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■ REVIEW ■

Oxygen-Dependent and -Independent Regulation of HIF-1alpha

Hypoxia-inducible factor-1 (HIF-1) is composed of HIF-1alpha and HIF-1beta, and is a master regulator of oxygen homeostasis, playing critical roles in physiological and pathological processes. Normally, the formation and transcriptional activity of HIF-1 depend on the amount of HIF-1alpha, and the expression of HIF-1alpha is tightly controlled by the cellular oxygen tension. Recent progress in the study of its regulation mechanism provided clues as to how HIF-1alpha is regulated by oxygen. It appears that HIF-1alpha is not regulated only by the oxygen tension, but also by various other stimuli, such as transition metals, nitric oxide, reactive oxygen species, growth factors, and mechanical stresses. In this review, we summarize the oxygen-dependent and -independent regulation of HIF-1alpha, and the respective physiological and pathological meanings.

Key Words : *HIF1alpha; Arnt; Cell Hypoxia*

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INTRODUCTION

Oxygen is essentially required by the aerobic metabolisms of most eukaryotic organisms. It functions as an electron sink to be reduced by four electrons and produce water in the final step of oxidative phosphorylation. Thus organisms and cells have developed numerous adaptive mechanisms that enable them to survive in oxygen-depleted conditions. At the organism level, hypoxic adaptation includes reflex hyperventilation, increased production of red blood cells, and new vessel formation, which in combination lead to increased oxygen delivery from the atmosphere to the tissues (1). At the cellular level, the adaptation involves a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis, increased glucose uptake, and the expression of stress proteins related to cell survival or death (1, 2). The regulation of most proteins required for hypoxic adaptation occurs at the gene level, which involves transcriptional induction via the binding of a transcription factor, hypoxia-inducible factor-1 (HIF-1), to the hypoxia response element (HRE) on the regulated genes (2, 3) (Fig. 1).

This review aims to summarize our current knowledge of the regulation of HIF-1.

DISCOVERY OF HIF-1

Before HIF-1 was found, HRE had been identified in the 3′-enhancer region of the erythropoietin gene, whose transcription is up-regulated by more than 100-fold by severe hypoxia (4-6). Semenza and Wang (7) defined a binding site critical for the hypoxia-inducible function, which involves a transcription factor induced by hypoxia. Subsequently, they purified a DNA-binding complex bound to the HRE by affinity-purification using oligonucleotide with the HRE sequence (8), and thus identified the encoding cDNAs (9). HIF-1 was found to be a heterodimer composed of two basic-helix-loop-helix (bHLH) proteins of the PAS family, HIF-1α and HIF-1β. Of these, HIF-1β had previously been identified as the aryl hydrocarbon nuclear receptor translocator (ARNT), which is dimerized with the aryl hydrocarbon receptor. However, HIF-1α was a newly defined protein and uniquely associated with the transcription of the hypoxia-inducible genes. Later, homology searches in the gene bank and cloning experiments found other members of this family, such as HIF-2α (also known as endothelial PAS protein-1) (10, 11) and HIF-3α (12). HIF-2α is also tightly regulated by oxygen tension and its complex with HIF-1β appears to be directly involved in hypoxic gene regulation, as is HIF-1α (13). However, although HIF-3α is homologous to HIF-1α, it might be a negative regulator of hypoxia-inducible gene expression (14).

STRUCTURE OF HIF-1α

HIF-1α is an 826-amino acid protein, as shown in Fig. 2. Its N-terminal half contains the basic domain (aa. 17-30), a helix-loop-helix domain (aa. 31-71), and a PAS domain (aa. 85-298), which are required for dimerization with HIF-1β and binding to the HRE DNA core recognition sequence

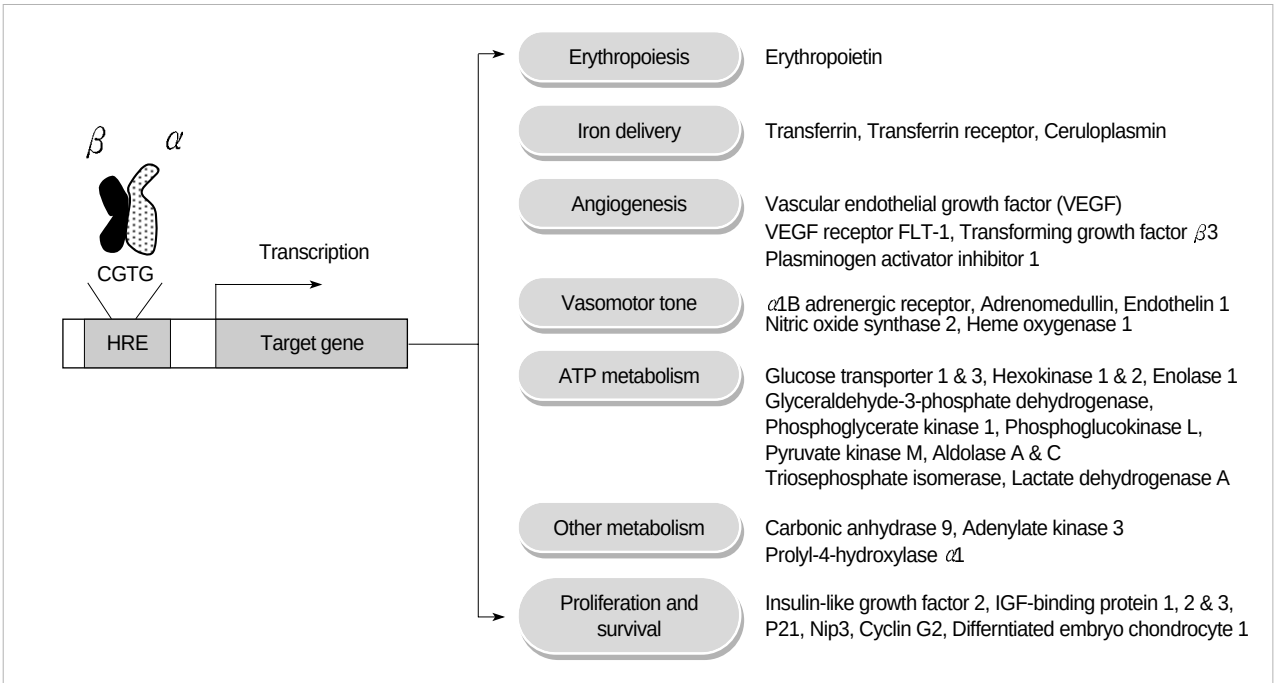

Fig. 1. Identified HIF-1 target genes. Abbreviations: α, HIF-1α; β, HIF-1β; HRE, hypoxia response *cis*-element.

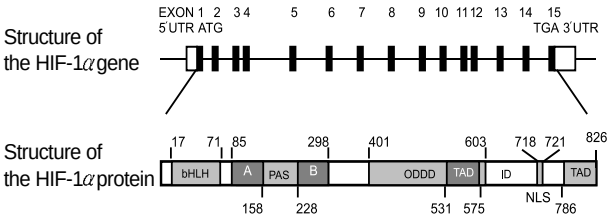

Fig. 2. Structure of the HIF-1α gene and protein. The HIF-1α gene is composed of 15 exons and 14 introns. The protein possesses basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain, which are involved in DNA binding and dimerization with HIF-1β. Its C-terminal part contains two transacting domains (TAD), an inhibitor domain (ID), and a nuclear localization signal (NLS). The oxygen-dependent degradation domain (ODDD) contains two subdomains targeted by the von Hippel-Lindau protein (pVHL) at its N-terminal and C-terminal ends.

(5′-RCGTG-3′). The PAS domain is divided into two subdomains, PAS-A (aa. 85-158) and PAS-B (aa. 228-298) (9). The C-terminal half of HIF-1α is required for transactivation. The transactivation domains (TADs) are localized to aa. 531-575 (N-terminal TAD) and aa. 786-826 (C-terminal TAD), which are separated by an inhibitory domain (15, 16). Nuclear localization signals (NLSs) are localized at N-terminal (aa. 17-74) and C-terminal (aa. 718-721) of HIF-1α (17). The C-terminal NLS motif plays a critical role in mediating hypoxia-inducible nuclear import of HIF-1α, whereas the N-terminal one may be less important. Moreover, the C-terminal half contains two PEST-like motifs at aa. 499-518 and 581-600 (9). The PEST motif contains a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T). Since this motif has been found in many proteins with half-lives of less than 2 hr, proteins containing the PEST motif tend to be targets for rapid intracellular degradation. HIF-1α is also a very unstable protein with a short half-life less than 10 min under normoxic conditions. Salceda and Caro (18) first revealed that HIF-1α is ubiquitinized under normoxic conditions, and then targeted by proteasome. Later, Huang et al. (19) clearly defined the domain responsible for the normoxic destruction of HIF-1α, and designated it the oxygen-dependent degradation domain (ODDD). The ODDD (aa. 401-603) contains PEST-like motifs, and controls HIF-1α degradation by the ubiquitin-proteasome pathway.

OXYGEN-DEPENDENT REGULATION OF HIF-1α

How does the ODDD regulate the ubiquitination of HIF-1α, in an oxygen tension dependent manner? Recently, several leading research groups shed light upon the answer to this question. The mechanisms of oxygen sensing and HIF-1α regulation are summarized in Fig. 3. Maxwell et al. (20) first demonstrated that the von Hippel-Lindau tumor suppressor protein (pVHL) binds directly to HIF-1α oxygen-dependently, and that this leads to the proteolysis of HIF-1α. Later, the role of pVHL in this process was clearly elucidated by biochemical and structural studies. pVHL participates in the ubiquitination of HIF-1α as a part of an E3 ubiquitin ligase protein complex (21, 22), in which the beta-domain of pVHL interacts directly with the ODDD of HIF-1α (23).

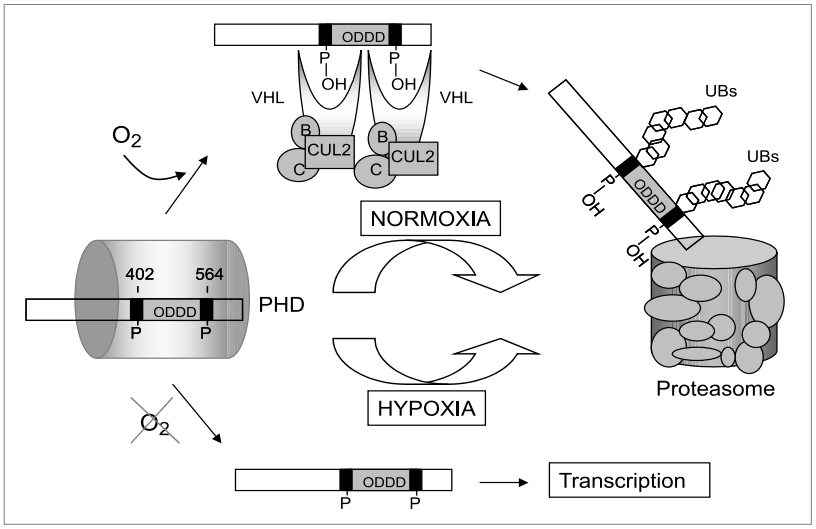

Fig. 3. Oxygen-dependent regulation of HIF-1α. Under normoxic conditions, HIF-1-prolyl hydroxylases (PHD) hydroxylate the prolyl residues at amino acid 402 and 564. These enzymes require dioxygen, Fe^{2+}, ascorbate, and 2-oxoglutarate for activity. The hydroxylated peptides interact with an E3 ubiquitin-protein ligase complex composed of VHL, elongin B&C, and Cullin 2 (CUL2), and then poly-ubiquitinized, resulting in HIF-1α degradation by the 26S proteasome. Under hypoxic conditions, HIF-1α is not hydroxylated because the major substrate, dioxygen, is not available. The unmodified protein escapes the VHL-binding, ubiquitination, and degradation, and then dimerizes HIF-1β and stimulates the transcription of its target genes.

pVHL associates with the peptides containing the ODDD extracted from cells cultured under normoxic conditions, but does not associate with the peptides from cells cultured under hypoxic conditions. However, another important question remains still. What determines the oxygen-dependent interaction between HIF-1α and pVHL? Recent insight comes from the demonstration that the pVHL-dependent ubiquitination of the N-terminal (aa. 390-417) (24) or the C-terminal part (aa. 549-582) (25, 26) within the ODDD is preceded by the hydroxylation of a proline residue (HIF-1α 402 and 564), present in each part. These proline residues are embedded within the amino acid motif LXXLAP, which is conserved in the HIF-1α proteins of other species and HIF-2α. Recently, three isoforms of HIF-1-prolyl hydroxylase (PHD1-3) were found to be able to hydroxylate the motif targeted by pVHL (27, 28). Sequence analyses imply that these PHD enzymes belong to a subfamily distinct from the procollagen prolyl hydroxylases, which hydroxylate proline residues in procollagen and stabilizes collagen. These enzymes use molecular oxygen as a substrate and ferrous ion as a cofactor, and generate carbon dioxide and succinate as by-products (29). Since the activities of these enzymes depend on the concentrations of oxygen and iron, depletion of these molecules might limit the hydroxylation of proline residues in HIF-1α, thereby precluding binding of pVHL to HIF-1α and stabilizing HIF-1α. Thus it presents a good scenario for explaining how HIF-1α is regulated by the level of oxygen or iron.

OXYGEN-INDEPENDENT REGULATION OF HIF-1α

Transition Metals

Transition metal ions, such as cobalt and nickel, stabilize HIF-1α under normoxic conditions, and induce HIF-1 activity and the expression of its downstream hypoxia-inducible genes (13, 30). Previously, cobalt and nickel ions were suggested to substitute for the iron atom in the heme moiety of the putative oxygen sensor protein, thereby locking the protein in its deoxygenated state (31). The presence of the putative oxygen sensor is also supported by experiments showing that carbon monoxide, on binding to heme, suppresses both the hypoxic accumulation of HIF-1α and erythropoietin production (32). Cobalt and nickel ions have been shown to be substrates for ferrochelatase, an enzyme responsible for the incorporation of iron into protoporphyrin IX, and are incorporated into heme (33). Cobalt or nickel protoporphyrin IX binds oxygen with a lower affinity than iron protoporphyrin IX (34) and in turn this poorer binding may mimic low oxygen tension, which results in HIF-1 activation. Besides these metal ions, zinc ion also can replace heme iron, and the resulting zinc protoporphyrin IX has a similarly low affinity to oxygen (35). A recent report demonstrated that zinc stabilizes HIF-1α under normoxic conditions, further supporting this hypothesis (36). However, other hypothesis about the metal ion-mediated induction of HIF-1α could be suggested from recent reports (27, 29). As mentioned in the previous section, PHD can participate in the HIF-1α stabilization by metal ions. Ferrous ion, a cofactor of PHD, is coordinated by two histidine residues and a carboxylated residue in PDH. If this metal binding is not tight, however, other metal ions could substitute for the ferrous ion bound to PDH, and inhibit its enzymatic action, which might stabilize HIF-1α even under normoxic conditions.

Nitric Oxide

Hypoxia stimulates nitric oxide (NO) production through the induction of inducible NOS, the transcription of which is enhanced by HIF-1 (37, 38). Conversely, NO affects the HIF-1-mediated induction of the hypoxia-inducible genes, such as the erythropoietin and vascular endothelial growth

factor genes. However, the effect of NO on HIF-1α expression is reciprocal, and may depend on the chemical structure and the concentration of the NO donor used. The effect of NO on HIF-1 is attributed to two research groups. They showed that NO at relatively high concentrations reduces the hypoxic induction of HIF-1-DNA binding and the transcriptional activity of HIF-1 (39, 40). Subsequently, Huang et al. (32) revealed that NO not only blocks the hypoxic stabilization of HIF-1α but also represses its transcriptional activity. Carbon monoxide, which has a well-known ability to block the induction of the hypoxia-inducible genes, showed similar effects on HIF-1. Based on this effect of NO, Chun et al. (41) found a new inhibitor of HIF-1α, YC-1, which also suppresses HIF-1 activity and the hypoxic induction of the erythropoietin and vascular endothelial growth factor genes. In addition, NO and YC-1 effects were suggested to be linked with a metal-related oxygen sensing pathway, and not with soluble guanylate cyclase stimulation. However, it was demonstrated later that NO under different conditions can induce the accumulation of HIF-1α even under normoxic conditions (42, 43). Why does NO show these reciprocal effects on HIF-1α, inhibition and stimulation? This question remains unanswered thus far. In our opinions, the direction of the NO effect may be determined by the concentration of NO itself. According to experiments demonstrating the inhibitory effects of NO, 100 μM sodium nitroprusside (SNP) and 3 mM S-nitrosoglutathione (GSNO) were used to generate NO. Since SNP releases NO quickly, 100 μM of SNP can produce a higher concentration of NO. We believe that NO was present at high concentrations under these experimental conditions. However, in experiments that demonstrated the stimulatory effects of NO, GSNO or other donors were used at the concentration of 100-200 μM. In terms of GSNO, the concentrations used for HIF-1 activation were 5 to 10-fold lower than that used for HIF-1 suppression. Moreover, Kimura et al. (44) showed that S-nitroso-N-acetylpenicillamine (SNAP), another NO donor, stimulates HIF-1 activity under normoxic conditions at concentrations of less than 0.5 mM, but that it inhibits HIF-1 activity under both hypoxic and normoxic conditions at concentrations of more than 0.5 mM. Therefore, we suggest that the effect of NO on HIF-1 depends upon its concentration.

Reactive Oxygen Species

In general, it is believed that reduced environmental oxygen tension causes a decrease in the cellular levels of reactive oxygen species (ROS), which leads to the stabilization of HIF-1α and the activation of HIF-1. Wang et al. (45) demonstrated that strong oxidizing reagents impair the expression of HIF-1α in hypoxic cells and the DNA-binding activity of HIF-1. Evidence of this redox-dependent regulation of HIF-1 has been provided by several experiments (46, 47). Recently, however, another hypothesis on the ROS production under hypoxic conditions has been introduced. The respiratory chain in mitochondria is one of the major ROS generation sites. Many reagents, which block the electron flow in this respiratory chain, cause an accumulation of electrons in the respiratory compartments prior to the compartment being blocked by an inhibitor, and then produce ROS because molecular oxygen can be reduced univalently by electrons leaking from the fully reduced compartments (48). In cases of oxygen deficiency, similarly, the last step in the respiratory chain is blocked because oxygen is essentially required for removing electrons. Electrons thus accumulate in the respiratory chain and produce ROS by reducing oxygen remaining in the mitochondria (49). Therefore, ROS production by mitochondria increases under hypoxic conditions, although the total level of intracellular ROS might be reduced because of a decrease in the ROS production by other oxidases. Based on this hypoxic increase in ROS generation from mitochondria, Chandel et al. (50) suggested that the mitochondrion acts as an oxygen sensor by increasing ROS production during hypoxia. They demonstrated that there is a positive relationship between ROS production and HIF-1 activation, and that mitochondria are essential for HIF-1 activation, by using Hep3B cells depleted of the mitochondria. Subsequently, this research group reported that ROS generated by the mitochondrial complex III participates in HIF-1α stabilization (51). In addition, it has been demonstrated that HIF-1α is up-regulated even under normoxic conditions by cytokines generating ROS (52, 53). Taken together, it is hard to draw a simple conclusion on the effect of ROS on HIF-1α. ROS seems to regulate the stability of HIF-1α in the opposite direction. The direction of ROS effect may be determined by the production amount and subcellular distribution of ROS.

Growth Factors

A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (54, 55). Immunohistochemical analyses show that HIF-1α is present at high levels in human tumors. Moreover, the expression levels of HIF-1α in the biopsies of various solid tumors correlate with tumor aggressiveness, vascularity, treatment failure, and mortality. In addition, tumor growth and angiogenesis in grafted tumors also depend on the HIF-1 activity or the expression level of HIF-1α. During tumor development, HIF-1 induces the expression of the gene products that promote angiogenesis, such as vascular endothelial growth factor, basic fibroblast growth factor, angiopoietin 2, and adrenomedullin (56). In addition, it aids tumor cell survival under hypoxic conditions via the expression of gene products that promote anaerobic ATP synthesis, such as glucose transporters (1, 3), a series of glycolytic enzymes (aldolase A and C, enolase 1, hexokinase 1 and 3, lactate dehydrogenase A, phosphofructokinase L, and phosphoglycerate kinase 1), and cell survival factors (insulin-like growth factor 2 and insulin-like growth factor

binding proteins) (57). In solid tumors, intratumoral hypoxia and genetic alterations that affect the expression of the VHL tumor suppressor gene are undoubtedly important mechanisms of the over-expression of HIF-1α. In addition, the growth factor-mediated activation of phosphatidylinositol-3-kinase (PI3K) contributes to the expression of HIF-1α in tumors. The growth factor-dependent expression of HIF-1α was first demonstrated in prostate cancer cells, in which HIF-1α is constitutively expressed even under normoxic conditions (58). Growth factors reported to elicit such effect, include insulin (59), insulin-like growth factor (59, 60), epidermal growth factor (61), and interleukin-1 (62). These growth factors are known to stimulate their receptors and activate the receptor tyrosine kinases, and in turn sequentially activate PI3K, a serine/threonine kinase AKT (also known as protein kinase B), and FKBP-rapamycin associated protein (FRAP). Finally, the FRAP stimulates the expression of HIF-1α under normoxic conditions (61) (Fig. 4). On the other hand, this pathway is negatively regulated by the PTEN tumor suppressor protein, which dephosphorylates the products of the PI3K (63). Therefore, the activation of the PI3K pathway and genetic alterations to affect the expression of PTEN are considered to be important mechanisms responsible for the normoxic expression of HIF-1α in tumor cells. Similarly, the increased activity of human epidermal growth factor receptor 2 (HER2, also known as neu) contributes to the over-expression of HIF-1α in breast cancer cells (64). HER2 has tyrosine kinase activity in the absence of any known ligand, and is known to stimulate the PI3K/AKT/FRAP pathway. Since genetic alterations that increase the HER2 activity occur in approximately one-third of breast tumors and are associated with increased tumor grade (65), the HER2-mediated induction of HIF-1α is considered to be an important mechanism for the development of breast tumors. Interestingly, Laughner et al. (64) also found that the HER2-mediated induction of HIF-1α is promoted by a novel mechanism. As described previously, the level of HIF-1α is regulated by a post-translational modification of prolyl hydroxylation. However, the HER2/PI3K/AKT/FRAP pathway regulates the level of HIF-1α at the translational level, and enhances the rate of HIF-1α protein synthesis from its mRNA. In this process, the 5′-untranslated region of HIF-1α mRNA may be targeted by the pathway (64). However, it is not clear whether this new mechanism occurs in other cells and under other experimental conditions.

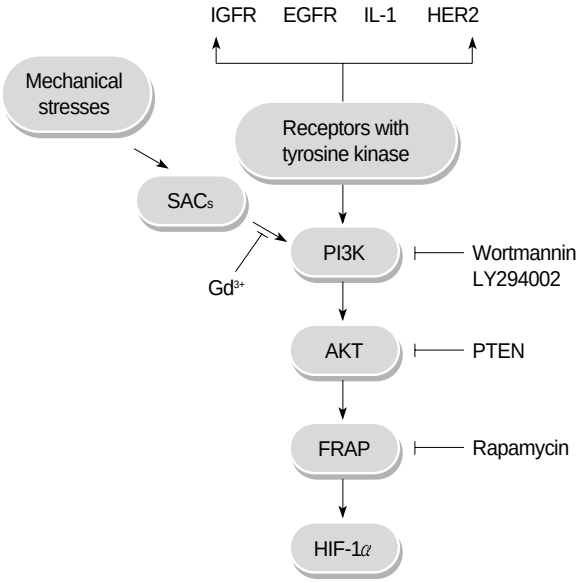

Fig. 4. Signal transduction pathways promoting non-hypoxic expression of HIF-1α. Even under normoxic conditions, HIF-1α can be induced by growth factors and mechanical stresses. The oxygen-independent regulation of HIF-1α is commonly mediated by the PI3K-AKT-FRAP pathway. The involvement of this pathway has been examined using specific inhibitors denoted at the right side.
Abbreviations: IGFR, insulin-like growth factor receptor; EGFR, epidermal growth factor receptor; IL-1, interleukin-1; HER2, human epidermal growth factor receptor 2; SACs, stretch-activated channels; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; FRAP, FKBP-rapamycin-associated protein; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

Mechanical Stress

Although HIF-1α is not usually detected in cancer cells cultured under normoxic conditions, HIF-1α is detected immunohistochemically in the nuclei of normal mouse tissues, such as brain, kidney, liver, and heart, and increased in response to whole body hypoxia (66). The existence of HIF-1α in normoxic tissues suggests that HIF-1α may be required for maintaining the basal expression of the essential genes regulated by HIF-1. Moreover, the accumulation and nuclear targeting of HIF-1α has been observed in ischemic tissues (67). Although the pathophysiology of ischemia is more complicated than that of hypoxia, reduced oxygen tension in ischemic tissues might be the major causative factor behind the stimulation of HIF-1α induction. Clinically, HIF-1α induction in the heart has great significance, as HIF-1α has been observed to be induced in the ischemic human myocardium. In biopsy specimens obtained from ischemic or infarcted myocardium, HIF-1α and VEGF proteins were detected by immunohistochemical staining (68). These results suggest that the early induction of HIF-1α mediates the transcription of the VEGF gene in the ischemic myocardium, which is one of the first adaptations of the human myocardium to ischemia. In the rat heart myocardial infarction model, however, VEGF expression was found to be induced in the normoxic myocardium remote from the infarct area (69). This suggests that stimuli other than ischemia might be responsible for the VEGF induction. Recently, Kim et al. (70) demonstrated that HIF-1α accumulated in the nuclei of cardiac myocytes in the non-ischemic myocardium, and found that this was followed by the induction of the VEGF gene in the same site.

They also found that wall stretch causes HIF-1α induction in the non-ischemic myocardium. Interestingly, the PI3K/AKT/FRAP pathway, which is activated during the early phase of wall stretch, stabilizes HIF-1α protein in the heart, as the pathway involves the HIF-1α induction in prostate cancer cells. Moreover, the stretch-mediated induction of HIF-1α and VEGF was suppressed by gadolinium (a stretch-activated channel inhibitor) (Fig. 4). These results imply that HIF-1α plays important roles not only in the adaptation to ischemia but also in the adaptation to mechanical stress. A similar in vivo finding was obtained in vascular smooth muscle of the aorta subjected to hypertension (71). In this case, after being subjected to hypertension for three days, cells positively stained with anti-HIF-1α and anti-VEGF antibodies appeared and increased further over seven days. As increased blood pressure causes the wall of the aorta to stretch, the associated mechanical stress may cause the induction of HIF-1α in the muscle cells of the aorta. Taken together, the mechanical stress-mediated, non-hypoxic induction of HIF-1α seems to be a common phenomenon, which occurs in the muscular tissues that compose the cardiovascular system. However, it is not clear how the muscle cells sense mechanical stress and signal the PI3K - HIF-1α pathway.

CONCLUSION

HIF-1 is a master regulator of oxygen homeostasis, and plays critical roles in physiological and pathological processes. To date, approximately four dozen genes targeted by HIF-1 have been identified. Moreover, both the expression of HIF-1α and its transcription activity are tightly controlled by cellular oxygen tension. Recent progress in the study of its regulation mechanism gives us clues to the manner in which HIF-1 is regulated by oxygen. In addition to HIF-1-mediated adaptation to hypoxia, HIF-1 also contributes to other cellular processes that occur under normoxic conditions, such as the development of normal tissues or tumors, the determination of cell death or survival, immune responses, and the adaptation to mechanical stresses. In these cases, the regulation of HIF-1α is not dependent on oxygen tension, but mediated by various stimuli. Finally, understanding the roles and regulation mechanisms of HIF-1α will open a new era in the development of therapeutic strategies against a variety of pathologic conditions, such as ischemic/hypoxic injuries, tumor growth, wound healing, and cardiovascular remodeling.

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