REVIEW

Hypoxia-Inducible Factor (HIF)-1 Regulatory Pathway and its Potential for Therapeutic Intervention in Malignancy and Ischemia

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Hypoxia-Inducible Factor (HIF)-1\textsuperscript{†} is a dimeric protein complex that plays an integral role in the body’s response to low oxygen concentrations, or hypoxia. HIF-1 is among the primary genes involved in the homeostatic process, which can increase vascularization in hypoxic areas such as localized ischemia and tumors. It is a transcription factor for dozens of target genes; HIF-1 is also essential for immunological responses and is a crucial physiological regulator of homeostasis, vascularization, and anaerobic metabolism. Furthermore, HIF-1 is increasingly studied because of its perceived therapeutic potential. As it causes angiogenesis, enhancement of this gene within ischemic patients could promote the vessel proliferation needed for oxygenation. In contrast, as HIF-1 allows for survival and proliferation of cancerous cells due to its angiogenic properties, inhibition potentially could prevent the spread of cancer. With a growing understanding of the HIF-1 pathway, the inhibition and stimulation of its transcriptional activity via small molecules is now an attractive goal. Gene therapy to achieve both vessel proliferation and tumor regression has been demonstrated in animal studies but requires significant improvement and modification before becoming commercially available. This review focuses on the potential of the HIF-1 pathway in therapeutic intervention for the treatment of diseases such as cancer and ischemia.

OXYGEN METABOLISM IN MAMMALS

Oxygen is required by the cells of most organisms to produce adequate amounts of ATP necessary for metabolic activities. Hypoxia, or oxygen deprivation, occurs within human tissues and cells due to a variety of conditions, including disorders of the heart and lungs, anemia, and circulatory problems. Depending on the severity, permanent damage to tissues and cells may occur [1].

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\†Abbreviations: HIF-1, Hypoxia-Inducible Factor; PAS, Per-ARNT-Sim; ODD, oxygen-dependent degradation; PHD-2, proline-hydroxylase-2; TAD, transactivation domains; CBP, CREB binding protein; C-TAD, carboxy-terminal transactivation domain; ARNT, aryl hydrocarbon receptor nuclear translocator; VEGF, vascular endothelial growth factor; FIH, factor Inhibiting HIF-1; VHL, von-Hippel-Lindau; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MMP2, matrix metalloproteinase 2; CATHD, cathepsin D; KRT, keratin; AAV, adeno-associated virus; 2OG, 2-oxoglutarate; CH1, calponin homology 1.
However, hypoxia also can play an important and beneficial role in human physiology and development. It is integral for proper embryonic development. Although the exact mechanisms are unknown, oxygen tension is related to closure of the neural tube, mediation of apoptosis, and proper morphological development during gestation. Such findings indicate that in addition to genetic cues, environmental conditions such as hypoxia serve as signals in embryonic development [2,3,4].

Many organisms have evolved adaptive mechanisms for hypoxic conditions. Changing oxygen levels can result in activation or repression of certain homeostatic regulatory genes, allowing for the survival of tissues and cells despite fluctuating environmental conditions. Genes such as HIF-1, whose activation is prompted by hypoxic conditions, can interact with enzymes and other transcription factors in order to control vascularization and tissue growth. While microenvironments surrounding cancerous tumors are extremely hypoxic, proliferation of such masses often is made possible by HIF-1 activation, which leads to increased angiogenesis and, thus, an increased oxygen supply to the area [5,6].

Given its prominent function, manipulation of HIF-1 activity within areas of ischemia and tumor masses has become a focus in the effort to develop noninvasive, pharmaceutical treatment options for cancer and heart disease patients. Although no such human protein has been successfully regulated by scientific means, control of HIF-1 activity is increasingly feasible as details of its structure, function, and genetic pathway are elucidated.

**HIF-1 DOMAIN STRUCTURE**

HIF-1 is a heterodimeric transcription factor consisting of a constitutively expressed β-subunit and an oxygen-regulated α-subunit. The HIF-1α and HIF-1β proteins both contain basic helix-loop-helix motifs that bind DNA and cause subunit dimerization [7,8,9]. Both subunits also have a Per-ARNT-Sim (PAS) domain, with similar functions. In the α-subunit, there is an oxygen-dependent degradation (ODD) domain, which is hydroxylated by proline-hydroxylase-2 (PHD-2), rendering the α-subunit vulnerable to proteasomal degradation under normoxic cellular conditions [10]. The structure of HIF-1α and HIF-1β is depicted in Figure 1.

The HIF-1α subunit also contains two transactivation domains (TAD), which regulate HIF-1 target genes. CREB binding protein (CBP) and p300, two transcriptional co-activators of HIF-1, interact with the carboxy-terminal transactivation domain (C-TAD) of HIF-1α.

Both activators are essential for HIF-1 transcription and are therefore targets in the effort to regulate HIF-1 expression; inhibition of HIF-1α C-TAD interactions by proline hydroxylation inhibits HIF-1 gene expression, preventing normal transcription and translation [11]. HIF-1β contains only one such analogous region, which is unnecessary for HIF-1 complex function [7,10,12]. Recent reports show that HIF-1β is identical to the previously discovered vertebrate protein, aryl hydrocarbon receptor nuclear translocator (ARNT) [12].
HIF-1 REGULATES OXYGEN HOMEOSTASIS

HIF-1 is a major regulator of oxygen homeostasis within cells. As a transcription factor, it affects and regulates the expression of dozens of genes involved in maintaining homeostasis as oxygen concentrations change [13]. HIF-1 further mediates cellular responses to hypoxia by regulating glucose uptake and anaerobic respiration in oxygen-depleted environments [5,2].

Transcriptional Regulation Controls Angiogenesis in Hypoxia

One important HIF-1 function is to promote angiogenesis; HIF-1 directs migration of mature endothelial cells toward a hypoxic environment [2,5]. This is done via HIF-1 regulation of vascular endothelial growth factor (VEGF) transcription. VEGF is a major regulator of angiogenesis, which promotes endothelial cell migration toward a hypoxic area. During hypoxia, HIF-1 binds the regulatory region of the VEGF gene, inducing its transcription and initiating its expression [12,15,16]. Such endothelial cells ultimately help to form new blood vessels, supplying the given area with oxygenated blood [14].

HIF-1 Regulates Shift to Anaerobic Metabolism

HIF-1 also can regulate anaerobic metabolism. When oxygen is available, most cells produce ATP via oxidative phosphorylation. However, in hypoxic environments, there is a shift to anaerobic metabolism for cellular energy production. HIF-1 is among the principal genes to coordinate this shift, by inducing a variety of glycolytic enzymes and glucose transporters such as aldolase A and pyruvate kinase M, which help cells efficiently produce energy in hypoxic environments [5,16]. In addition to increasing the expression of these enzymes, HIF-1 decreases mitochondrial oxygen consumption by activating pyruvate dehydrogenase kinase I and halting the citric acid cycle [17].

CANCER, INFLAMMATION, AND HYPOXIA

Environments surrounding metastasizing tumor masses are often hypoxic. HIF-1 is a crucial protein in such masses; it enables tumor progression by inducing alternative metabolic pathways within cancer cells, as discussed above in the context of physiologic hypoxia.

Tumor Proliferation

Due to its role in hypoxia, HIF-1 plays a critical part in tumor proliferation [18]. As a tumor develops and grows, a hypoxic environment is created because of the extreme energy demands of the numerous, rapidly dividing cells. Angiogenesis is often induced by such cellular masses to meet the needs for increased oxygen, energy, and blood supplies [5,16]. Concurrently, HIF-1 contributes to the shift to anaerobic metabolism. The importance of this transcription factor in tumor cell survival is reflected in the finding that the levels of HIF-1α in glioma tumor cells increase proportionally with the grade of the tumor [19].

The mechanisms of HIF-1 mediated tumor survival have been partially revealed by the work of Semenza et al. on VHL-lacking renal carcinoma cells. HIF-1 was found to decrease oxygen consumption in these cells by inhibiting C-MYC, a transcription factor that regulates mitochondrial mass and oxygen consumption and is known to be down-regulated in a variety of human cancers. Semenza et al. report that HIF-1 decreases levels of C-MYC by increasing the transcription of MXI1, a repressor of C-MYC, and by increasing the rates of proteosome degradation of the C-MYC protein. The decreased levels of C-MYC in these cancer cells were found ultimately to lead to increased glycolysis and decreased mitochondrial respiration, crucial characteristics of cancer cells that survive and proliferate in the hypoxic conditions of the tumor microenvironment [20].

Overexpression of HIF-1 Causes Apoptosis

A multitude of studies currently are being conducted on the role of HIF-1 in hy-
poxia-induced apoptosis of various cell types. For example, Kricket al. recently reported that overexpression of HIF-1 in alveolar epithelial cells resulted in increased apoptosis [21]. Although the exact pathways and mechanisms involved in this process remain unclear, data suggest that in hypoxic conditions the tumor suppressor p53 is activated. Via interaction with the HIF-1 protein, p53 is stabilized and begins to activate genes such as p21, which, in turn, cause cell death [5,21].

**HIF-1 Supports Inflammatory Responses and Hypoxic Recovery**

In addition to its other roles in adaptation to hypoxia, HIF-1 has been shown to play a role in inflammation. Cramer and coworkers demonstrated that HIF-1 is necessary for metabolism within myeloid cells [22]. Overexpression of HIF-1 in vivo resulted in increased localized inflammation, while loss of the HIF-1 gene decreased the ability of myeloid cells to aggregate, migrate, and promote bactericidal responses. This dependence of myeloid cells on HIF-1 may be related to their reliance on anaerobic respiration as a means of energy production. Myeloid cells lacking this gene are unable to efficiently produce ATP, effectively migrate to injured tissues, or destroy foreign invaders [22]. In addition, HIF-1α expression plays a role in the differentiation of myeloid cells into monocytes and macrophages [23].

In contrast, HIF-1 can function to prevent tissue and cardiac damage caused by ischemia, which may result in a variety of long-term cardiac problems. Overexpression of HIF-1 in such tissues can cause angiogenesis and thus increase oxygenation of the area [24,25]. This serves as the basis for the cur-
rent efforts to find pharmaceutical and other non-invasive treatments for ischemia and related diseases.

**ACTIVATION AND SUPPRESSION PATHWAYS**

**Normoxia Causes Degradation; Hypoxia Allows Activation**

Under normoxic conditions, HIF-1α is degraded by proteasomes. The HIF-1α subunit is “marked” for such degradation by proline-hydroxylase-2 (PHD-2) and by von-Hippel-Lindau (VHL)-ubiquitin ligase complexes. Therefore, HIF-1 does not function in the presence of sufficient oxygen [10,26]. Also contributing to HIF-1 inactivation in normoxic conditions is Factor Inhibiting HIF-1 (FIH) protein, which hydroxylates HIF-1, preventing interaction of this subunit with the co-activators p300 and CBP. Expression and stabilization of the HIF-1 complex is regulated via feedback inhibition, as PHD-2 itself is activated by HIF-1 [12].

However, in hypoxic conditions, HIF-1 protein is stable and active as hydroxylases, VHL proteins, and FIH are all inhibited by a lack of oxygen. Then HIF-1 is able to interact with its co-activators and can dimerize with its constitutively expressed β-subunit. Once stabilized, the HIF-1 protein can bind to the regulatory regions of its target genes, inducing their expression [7,10,27] (Figure 2).

**Oxygen-Independent Stimuli**

A variety of HIF-1 stimuli function independently of oxygen concentration. These stimuli are primarily proteins, which regulate HIF-1 *transcription*, contrasting sharply with the hypoxic stimuli of this gene, which act upon the already expressed α-subunit. Protein kinase C (PKC) increases the rate of HIF-1α transcription of and functions in conjunction with the phosphatidylinositol 3-kinase (PI3K) pathway, which also enhances HIF-1α translation. The PKC pathway activates expression of the S6 ribosomal protein, which specifically recognizes mRNA transcripts such as HIF-1α. Via phosphorylation of the S6 protein in normoxic conditions, the rates of HIF-1α mRNA translation can be greatly increased, effectively countering the effects of the proteasome degradation of this subunit and increasing levels of the HIF-1 complex within the cell. The PI3K pathway has been identified as the primary means by which various mediators, such as lipopolysaccharides, affect activation of HIF-1α in vascular smooth muscle cells and macrophages [12,27].

**THERAPEUTIC TARGETS IN THE HIF-1 PATHWAY: ISCHEMIA**

**Overexpression**

In the case of ischemia treatments, HIF-1α upregulation may stimulate angiogenesis and increase blood flow. Many genes involved in angiogenesis, such as VEGF, matrix metalloproteinase 2 (MMP2), cathepsin D (CATHD), and keratin (KRT), are targets of the HIF-1 transcription complex. It is believed increased HIF-1 levels lead to proportional increases in these proteins [12,28]. In several recent studies, mice injected with HIF-1α DNA lacking an ODDD displayed increased blood supply to wounded or ischemic areas, suggesting increases in HIF-1α levels can aid in supply of blood, oxygen, and nutrients to areas of focal ischemia [29,30].

Introduction of a constitutively stable HIF-1α hybrid to rat cardiomyocytes resulted in decreased ischemic damage. This hybrid consisted of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain of HSV VP16 protein [31]. HIF-1α overexpression in mouse myocardial infarction models reduces infarct size, thereby preserving cardiac function [32]. Increasing HIF-1 expression may prove to be a successful pharmaceutical treatment for ischemic patients on whom surgery cannot be performed.

**Direct HIF-1 Modifications**

Direct phosphorylation of the HIF-1α subunit itself can increase HIF-1 activity, presumably by impeding proteasome/VHL recognition. Although very little is known
about phosphorylation of HIF-1α, p42/p44 mitogen activated protein kinases phosphorylate this protein in vitro. In vivo, such phosphorylation is necessary for HIF-1 function. Activation of the p42/p44 pathway results in increased levels of HIF-1α transcription. This phosphorylation may be an optimal step in the HIF-1 pathway to induce overexpression [33].

The HIF-1 hydroxylases consist of several related molecules, including Factor Inhibiting HIF (FIH) and Prolyl Hydroxylase Domain (PHD) proteins. As VHL mediates proteasome degradation of hydroxylated HIF-1α, the levels of HIF-1α can be increased by silencing HIF-1alpha-prolyl-4 hydroxylase-2 (PHD2). Inhibition of PHD2 via siRNA also results in a decrease in the cardiac infarction size in mice. These pathways may be amenable to pharmacological approaches [34].

Small Molecule Inhibitors

Several small molecules, such as dimethyl oxalylglycine, a prolyl hydroxylase inhibitor, activate HIF-1. Hydroxylase activity can be rescued by mutating specific regions or by adding cobalt ions to the cell, which presumably compete for iron binding sites [35]. Some hydroxylases in the prolyl family can be selectively inhibited by Adriamycin in vitro [36]. Cobalt (II) and Nickel (II) ions in cells increase HIF-1 activity, presumably as such ions displace the iron from the active sites of 2OG hydroxylases.

Small molecule therapy may be useful not only in HIF-1 suppression, but also in its activation for the treatment of ischemic diseases [7]. Hormones such as angiotensin II and platelet-derived growth factor stimulate the HIF pathway by increasing HIF-1α protein levels via production of reactive oxygen species (ROS) within the cell. Although the exact mechanism is unclear, it appears to be entirely distinct from the hypoxia pathways. Thrombin and other growth factors increase angiogenesis via HIF-1α protein agonist mechanisms [14,33]. Insulin likewise activates HIF-1α by activating multiple protein kinases necessary for expression and function [37].

In another study on HIF-1 activation, homozygous deletion of the p53 gene led to HIF-1 activation [38]. Therefore, p53, responsible for promoting ubiquitination of HIF-1α, may be another possible target.

Gene therapy eventually may be used to increase HIF-1 levels and relieve complications of ischemia. For example, delivery of a stabilized, recombinant form of HIF-1α via adeno-associated virus (AAV) in order to overexpress HIF-1 in skeletal muscle resulted in significantly increased capillary numbers [38,39]. While gene therapy approaches aimed at the process and effects of angiogenesis continue to be developed and studied, higher levels of success in preclinical trials currently are being sought before clinical applications are pursued. Among the most prominent of the remaining obstacles in gene therapy is the mode of delivery [38]. The search for the most efficient delivery vector continues.

THERAPEUTIC TARGETS IN THE HIF-1 PATHWAY: CANCER

With a clearer understanding of the HIF-1 pathway, efforts are directed at manipulation of this complex genetic process in order to ultimately increase or decrease cellular HIF-1 levels. Although a human transcription factor has yet to be successfully controlled through external manipulation, such a goal remains attractive as it may lead to non-invasive treatment methods for diseases such as cancer and ischemia. Optimal points in the HIF-1 pathway for such therapeutic intervention are currently under investigation.

The nature of cancer growth and metastasis provides multiple phases at which therapeutic intervention is possible. As HIF-1 has been found to regulate the shift within the tumor cells to anaerobic metabolism and to activate VEGF and angiogenesis, downregulation of the HIF-1 complex may suppress cancer progression. Among the methods considered for HIF-1 downregulation is the activation of hydroxylases, which target the HIF-1 complex for eventual degradation. Such hydroxylases are members of
the 2-oxoglutarate (2OG)-dependent oxygenase superfamily and require a ferrous ion at the active site. Although the exact mechanism remains unknown, HIF-1α degradation is increased by treating cells with iron and ascorbate, which most likely decrease levels of HIF-1 protein and of HIF-target genes by activating the hydroxylases [40].

Specific Therapeutic Targets: Proteins

HIF-1α binding to its co-activators has also been a target for HIF-1 inhibition. The CAD of HIF-1 must bind to the calponin homology 1 (CH1) domains of the co-activators CBP and p300 proteins to initiate transcription [41,42,43]. When excess HIF-1α-CAD polypeptides are transfected into cells, the expression of hypoxia-inducible reporters decreases [41,42]. These peptides compete with HIF-1α for the CBP site, disrupting the interaction, which is crucial for downstream effects.

Stephen et al. have shown that p300/CBP interaction with HIF-1α can be prevented via stimulation of the CITED4 protein. CITED4 is a transcription factor that binds to the CH1 domain of the p300/CBP co-activators, thus competing with co-activators for HIF-1α. In this study, it was demonstrated that HIF-1α levels decrease with stimulation of CITED4 expression and tumor cells are capable of decreasing the levels of CITED4, leading to an increase in the amount of HIF-1α [33].

Alteration of specific HIF-1α amino acid residue oxidation-reduction status can suppress HIF-1 expression. Pleurotin and PX-12 inhibit thioredoxin-1, which reduces a residue in the C-TAD of HIF-1α, in turn increasing the effectiveness of HIF-1 as a transcription factor. Such inhibitors simultaneously decrease levels of VEGF within the cell, as well as angiogenesis in tumors by a mechanism unrelated to HIF-1 inhibition [28]. The underlying mechanisms of these effects remain unclear [44].

Specific Therapeutic Targets: Small Molecules/RNA

Several small molecule inhibitors of HIF-1 activity have been identified and are currently being studied. Topotecan, a topoisomerase inhibitor, decreases cellular accumulation of HIF-1α [31]; it may alter the ribosome entry site on the HIF-1α mRNA molecule, preventing translation [44,45]. 103D5R is another small molecule that recently has been found to have HIF-1α inhibiting properties. The molecular mechanisms and pathways behind the repression of HIF-1α by 103D5R are also unknown, but it may have an action similar to topotecan [42]. However, only in vitro studies of 103D5R have as yet been completed.

HIF-1-HIF-1α inhibition via gene therapy has been investigated using both siRNA and antisense RNA in various cell types. Jensen et al. transfected glioma cells with HIF-1α siRNA, which decreased cell growth both in vivo and in vitro [46]. Similar results were obtained by Sun et al.; plasmid delivery of antisense HIF-1α led to decreased microvessel densities in EL-4 tumors in mice [47]. Chang et al. recently demonstrated that transfection of BxPc-3 cells with antisense HIF-1α results in decreased progression and metastasis of pancreatic cancer [48]. Although such results have yet to be replicated in humans, they have significant implications for the future of cancer treatment. While less studied, the VHL protein, which marks HIF-1α for degradation, is another potential therapeutic target. The VHL tumor suppressor gene becomes inactivated in many types of carcinomas, which causes increased HIF-1α levels and allows for survival in hypoxia. As the promoter regions in VHL in which transcription factors bind now have been identified, silencing may one day be possible [49].

CONCLUSIONS

As more is learned about functions, target genes, and activation pathways of HIF-1, novel treatments of diseases such as cancer and ischemia can be developed. Despite limited success in mouse ischemic models and human clinical trials, significant advances in manipulating HIF-1 expression continue to be made [39]. Such results further stress the need to target the HIF-1 path-
way directly, as opposed to merely its effects, to truly develop efficient pharmaceutical therapies. In this respect, advances in gene therapy may allow for a greater ability to directly influence intracellular HIF-1 levels.

In the search to find a non-invasive treatment for ischemia, it appears inhibition of 2OG hydroxylases via small molecule therapy is the most promising way to stimulate HIF-1 activity, assuming that selective suppression of such hydroxylases ultimately can be achieved. Selective inhibition of collagen prolyl hydroxylases [7,12] and in clinical trials of a 2OG-related enzyme, cyclooxygenase II, have been successful [50]. Suppression of HIF-1 in the treatment of cancer is also a focus, as the significant role of this transcription complex in tumor progression is now recognized [15,16,19].

The entire spectrum of HIF-1 function is not yet understood. Accordingly, regulation of transcription factor activation in humans has not yet been achieved. That the various HIF-1α hydroxylases affect and regulate levels of this transcription factor differently only recently was described. Much remains to learn about the selectivity and properties of factors influencing the HIF-1 pathway [51]. Although current efforts to manipulate the HIF-1 pathway have been principally centered on the need for treatments of cancer and ischemia, it has been suggested that HIF-1α may play a role in the onset of pulmonary fibrosis [52]. As the molecular mechanisms leading to the onset of this disease remain unknown, HIF-1α inactivation in respiratory cells may be a fruitful line of inquiry. Despite such gray areas in the knowledge of HIF-1, control of this transcription factor does in fact remain a high-priority target. Modulation of the HIF-1 pathway promises to have significant effects on cancer and cardiac treatments, potentially affecting the lives of millions who are afflicted with such diseases.

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