF pathway. To this end, it has been reported that moderate induction of the hypoxia marker CAIX by pericellular hypoxia generated in dense cultures of certain transformed cells is HIF-1 α -dependent, yet it occurs without appreciable accumulation of HIF-1 α [54]. Although this phenomenon could be cell-type specific and the mechanism is not fully understood, it would suggest that it is possible to selectively up-regulate HIF transcriptional activity (presumably via inhibition of FIH-1) without significantly affecting the stability of HIF- α (activity of PHDs).

HIF target genes

Historically, the HIF target genes have been identified on the basis of one or more of the following strategies: 1. identification of a functional HRE containing a HIF-binding sequence (HBS); 2. comparison of patterns of gene expression in HIF- α wild-type and null cells (or cells treated with siRNA targeting HIF- α); 3. screening for increased gene expression using VHL-null cells or cells transfected with a HIF- α expression vector [6]. Expression profiling experiments indicated the heterogeneity of gene subsets induced by hypoxia, some of which could be explained by different cell types examined and the relative level or duration of hypoxia [55]. However, the differences in hypoxia-responsive profiles cannot be explained by the genetic instability of the tumor cell lines used, as normal stromal and epithelial cells also display distinct hypoxic profiles [55]. The consensus from various studies is that there is a core set of genes that are consistently induced by hypoxia and then there are genes that exhibit cell-type specific induction. This conclusion underscores the importance of studying hypoxic gene expression in a cell-type-dependent context [55]. Hypoxia induces HIF activity in almost all cell types and, therefore, HIF alone cannot account for the cell-type specific gene expression. Instead, the cell-type specific induction by hypoxia appears to be determined by functional interactions of HIF with other transcription factors [6,56].

Estimates of the total number of HIF target genes induced by hypoxia in one or more cell types vary, from more than 200 [56] to as many as 1-5% of all human genes [6], although not all of them may be regulated directly through HREs. Other transcription factors have also been implicated in hypoxia-inducible gene expression, e.g. HIF-activated transcription factors (DEC 1 and 2, ETS-1) also contribute to the pool of hypoxia-induced genes. General stress-responsive transcription factors, such as AP-1, NF- κ B, and Egr1 are also upregulated by hypoxia, although their sensitivity to mild hypoxia and the duration of their transcriptional response is much less than that of HIF [55].

The HIF pathway directly activates an array of at least 70 genes in which functional HREs were experimentally confirmed (reviewed in [6,56]). In general, HIF induces expression of proteins that specifically help meet the metabolic and survival needs of hypoxic cells. For practical purposes, these proteins are grouped according to their function (reviewed in [6,56]) and the major groups with selected representatives are shown in Table 1. Interestingly, HIF activates genes coding for the pro-apoptotic proteins NIP3 [72], BNIP3 [73], and Noxa [74] as well as the anti-apoptotic protein Mcl-1 [75]. This has led to the concept that the ratio of pro- and anti-apoptotic factors could play a role in the differential response of various cell types to hypoxia. In the context of solid tumors, it could contribute to the process in which the most malignant cells are selected [55]. The presence of HREs in the regulatory regions of *CITED2/p35srj* [33], *PHD2* [76], and *PHD3* [77] allows activation of corresponding proteins by hypoxia and provides a negative feedback on HIF activity.

Hypoxia Responsive Elements (HREs)

In contrast to our knowledge of the key events and players regulating the HIF system, relatively little progress has been made towards understanding the fundamental structural features of HREs, the minimal cis-regulatory elements mediating hypoxic transactivation. HREs are composite regulatory elements, comprising the conserved HBS with a core A/GCGTG sequence, and a highly variable flanking sequence [56]. There are many more sites with the core A/GCGTG sequence in the regulatory regions of mammalian genes than are actually used by HIF to regulate gene expression and at the moment we do not understand what distinguishes HBSs in the functional HREs from the putative, non-functional HBSs that contain the identical core sequence. Thus, it would seem reasonable to assume that the variable flanking sequences (there is no consensus sequence) must play some critical “helper” function. Another possibility

is that the core HBS contains a CpG dinucleotide that can be methylated by DNA methyltransferases. It has been shown that a hemimethylated HBS abolishes HIF-1 binding, and it has been proposed that CpG methylation of the erythropoietin HBS might play a selective role in regulation of its expression [78]. Whether this is a more general selective mechanism remains to be seen. Our limited understanding of the concept of what constitutes an optimal HRE is documented by the lack of published systematic analyses. The few earlier studies dedicated to such analysis focused mainly on side-by-side comparison of various endogenous HREs and their multimers (e.g. [79]).

In addition to the obligatory core HBS sequence, compilation of data from more than 100 functionally verified HBSs revealed that certain nucleotide positions, other than the core A/GCGTG sequence, show a non-random character. Thus, A in the -1 position is 4.5 times over-represented, whereas T in the -3 position is 4.2 times under-represented (numbering of HBS positions is shown in Figure 3A) [56]. However, the functional consequence of this non-random distribution is not clear. A few reports related the HRE sequence to its activity, e.g. mutational analysis of the -2 position of HBS revealed that hypoxic induction decreased in the T>>G>C order [80,81]. The low induction of the palindromic CACGTG sequence (E-box) by hypoxia could be explained by competitive binding of other factors, such as USF, c-Myc/Max and Rox/Max heterodimers, or HIF-1 β homodimers, some of which repress transcription [82]. The low activity of the *endothelin-1* and *Flt-1* HREs with the AACGTG sequence also supports the importance of the -2 position of HBS [83].

A single HBS is necessary but not sufficient for activation by hypoxia and it is generally accepted that the non-conserved but functionally critical flanking sequences are an integral part of the HRE. This suggests that, in order to be transcriptionally active, the HBS-bound HIF needs to cooperate with juxtaposed transcription factors. These factors are not necessarily hypoxia-inducible but are required to amplify the hypoxic response or render the HRE tissue-specific [56]. HIF cooperates with a variety of transcription factors, e.g. ATF-1 and CREB-1 in the murine *LDHA* promoter [64], AP-1 binding factors in the *VEGF* promoter [84], hepatic nuclear factor 4 in the *EPO* enhancer [85], or SP1 in the *CA9* promoter [86]. In some, but not all, genes the HBS cooperates with the HIF-1 ancillary sequence (HAS, Figure 3B), a functionally poorly characterized non-conservative regulatory element located 8 bp 3' from the HBS [81]. HBSs arrayed in antiparallel or parallel orientation (Figure 3C) can also form a functional HRE, e.g. in *transferrin* [57], *CITED2/p35srj* [33], murine *PGK-1* and *LDHA* genes [87]. The complexity of regulation by hypoxia was highlighted in the murine *LDHA* promoter where mutations at three separate sites abolished hypoxic induction [64]. Moreover, concatamerization of any of the three functionally critical sites did not confer high-level hypoxic induction, leading to the conclusion that each site is necessary but not sufficient on its own for hypoxic induction [64].

Why is it that endogenous HREs come in many different “flavors”, so much more divergent from each other than binding sites for other transcription factors? Direct comparison of activity of isolated HREs revealed their different strength [79], although it should be kept in mind that the environment of HREs in reporter constructs does not reflect the much more complex situation in the endogenous location in the mammalian genome (for example, effects of chromatin structure, distal regulatory elements). Apparently, each flavor evolved to respond uniquely to hypoxia and the strength of this response is determined by the flanking sequences. Although the governing principles are poorly understood, it appears that the sequence and/or organization of these flanking sequences determines how much of the hypoxic signal will be integrated into the transcriptional response of a particular gene. There are several explanations for the varying strength of endogenous HREs. There could be an “optimal” sequence conferring maximal induction and where the strength of individual HREs will be determined by how closely the flanking sequence matches the optimal sequence. Alternatively, a modular approach

could be in place where certain combinations of HBS and regulatory elements binding other transcription factors and transcriptional cofactors create the HRE. It cannot be ruled out that both strategies are used, resulting thus in the complex organization of HREs with seemingly random flanking sequences.

The fact that HREs function in the context of heterologous promoter/gene constructs made possible construction of hypoxia-inducible vectors for therapeutic or monitoring purposes. In the first such report, a trimer of the murine *PGK-1* HRE was used [88]; later, multimers of HREs of various length from other genes were used, most frequently *EPO* and *VEGF* [89, 90]. Multimers of the *VEGF* HRE were used to generate hypoxia-sensing transgenic rats and this model was employed to study the effects of physiological hypoxia in tissues of live animals. Interestingly, expression of the hypoxia-responsive transgene increased throughout the observation period, whereas expression of vascular endothelial growth factor showed a mild decrease, reflecting that the two genes, despite sharing the same HRE, behave distinctly [91]. To date, all published hypoxia-inducible constructs in use are based exclusively on multimers of naturally occurring (endogenous) HREs. Apparently, the progress towards artificial, optimized HREs has been hampered by two major factors: the lack of conservation in the flanking sequences [56] and the report that concatamers of individual regulatory elements were not significantly activated by hypoxia [64]. However, further systematic studies should improve our understanding of the minimal structural requirements of HRE and facilitate the design of new hypoxic enhancers.

Regulation of target genes by HIF-1 α and HIF-2 α

The existence of three members within the HIF- α family, HIF-1 α , HIF-2 α (also called EPAS1, MOP2 or HLF), and HIF-3 α , raises questions about the role of individual isoforms in regulation of hypoxic transcription. All α subunits exhibit high conservation at the protein level, domain structure, and hypoxia-dependent mechanisms of regulation, they heterodimerize with HIF-1 β and bind to the same cis-element – HBS [7]. Yet, despite similar biochemical properties, distinct patterns of cellular expression appear to be responsible for distinct physiological roles of HIF-1 α and HIF-2 α [7]. For instance, both isoforms are abundantly expressed in the kidney but in different types of cells: HIF-1 α is predominantly expressed in epithelial cells whereas HIF-2 α is predominantly detected in interstitial fibroblast and endothelial cells [92]. The fact that neither *HIF-1 α ^{-/-}* nor *HIF-2 α ^{-/-}* embryos can survive suggests that HIF-1 α and HIF-2 α are functionally non-redundant and unable to functionally complement each other [7]. Inactivation of HIF-1 α or HIF-2 α by siRNA elicited remarkably different cell-specific effects: hypoxia-inducible gene expression was critically dependent on HIF-1 α in endothelial and breast cancer cells whereas in renal carcinoma cells it was critically dependent on HIF-2 α [93]. HIF- α isoforms displayed unexpected suppressive interactions in renal cell carcinoma, where up-regulation of HIF-2 α suppressed HIF-1 α and vice-versa [94].

Several studies have suggested that HIF-1 α and HIF-2 α differ in their capability to transactivate hypoxia-inducible genes. An earlier comparative study found that some genes were transactivated exclusively by HIF-1 α (notably genes coding for glycolytic enzymes), some genes were transactivated by both isoforms but no genes were transactivated only by HIF-2 α [95]. In a different study, using siRNA, a small group of genes was found to be preferentially regulated by HIF-2 α . Promoter analysis revealed that these genes have binding sites for the ETS family of transcription factors in common, and knock-down of ELK-1, the most abundant member of ETS family, significantly reduced hypoxic induction of the HIF-2 α – dependent genes [96]. It has been reported that the VEGF receptor *Flk-1* is apparently regulated specifically by HIF-2 α but the comparison of the *Flk-1* HBS with HBSs of HIF-1 α target genes revealed no obvious difference [97]. Intriguingly, despite the fact that HIF-1 was originally identified by affinity purification with *EPO* HRE [98], recent studies indicated that HIF-2 is

the physiological regulator of EPO production [99,100]. This notion is further supported by a linkage of familial erythrocytosis with a gain-of-function mutation in *HIF-2 α* [101].

In summary, the available data support the notion that, of the two HIF- α isoforms, HIF-2 α is the more selective activator of hypoxia-inducible genes. However, the question how HIF-1 and HIF-2 discriminate between the target genes is far from settled. The observed significant difference in the magnitude and/or specificity of activation of target genes does not appear to depend on the sequence of the relevant HBS. Instead, these differences could be accounted for by preferential cooperation of one of the isoforms with certain subsets of transcription factors, coactivators or corepressors, and/or tissue-specific expression of HIF- α isoforms [56].

Conclusions and perspectives

In this review we have summarized the key steps involved in the transcriptional response to hypoxia. This process begins with activation of the HIF pathway that is mediated by two molecular switches controlling the abundance of the α subunits and their transcriptional activity. In most cases these two switches are coregulated, however, there are situations when they become uncoupled, thus providing a means for differential modulation of HIF activity by a variety of physiological and pharmacological stimuli. The main task of the HIF pathway is to increase production of the functionally diverse proteins catering for the specific needs of hypoxic cells. Transcriptional induction of genes coding for these proteins is mediated through HREs. The structure of these HREs primarily defines how much of the HIF signal is integrated into the transcriptional output of individual genes. However, the transcriptional output will also depend on the availability of other transcriptional factors cooperating with HIF in the context of the HRE. As HIF- α isoforms differ in their transactivation properties, their individual levels of abundance can also affect the transcriptional response.

Overall, considerable progress has been made in deciphering how the hypoxic signal is transformed into transcriptional response. However, we certainly do not have a complete understanding of the whole process. It is likely that further study of the processes that uncouple stabilization and transcriptional activation of HIF- α will lead to new concepts with significant implications for human pathogenesis. With respect to the hypoxic transcriptome, the majority of the genes that are strongly transcriptionally activated by hypoxia may have already been identified. However, it is likely that not all of the genes that are activated weakly or in a cell-type specific manner have been identified. Another complicating, but immensely interesting facet of HIF activity, is the possibility that expression of a specific group of small non-coding regulatory RNAs may be regulated by HIFs, thus extending the repertoire of HIF targets beyond translated genes [102]. The completion of the hypoxic transcriptome would be greatly aided by the progress in our understanding of HREs that would in turn allow the development of algorithms for predicting new HREs. In the category of HRE-related problems is whether the “optimal” HRE exists. Most likely, the answer to this question is yes but it may not be present in mammalian genomes. Design of these “optimal” HREs would also require improved knowledge of the HRE structure-activity. Another outstanding question is how individual HIF- α isoforms discriminate between their transcriptional targets, to what extent this discrimination is brought about by cis-acting elements (structure of HREs and the flanking sequences) and which domain(s) of HIF- α isoforms are implicated.

Finding answers to the outlined problems will not only significantly increase our knowledge of hypoxic regulation but also provide tools for more efficient therapeutic intervention in hypoxic processes occurring in patho-physiological conditions.

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Abbreviations

bHLH

basic loop-helix-loop

BTM

basic transcriptional machinery

CAIX

	carbonic anhydrase IX
CAD	C-terminal activation domain
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EPO	erythropoietin
GAPDH	glyceraldehyde phosphate dehydrogenase
HAS	HIF-1 ancillary sequence
HBS	HIF-binding site
HRE	hypoxia-responsive element
FIH-1	factor inhibiting HIF-1
LDHA	lactate dehydrogenase A
NAD	N-terminal activation domain
NLS	nuclear localization signal
ODDD	O ₂ –dependent degradation domain
2OG	2-oxoglutarate
PAS	PER-ARNT-SIM
PHDs	prolyl hydroxylase domain proteins
PGK-1	phosphoglycerate kinase 1
PI3K	phosphatidylinositol-3-kinase

Ub	ubiquitin
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau protein

Figure 1. HIF-1α: schematic outline of the domain structure, function of individual domains, and the sites of post-translational modifications critically affecting its function

bHLH, basic loop-helix-loop; CAD, C-terminal activation domain; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF 1; NAD, N-terminal activation domain; NLS, nuclear localization signal; ODDD, O₂ – dependent degradation domain; PAS, PER-ARNT-SIM; PHDs, prolyl hydroxylases.

Figure 2. The outline of regulation of stability and transcriptional activity of HIF- α

In the presence of O_2 and cofactors Fe^{2+} and 2OG, PHDs hydroxylate HIF- α , allowing its recognition by the E3 complex that is followed by E2/E1-mediated ubiquitylation and degradation in the 26S proteasome. FIH-1-mediated N-hydroxylation prevents recruitment of p300/CBP transcriptional coactivators. In the absence of O_2 , PHDs and FIH-1 are inactivated, non-hydroxylated HIF- α translocates to the nucleus, dimerizes with HIF- β , recruits p300/CBP, and induces the expression of its target genes via binding to the HRE. BTM, basic transcriptional machinery; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF-1; HRE, hypoxia-response element; 2OG, 2-oxoglutarate; PHDs, prolyl hydroxylases; Ub, ubiquitin; VHL, von Hippel-Lindau protein.

Figure 3.

(A) Numbering of the nucleotide positions in HBS (the core sequence in bold); **(B)**. Schematic outline of HBS and HAS in the *EPO* HRE; **(C)** Schematic outline of the two fundamental arrangements of HBSs present in the *mLDHA* (antiparallel) and *mPGK-1* (parallel) HRE. Arrows indicate the orientation of HBSs.

Table 1

Selected HIF target genes grouped according to their function

Group	Gene	Protein function	Ref.
O ₂ supply	<i>transferrin</i>	iron transport	57
	<i>transferrin receptor</i>	iron transport	58
	<i>VEGF</i>	angiogenesis	59
	<i>Flt-1</i> (VEGF receptor)	angiogenesis	60
	<i>plasminogen activator inhibitor 1</i>	blood flow	61
	<i>erythropoietin</i>	erythropoiesis	62
cellular metabolism	<i>enolase 1</i>	Glycolysis	63
	<i>lactate dehydrogenase A</i>	Glycolysis	64
	<i>phosphoglycerate kinase 1</i>	Glycolysis	63
	<i>GAPDH</i>	Glycolysis	65
	<i>phosphofructokinase L</i>	Glycolysis	63
	<i>glucose transporter 1</i>	glucose uptake	66
	<i>carbonic anhydrase 9</i>	pH regulation	67
transcription	<i>MDR1</i>	xenobiotic metabolism	68
	<i>DEC 1 and 2</i>	transcription factors	69
	<i>ETS-1</i>	transcription factor	70
	<i>ID2</i>	transcriptional repressor	71
apoptosis	<i>CITED2/p35sri</i>	transcriptional cofactor	33
	<i>NIP3</i>	pro-apoptotic	72
	<i>BNIP3</i>	pro-apoptotic	73
	<i>Noxa</i>	pro-apoptotic	74
other	<i>Mcl-1</i>	anti-apoptotic	75
	<i>PHD2</i>	O ₂ sensing	76
	<i>PHD3</i>	O ₂ sensing	77