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REVIEW

Mechanisms of Regulation of Transcription Factor HIF under Hypoxia

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Abstract—The importance of transcription factor HIF in cellular response to hypoxia and various regulatory levels determining its functioning within cells are considered. Special attention is paid to important links of the hypoxia-triggered HIF-mediated mechanism. The review contains retrospective analysis of the search for cellular primary oxygen sensors and their interactions with HIF. It also deals with the interrelation of HIF-mediated and other hypoxia-activated mechanisms and outlines importance of HIF subunits in the cell.

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Significant progress has been made in the knowledge on cell sensitivity to decreased oxygen concentration and mechanisms underlying this sensitivity. It is known that oxygen detection involves not only the systemic level employing special chemoreceptor cells mediating immediate adaptive physiological reactions, first of all that realized by cardiovascular and respiratory systems due to activation of nerve-mediated pathways. Convincing evidence now exists that any cell is sensitive to changes of oxygen content in the surrounding medium. This change is a physiological stimulus, which triggers certain intracellular mechanisms responsible for cell functioning under new conditions.

Altered activity of the transcription factor HIF in the cell is a universal event associated with reduced oxygen concentration in the medium; the role of HIF in expression of hypoxia-activated genes is now generally recognized [1-3]. The number of known HIF target genes constantly increases and includes more than one hundred genes [4]. A certain proportion of these genes are activated in all hypoxic cells, whereas expression of some HIF target genes is specific for particular cell types [5]. The genes encode proteins that play an important role in cell adaptation to hypoxia [2, 6-8]. These include:

1) proteins determining cell functioning under hypoxic conditions (aldolase A, aldolase C, enolase-1, glyceraldehyde-3-phosphate dehydrogenase, hexokinase-1, hexokinase-2, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase-1, pyruvate kinase M, triosephosphate isomerase, glucose phosphate isomerase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3, glucose transporters 1 and 3, carbonic anhydrase);

2) proteins increasing tissue oxygen content due to stimulation of erythropoiesis, angiogenesis, and vascular tone regulation (vascular endothelial growth factor (VEGF) and its receptor FLT-1, plasminogen activator inhibitor, NO synthase-2, heme oxygenase-1, endothelin-1, α-1β adrenoreceptor, adrenomedullin, angiopoietins 1 and 2, erythropoietin, ceruloplasmin, transferrin, and transferrin receptor);

3) proteins influencing cell viability and proliferation (transforming growth factors TGF-β and TGF-α, p21, proapoptotic proteins NIP3, NIX, and RTP801, insulin-like growth factor binding proteins (IGFBP1, -2, -3), insulin-like growth factor-2 (IGF-2), cyclin G2, platelet-derived growth factor (PDGF) β receptor, leptin, placental growth factor (PGF), chemokine receptor (CXCR4));

4) cytoskeletal proteins (keratins 14, 18, and 19, vimentin);

5) extracellular matrix proteins and enzymes (matrix metalloproteinase MMP2, cathepsin D, collagen prolyl 4-hydroxylase, α1 fibronectin 1, collagen V α1 subunit).

Abbreviations: CTAD, C-terminal TA domain; NTAD, N-terminal TA domain; ODDD, oxygen-dependent degradation domain; ROS, reactive oxygen species.

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MECHANISMS DETERMINING CELL SENSITIVITY TO ALTERED OXYGEN LEVEL

Results obtained during last 10-15 years suggest that HIF activation is a universal cell response to decreased oxygen. However, until recently cell processes linking HIF activation with altered oxygen content represented the less studied component among hypoxic signaling processes in cells.

Earlier several models explaining cell sensitivity to oxygen level in the surrounding medium have been proposed. The simplest of them considered the mechanism of oxygen binding to some sensor molecule, and the decrease in oxygen concentration was accompanied by the decline in ligand binding to such sensor. For example, in the bacterium *Rhizobium meliloti* the oxygen detector consists of a two-component system; it includes the sensor hemoprotein kinase FixL (active only in a deoxygenated state) and the transcription factor FixJ, which is activated during phosphorylation by the FixL kinase. Thus, under normoxia the hemoprotein kinase is oxygenated and inactive; decrease in the oxygen level causes protein deoxygenation and its activation. The activated protein can react with a transcription factor and thus participate in hypoxic signaling. The same scheme of functioning of oxygen hemoprotein sensors is also applicable for mammalian cells [1, 6].

In accordance with the theory on the heme nature of oxygen sensors, several candidates were considered: the b558/NADPH oxidase complex found in many (non-immune) cells and mitochondrial cytochromes [6, 9]. Besides hemoproteins, the list of putative oxygen sensors also included iron-sulfur proteins, which are inactive in the oxidized state (in the presence of oxygen) and active in the reduced state (during oxygen deficit), when they are able to interact with other molecules and thus transduce a hypoxic signal [1]. In some experiments heme synthesis inhibitors suppressed expression of the erythropoietin gene [6]. However, in other studies heme synthesis inhibitors insignificantly influenced the hypoxic response [10]. Thus, although many experimental data were consistent with the theory on the heme nature of mechanisms of oxygen detection in the cell, some experimental results were inconsistent with it [6].

Two other models considered reactive oxygen species (ROS) as the key component mediating the effect of altered oxygen content on the cells. Direct ROS interaction with effector proteins (e.g. components of potassium channels or HIF subunits) of ROS dependent activation of some kinase cascades, causing activation of effectors, was considered as the final element of both models. The major difference between these models, considering ROS as the trigger of the hypoxic signal, is changes in ROS concentration: one model postulates the decrease whereas the other suggests the increase in ROS concentrations as the triggering factor. However, there are experimental data supporting both models. These include cell culture studies of the effects of pharmacological inhibitors of proteins playing a key role within each theory (NADPH-oxidoreductase or electron transport chain components) on expression of HIV and its target genes and also assays of ROS concentrations using various fluorescent probes.

Superoxide anion radical ($O_2^{\cdot-}$) and other ROS are usually considered either as the product produced by NADPH oxidase or mitochondrial electron transport chain (under hypoxic conditions). It is known that the membrane bound enzyme, NADPH oxidase (various forms of this enzyme are present in many tissues) converts molecular oxygen into $O_2^{\cdot-}$, which is then utilized by superoxide dismutase yielding hydrogen peroxide. According to one of the models, ROS generation by NADPH oxidase is proportional to cell oxygen content: the decrease of oxygen in the external medium causes the decrease in ROS generation, which can thus mediate the hypoxic signal [6, 9, 11]. This was experimentally validated [10, 12].

According to the other model, complex IV of the mitochondrial electron transport chain (cytochrome oxidase) plays the major role in detection of the oxygen level. This hypothesis postulates inhibition of the reduction of molecular oxygen to H_2O under hypoxic conditions. Electrons "unused" in oxygen reduction are utilized by complex III of the mitochondrial electron transport chain for generation of superoxide anion radical, which undergoes further conversion into hydrogen peroxide. Thus, hypoxia increases cell concentration of ROS generated by mitochondria and this might also mediate the hypoxic signal [1, 6]. Experimental data supporting this hypothesis have been obtained using various (including human) cells. For example, impairments in HIF activation under hypoxic conditions were found in cells treated with an inhibitor of complex I of the electron transport chain (due to decreased concentration of $O_2^{\cdot-}$), and also in cells lacking mitochondrial DNA (and unable to increase production of mitochondrial ROS); this suggests the major role of mitochondrial ROS in activation of gene transcription under hypoxia [11, 13-15]. Experiments on mice with inactivated superoxide dismutase gene revealed more than a 100-fold increase in the erythropoietin gene expression; this suggests a trigger role of superoxide anion radical in cell response to hypoxia [16].

However, other experiments demonstrated maintenance of the HIF response in hypoxia regardless to functioning of the mitochondrial electron-transport chain and ROS; this is inconsistent with the mitochondrial hypothesis of oxygen detection. For example, cyanides did not influence HIF activation and expression of hypoxia inducible genes [17]. Later experiments on cell lines with specific genetic defects in the mitochondrial electron transport chain and with cell lines lacking mitochondrial DNA demonstrated that during hypoxia the activation of HIF and gene expression was maintained in

all cell lines [10, 18]. Increased expression of such ROS inhibitors as mitochondrial and cytosolic catalase did not cause changes in the hypoxic response; this suggests lack of ROS effect on HIF activation [10, 19].

Thus, although the role of ROS as the signaling pathways trigger of the cell response to hypoxia is generally accepted [20-26], results of experimental studies of HIF activation are contradictory. It is possible that such inconsistency is attributed to the use of various models, methodological differences in ROS assay, and types of hypoxia [4]. The hypothesis postulating dependence of involvement of ROS (of mitochondrial and non-mitochondrial origin) in HIF activation on severity of the hypoxic effect seems to reconcile contradictory results of such experiments. According to results of some studies, at the oxygen concentration in the medium of 1-2%, HIF stabilization requires normal functioning of the respiratory chain; this is not observed in anoxic conditions, when blockade of the respiratory chain is not important [14, 15, 27].

Below we consider the generally accepted theory postulating that hydroxylases involved into degradation of one of subunits of the transcription factor HIF and modulation of transcriptional activity are the primary sensors for decreased oxygen tension in the medium [1-3, 7, 8, 28-30].

STRUCTURE OF TRANSCRIPTION FACTOR HIF AND MECHANISM OF ITS STABILIZATION IN HYPOXIA

The transcription factor HIF is a heterodimer that consists of one of three existing α-subunits (HIF-1α, HIF-2α, HIF-3α) and β-subunit (HIF-1β), also defined as ARNT (aryl hydrocarbon receptor nuclear translocator). The latest studies revealed several variants (ARNT1, ARNT2, and ARNT3), which are characterized by certain homology and some tissue specificity of their expression also recognized for β-subunit; however, all organs express only ARNT that can bind all variants of α-subunit during dimer formation [30, 31].

Both subunits contain the bHLH domain (basic helix-loop-helix domain) responsible for transcription factor binding to DNA; it is also involved in subunit dimerization by their PAS domains. Recruitment of transcription coactivators during transcription complex formation (a transcription initiation complex) involves transactivation domains. The C-terminal TA domain (CTAD) presented in both subunits (except HIF-3α) is the most important for the transactivation process. The N-terminal TA domain (NTAD) typical for all variants of α-subunit also neighbors or even overlaps the oxygen-dependent degradation domain (ODDD). The latter is required for α-subunit degradation in an oxygenated medium; it is absent in β-subunit, and this determines its stability regardless of oxygen concentration in the medium, whereas α-subunit content is determined by intracellular oxygen concentrations [30, 31].

In the presence of oxygen, HIF α-subunit is degraded by a specific complex, which contains several components including von Hippel–Lindau tumor suppressor protein (pVHL). Its binding to the α-subunit involves a determinant localized within the ODDD domain; this causes subsequent ubiquitination, destabilization, and proteasome degradation of this subunit. Interaction between pVHL and the α-subunit ODDD domain is determined by hydroxylation of two highly conservative proline residues of the ODDD domain (Pro402 and Pro564); these residues form two hydrogen bonds with side chains of the pVHL domain responsible for substrate recognition [2].

Hydroxylation of proline residues of mammalian HIF α-subunits involves one of three hydroxylases (EGLN1, EGLN2, EGLN3, or PHD2, PHD1, PHD3, respectively, or HPH-2, HPH-3, HPH-1, respectively). The enzymatic hydroxylation is an oxygen-dependent reaction, because the oxygen atom used for hydroxyl group formation comes from molecular oxygen. The rate of hydroxylation is determined by the oxygen concentration [3, 7, 31].

It should be noted that hydroxylases require iron ions (Fe^{2+}) as cofactors (this explains the hypoxia mimetic effect of iron antagonists) and 2-oxoglutarate (because the hydroxylation reaction is coupled to its decarboxylation and conversion into succinate, which accepts the remaining oxygen atom). Thus, regulation of prolyl hydroxylase functioning by mitochondria can involve not only ROS generated under hypoxic conditions, but also by the Krebs cycle (due to ratio between anaerobic and aerobic pathways of glucose metabolism), which supplies prolyl hydroxylases with its intermediate, 2-oxoglutarate. Indeed, it has been shown that the tricarboxylic cycle intermediates play an important role in regulation of all three types of PHD [30]. In addition, changes in concentrations of mitochondria-generated ROS can cause imbalance between Fe^{2+} and Fe^{3+}; this influences hydroxylase activity and therefore stabilization of the HIF α-subunit. ROS can directly inactivate hydroxylases due to oxidative damage. On the other hand, certain evidence also exists that the decrease in ROS concentrations can also result in HIF stabilization. Thus, mitochondria play a significant role in the regulation of HIF α-subunit stabilization, and this has been experimentally demonstrated by using various mitochondrial inhibitors [4, 5].

Mitochondrial oxidative processes not only regulate HIF, but also represent targets for this transcription factor. The latter stimulates expression of a kinase inhibiting pyruvate dehydrogenase; this causes the decrease in acetyl-CoA formation (the starting product for the citric acid cycle) from pyruvate. This results in the decrease of mitochondrial oxygen consumption and ROS generation

under hypoxia. There are other mitochondrial oxidation processes modulated by HIF [32].

Thus, oxygen-dependent hydroxylation of proline residues in the HIF α-subunit catalyzed by specific hydroxylases is one of mechanisms responsible for the detection of oxygen level by mammalian cells; this is the recognition signal for subsequent binding of the hydroxylated sites by the pVHL protein, the components of a specific complex that performs subsequent degradation of the HIF α-subunit.

The question whether prolyl hydroxylases are actual primary sensors of altered oxygen concentration in the medium requires further studies. A hypothesis explaining contradictory results of experiments on elucidation of the role of ROS in HIF stabilization suggests that hydroxylases act as the primary sensors of oxygen only under anoxic conditions (or oxygen concentrations close to anoxic). Under hypoxia hydroxylases can be the targets of mitochondrial and non-mitochondrial ROS [14].

MULTILEVEL REGULATION OF HIF FUNCTIONING

Evidence now exists that besides stabilization and degradation of the α-subunit, regulation of HIF functioning also involves transcription and translation of the HIF α-subunit, its translocation into the nucleus, subunit dimerization, transcription factor binding to coactivators, HIF interaction with other transcription factors, and assembly of the competent transcription complex and its interaction with DNA [4, 7, 33].

It should be noted that many (including oxygen-independent) events that could change equilibrium towards either accumulation or degradation of the α-subunit have been identified among process of protein stabilization and degradation. For example, pVHL represents a potential target for regulatory molecules. It was demonstrated that various regulators can modulate its stability, affinity for HIF1α, or components of the complex degrading HIF. In addition to HIF hydroxylated proline residues other signals also exist for pVHL: hypoxia inducible ubiquitin-like proteins can influence hydroxylation-independent processes of HIF-1α stabilization and degradation. There is a protein influencing HIF α-subunit ubiquitination and basically canceling proteasomal degradation of HIF-1α [4, 5].

There is evidence that the transcription factor NF-κB, which interacts with HIF during transcription of hypoxia-activated genes, acts as a direct modulator of HIF expression [33].

Hypoxia-dependent expression of HIF mRNA was demonstrated for some cell types [7]. It is known that in order to decrease energy consumption, the intensity of protein synthesis decreases under hypoxic conditions. However, regardless of significant suppression of protein biosynthesis, translation of HIF-1α and some other proteins is not only maintained but also increased under hypoxic conditions. Mechanisms of this phenomenon remain to be clarified [34].

The HIF transcriptional activity associated with coactivator recruitment by the transactivator domains NTAD and CTAD is the most important and the best-studied regulatory mode. However, due to overlapping of the NTAD and ODDD domains, it is hard to discriminate the NTAD-specific regulation of HIF by coactivator binding and the ODDD-dependent regulation of α-subunit degradation. Nevertheless, stabilization-independent control of the α-subunit by NTAD-mediated regulation is possible, but its contribution to the transcriptional activity is rather small [30]. On the contrary, the CTAD domain significantly contributes to regulation of the transcription activity of most HIF target genes.

The HIF transactivation domains determine transcription activation due to binding with such coactivators as CBP/p300. Binding to CBP/p300 stabilizes the HIF CTAD domain so that HIF can activate transcription (and this occurs under hypoxic conditions). An increase in oxygen concentrations causes hydroxylation of an asparagine residue (Asn803 and Asn851 in HIF-1α and HIF-2α, respectively) in the CTAD domain catalyzed by specific hydroxylase FIH-1; this prevents both HIF CTAD binding to the coactivator CBP/p300 and transcription activation. Experiments on blockade of CBP/p300 coactivator binding with HIF-1α have demonstrated inhibition of transcription of HIF-activated genes; this indicates the importance of HIF interaction with the coactivator [3, 31]. Replacement of asparagine resulted in constant activation of CTAD and its binding to CBP/p300 regardless of oxygen levels; this suggests importance of the hydroxylation reaction for suppression of CTAD activity under normoxia [30].

Some experimental data indicate that the FIH-1 hydroxylase exhibits higher affinity to oxygen than PHD hydroxylases. Consequently, increase of hypoxia initially blocks prolyl hydroxylases, and this results in HIF stabilization and transcription activation by NTAD followed by inactivation of the asparagine hydroxylase FIH-1; this determines functioning of the CTAD domain required for full HIF transcriptional activity [30]. This is consistent with the fact that CTAD is involved in transcription activation of most but not all HIF target genes. Gene transcription ultimately dependent only on the NTAD (and independent of CTAD) domain should be activated at oxygen concentrations sufficient for FIH-1 functioning but already insufficient for activity of PHD hydroxylases. Moreover, sensitivity of the FIH-1 hydroxylase to oxygen can vary in different cell types [30].

These recent results give plausible explanations to earlier experimental data that questioned possible contribution of asparagine hydroxylation of the HIF CTAD domain to its inactivation. They also support a hypothesis

that modulation of CTAD activity by the hydroxylase FIH-1 is tissue specific and is important for some HIF target genes [3]. According to some viewpoints, the FIH-1 hydroxylase pathway of transcription inactivation exists as a gentler mechanism of regulation of HIF concentration (in addition to the main PHD-hydroxylase based mechanism) [3]. Different sensitivity of the PHD and FIH-1 hydroxylases to oxygen implies that under certain oxygen concentrations HIF is stable due to PHD inactivation, but transcriptionally inactive due to FIH-1 functioning. This suggests the possibility of HIF transcriptional activity reduction without changes in its stabilization/degradation under hypoxic conditions [5].

Besides hydroxylation, other mechanisms of post-translational protein modification are also crucial for regulation of HIF activity. These include phosphorylation of certain amino acid residues, which may be important at various levels of HIF regulation. It has been demonstrated that phosphorylation of the α-subunit plays a certain role in the HIF transcriptional activity, but not in stabilization of this protein, because substitution of the potential phosphate acceptor site in the ODDD domain did not influence protein stabilization under hypoxic conditions [7]. Importance of phosphorylation for regulation of HIF activity was demonstrated for the CTAD domain: phosphorylation of one amino acid residue by one of the kinases impaired hydroxylation by FIH-1 and increased domain interaction with transcription coactivators. It was also demonstrated that phosphorylation of certain amino acid residues can be important not only for HIF-mediated transcription but also for translocation of the α-subunit into the nucleus. Results of experiments evaluating the role of phosphorylation, nitrosylation, acetylation, and other chemical modifications of HIF for its functioning are contradictory: in various experiments the same modification caused either stimulation or suppression of HIF functioning [30].

After hypoxia-induced stabilization, translocation into the nucleus, ARNT-dimerization, and cofactor recruitment HIF binds to DNA at HRE (hypoxia response element) promoters of many oxygen-regulated genes. Structure, functioning, conservativeness, and variability of these elements and also mechanisms underlying their differences in various genes are investigated now [5]. In addition to CBP/p300, assembly of the transcriptionally competent complex (that occurs during transcriptional activation of hypoxia induced genes) involves HIF cooperation with other coactivators and transcription factors. These include TIF-2, SRC-1, TRIP 230, NEMO, Smad3, STAT3, HNF-4, ATF-1/CREB-1, AP-1, SP-1, and NF-κB [30, 34-38]. Such multiprotein complexes including HIF, coactivators, and also other transcription factors differ in various oxygen regulated genes [35, 36]; they also differ in composition, which depends on HIF isoforms or tissues [30]. This contributes to specificity of the hypoxic response of particular cells and unique features of its regulation. Possibility of direct interaction of some hypoxia-induced transcription factors has been demonstrated in experiments [34, 37, 38].

Thus, HIF functioning in hypoxia can be regulated at various levels. The most studied (and highlighted in the literature) mechanisms of HIF activation under hypoxic conditions include stabilization of its α-subunit and also cofactor recruitment. These mechanisms are associated with oxygen sensitive hydroxylases.

Results of studies of physicochemical characteristics of recombinant hydroxylases suggest that endogenous hydroxylases can play the role of oxygen sensors for detection of changes in the physiological oxygen concentrations *in vivo* [29, 30]. It should be noted that at 0.5% O_2 (this concentration corresponds to hypoxia *in vivo*) the HIF content reaches maximal level [17].

Although hydrolases play the key role in regulation of HIF stabilization and functioning, and some proteins and signaling pathways determining their stability and regulating their transcription have been recognized [4, 30], the question on regulation of these hydroxylases and mechanisms of their control still requires further investigation.

HIF-1, HIF-2, AND HIF-3: SPECIFICITY IN ADDITION TO OVERLAPPING EFFECTS

In addition to HIF-1α, two other members of this family are known: HIF-2α and HIF-3α. Comparative characterization of HIF-1α and HIF-2α revealed many similar features in their genetic organization, protein structure, stabilization mechanisms, heterodimerization with ARNT, recognition and binding to DNA, and also transactivation of known genes. Nevertheless, spectra of target genes activated by HIF-1α and HIF-2α do not completely coincide and certain genes can be regulated only by one type of HIF [39]. For example, specific regulation by HIF-2α has been reported for *OCT-4*, which is essential for cell differentiation processes [40].

Importance of a certain subunit (HIF-2α or HIF-1α) for expression of target genes can depend on activation mechanisms of this expression (by hypoxia, growth factors) [41]. According to an experimentally proved hypothesis, mechanisms of regulation of HIF-1α and HIF-2α can differ in some cell types. It should be mentioned that HIF-1α, but not HIF-2α responds to classical induction by hypoxia. Thus, for some cells only one type of HIF is transcriptionally active [42]. Specificity of HIF-1α and HIF-2α target genes is obviously determined by NTAD domain functioning [30].

The role of HIF-1α and HIF-2α in activation of hypoxia-induced genes can also depend on cell type [43]. In contrast to HIF-1α, which is expressed in all cells, expression of HIF-2α or HIF-3α is detected in a narrower range of tissues [8]. Specificity of the HIF-dependent

cell signaling in certain cell types can be illustrated by variability of HIF-1α expression. This was found in one study of rat organs (heart, liver, brain) by means of Western-blot and monoclonal antibodies. Although basal level was the same and cytosolic HIF-1α increased in all these organs, the rate and the magnitude of this increase varied. Maximal increase (by 100%) and the fastest response (after 3 h) were found in the heart. The liver was characterized by 50% increase in HIF-1α, which was registered 6 h after hypoxia. Slight HIF-1α induction in the brain was observed only after 12 h [44].

The mRNA levels of various HIF subunits are obviously tissue specific; the lowest level is typical for HIF-3α mRNA [7, 45]. However, one cell type responded to moderate hypoxia by the increase of HIF-3α mRNA (but not HIF-1α and HIF-2α), and the increase in HIF-1α and HIF-2α was detected after severe hypoxia [45]. Thus, HIF-3α can represent the most reactive and sensitive component of cell response to hypoxia; it provides adaptation to a small decrease of oxygen concentration in the medium or the earliest hypoxic changes [45]. On the other hand, it has been shown that the protein product of *HIF-3α* gene, formed due to alternative splicing and known as IPAS (inhibitory PAS domain protein), can bind to the β-subunit and prevent both complex dimerization and HIF-mediated transcription. Since hypoxia induces IPAS formation, it is considered as the factor responsible for negative feedback regulation of HIF-dependent transcription [30].

Recently another difference between HIF-1α and HIF-2α significant for *in vitro* studies has been recognized: it appears that HIF-2α functioning is determined not only by cell type but also conditions of their cultivation; this is not typical for HIF-1α [46].

Existence of three HIF α-subunits with partially overlapping functions is not redundant. Experiments with HIF-1α and HIF-2α knockout mouse embryos have shown that HIF-1α is required for survival of mesenchymal cells during embryonic development. HIF-1α knockout embryos were characterized by defects in formation of the cardiovascular system and neural tube, impairments of adipo-, chondro-, and osteogenesis, and also development of B-lymphocytes; this caused their death. Mice with one defective HIF-1α allele exhibited normal development and a weaker response to chronic hypoxia. An important role of HIF-2α for embryogenesis has been also demonstrated using HIF-2α knockouts. Thus, there is convincing experimental evidence that HIF acts not only as a trigger of cell and systemic response to hypoxia, but also as the regulator of many processes during embryogenesis and in adult organisms [6-8]. This is consistent with detection of HIF in various normoxic tissues. For example, HIF-1α is detected in brain, kidneys, liver, heart, and skeletal muscles of normoxic mice, and its concentration increases in response to hypoxia [47].

HIF, CELL SIGNALING PATHWAYS, AND HYPOXIA

There is evidence that HIF activation can be mediated not only by a decreased cellular oxygen level, but also by many growth factors and cytokines under normoxia. These include insulin, insulin-like growth factors 1 and 2, epidermal growth factor, fibroblast growth factor, interleukin-1β, tumor necrosis factor α, angiotensin II, thrombin, transforming growth factor β1, platelet derived growth factor, and hepatocyte growth factor. Their effects are obviously mediated through common kinase signaling pathways, activated by specific receptors [4, 7].

The signaling pathways involved in HIF regulation can include cascades of mitogen activated protein kinases (MAPK); however, mechanisms of their regulatory effects remain unclear. These kinases might regulate HIF functioning via phosphorylation of its α-subunit [2, 6, 7, 30, 38]. It is suggested that the effects of this signaling cascade can be determined by HIF interaction with coactivators. In this case, modulation of HIF transcriptional activity but not HIF stabilization is proposed [5]. On the other hand, the MAPK cascade might be involved in regulation of HIF-1α translation [4]. Interaction of many growth factors involves tyrosine kinase receptors and subsequent activation of the Ras/Raf-MEK1-p42/p44 pathway (the classical MAPK cascade). Another mode of modulation of HIF-1 activity can presumably involve activation of the PI3-Akt cascade. For example, inhibition of PI3-kinase caused suppression of many HIF-1-dependent cell responses. It is suggested that Akt-1 induces HIF-1 translation, but data on possible effect of this signaling pathways on protein stabilization are rather contradictory [4, 5, 7]. However, oxygen-independent stabilization of α-subunit is achieved by its binding to heat shock protein Hsp90, and HIF-1α interaction with RACK1 (regulated by intracellular calcium) causes oxygen-independent degradation of α-subunit [48].

Oxygen-independent destabilization of HIF-1α can be also achieved by its binding to p53 protein due to mechanisms determining p53 degradation [4, 5].

Thus, not only decreased oxygen concentration in the medium, but also some signaling pathways (activated by various growth factors) can be involved into regulation of HIF functioning. However, involvement of these signaling pathways in HIF stabilization requires further investigation because it remains unclear what mechanisms are responsible for HIF stabilization under normoxia, when normal oxygen level determines hydroxylase activity.

It is known that besides the main "target", HIF, hypoxia can activate certain signaling pathways. These pathways are also associated with such cell responses to hypoxia as altered proliferative activity or modulation of its viability (with involvement of pro- or antiapoptotic factors). For example, some experiments have demon-

strated activation of the classical MAP kinase cascade under hypoxia [49, 50], but now the possibility of this event is doubtful [38, 51].

In contrast to the classical MAP kinase pathway, the SAPK cascade usually activated under stress conditions (induced by UV irradiation, toxins, some "stress" cytokines such as interleukin-1 or tumor necrosis factors) was also activated by hypoxia. For example, experiments on fibroblasts, smooth muscle cells, and endothelial cells derived from pulmonary arteries of adult cows have shown that hypoxia stimulates JNK- and p38-kinase cascades without any significant stimulation of the p42/p44 MAP kinase pathway [52]. Activation of the JNK/SAPK p38 kinase pathways under hypoxic conditions has been demonstrated in many other cells [21, 37, 38, 50, 53-55]. Some studies revealed an important role of ROS in activation of these signaling pathways under hypoxia [21, 25]. Hypoxic conditions also caused activation of protein kinase C [56-58] and the PI3K/Akt pathway [59, 60].

Although under decreased oxygen level in the cell the hypoxic signal triggers several cascades that might potentially control HIF, the probability of their involvement in the HIF induction (i.e. indirect activation of HIF by some hypoxia-activated signaling pathways) seems questionable because in contrast to rapid activation of HIF by hypoxia, activation of kinases takes some time.

According to the latest data, HIF-1 induction in response to hypoxia occurs almost immediately. For example, experiments on the HeLaS3 cell line have shown that under hypoxic (0.02-5% O_2) or anoxic (0% O_2) condition HIF-1α could be detected in cell nuclei by Western blot within 2-min exposure. HIF accumulation in the nucleus occurred rapidly within 30 min, then the accumulation rate decreased; maximal accumulation was observed at the 60th min of the exposure. At the same time, the effect of hypoxia or anoxia on the nuclear content of such redox sensitive factors as NF-κB, c-Fos, c-Jun, Ref-1 (redox factor-1) was not detected during 60 min of the exposure [61].

It should be noted that even this rate of HIF degradation is overestimated because it includes time required for oxygen diffusion through the medium. Using perfused and ventilated lung preparations subjected to hypoxia/reoxygenation, it was demonstrated that the half-life of HIF does not exceed one minute: this is the shortest half-life among proteins [6]. This means that HIF accumulation in cell nucleus under hypoxia occurs almost immediately and represents an urgent mechanism of cell survival. Results of study [62], demonstrating that activation of the pathway PI3 kinase/Akt occurs after HIF induction or in parallel with it, are consistent with these observations.

Thus, decrease in oxygen content around the cell activates various mechanisms responsible for the development of adaptive reactions including changes in gene expression. Activation of the transcription factor HIF represents one such mechanism. Its key and universal role in the cell response to hypoxia is generally accepted, and important links and many details of the HIF-mediated mechanism of cell adaptation to hypoxia have been investigated. However, many questions on this pathway of realization of the hypoxic effect on the cell require further investigations. For example, it remains unclear how intensity of the hypoxic treatment determines mechanisms of HIF activation; whether hydrolases always act as primary oxygen sensors and which molecule use them as the targets; what is the role of ROS and mitochondria in HIF induction; how wide is the spectrum of regulatory mechanisms controlling HIF functioning and what is the role of various signaling pathways in this regulation; where cross-talk between the HIF-mediated mechanism and other hypoxia-activated signaling pathways is possible and how the HIF-dependent pathway is regulated at these cross-talk points; how overlapping and unique are the effects of various HIF subunits; what determines specificity of the HIF-dependent pathway in certain cell types and tissues, etc.

It is clear that hypoxia causes activation of various (universal and specific) signaling pathways in cells, which can be interrelated and interdependent, and this determines the overall cell response during each time interval. Specificity and mode of such response can depend on numerous factors such as tissue or cell culture specificity, intensity of the hypoxic treatment, cultivation conditions, etc. Only complex analysis of all these factors can systematize experimental results of studies of cell responses to hypoxia and specify mechanisms determining these responses.

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