Cellular Redox Status Regulates Hypoxia Inducible Factor-1 Activity. Role in Tumour Development

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The transcription factor hypoxia-inducible factor-1 (HIF-1) regulates the expression of more than 100 genes involved in cellular adaptation and survival under hypoxic stress. Activation of HIF-1 is associated with numerous physiological and pathological processes that include tumorigenesis, vascular remodelling, inflammation, and hypoxia/ischemia-related tissue damage. Experimental data support the concept that modulation of Reactive Oxygen Species (ROS) levels have an important impact on the hypoxic response mediated by HIF-1?. However, ROS generation, the exact kinetics and conditions of ROS production and their specific relevance to HIF-1? activation are issue still to be clarified.
Clinical studies suggested that HIF-1 activation correlates directly with advanced disease stages and treatment resistance among cancer patients. Preclinical studies support the inhibition of HIF-1 as a major molecular target for anti-tumour drug discovery. Considerable effort is underway to identify therapeutically useful molecule HIF-1 inhibitors. Most of the compounds discovered to inhibit HIF-1 are natural products or synthetic compounds with structures that are based on natural product leads. Natural products have also served a vital role as molecular probes to elucidate the pathways that regulate HIF-1 activity. Many of the substances found to inhibit HIF-1 are non-druggable compounds that are too cytotoxic to serve as drug leads. The application of high-throughput screening methods, complementary molecular-targeted assays, and structurally diverse chemical libraries hold promise for the discovery of therapeutically useful HIF-1 inhibitors.

Key Words: Hypoxia inducible factor, Oxidative stress, Free radicals, Antioxidants, Tumour hypoxia, HIF-1

In the human body, oxygen is delivered along a concentration gradient from the site of uptake in the lung capillaries to the site of consumption. Tissue oxygenation represents a steady state based upon O_2 consumption, primarily by mitochondrial oxidative phosphorylation, and O_2 delivery via erythrocytes travelling through tissue capillary networks (1, 2). Interruption of blood flow, reduction in oxygen tension, decreased oxygen carrying capacity, and failure to transport oxygen from the microvasculature to cells can all lead to an insufficient oxygen supply (hypoxia) to meet the metabolic requirements of specific tissues. In solid tumours, rapid tumour growth outstrips the capability of existing blood vessels to supply oxygen and hypoxic regions commonly occur (3-6). Unlike normal cells from the same tissue, tumour cells are often chronically hypoxic. Hypoxia triggers tumour angiogenesis and the newly formed tumour blood vessels often fail to mature. As a result, certain tumour regions are constantly under hypoxic stress due to sluggish and irregular blood flow. The extent of tumour hypoxia correlates positively with advanced stages and poor prognosis (3-6). Hypoxic tumour cells are more resistant than normoxic tumour cells to radiation treatment and chemotherapy and these hypoxic cells are considered an important contributor to disease relapse (7). Hypoxia also exerts indirect effects on tumour cells by inducing the expression of genes that promote hypoxic adaptation and survival. For example, hypoxia provides a physiological pressure and selects for cells with diminished apoptotic potential in oncogenically transformed cells (8).

Metabolic shifts in the evolution from anaerobic toward aerobic organisms conferred the ability to sense a decrease in environmental oxygen concentrations through a well conserved hypoxic response pathway (9,10). This pathway facilitates acclimation to hypoxia-evoked physiological stress by modulating the

expression of specific genes, and plays a central role not only in various physiological events during ontogenetic processes, including neovascularization and angiogenesis (11), but also in pathophysiological events such as tumourigenesis (12,13).

The studies of hypoxia response element of the erythropoietin (EPO) gene leads to the discovery of hypoxia-inducible factor-1 (HIF-1). HIF-1 was identified by its binding at the 39 region of the hypoxia response element (HRE) of the erythropoietin gene after hypoxic exposure (14,15). This information increased our understanding of molecular acclimation to a wide range of alterations in environmental oxygen concentrations (9,16). At present, many processes involved in oxygen homeostasis are believed to be mediated by a family of HIFs, which transcriptionally regulate the expression of more than 100 target genes (1,7). These genes encode proteins involved in angiogenesis, erythropoiesis, glucose metabolism, and cellular proliferation.

Structure of HIF-1

HIF-1 was found to be conserved from *Caenorhabditit elegans* via *Drosophila melanogaster* to *Homo sapiens* (1,17) suggesting that the HIF-1 system played an essential role during evolution. HIF-1 is a member of the basic helix-loop-helix (bHLH) superfamily of eukaryotic transcription factors in which the HLH domains mediate subunit dimerization and the basic domains bind to DNA. A family of bHLH proteins that is found only in animals contains an auxiliary dimerization interface known as the PAS domain, an acronym that derives from the first three proteins in which it was discovered, PER, ARNT and SIM (18). The bHLH-PAS proteins are heterodimers composed of a class I subunit, either ARNT (aryl hydrocarbon receptor nuclear translocator; also known as HIF-1α), ARNT2, ARNT3 (also known as BMAL1/MOP3) or MOP9, and a Class II subunit, which includes AHR (aryl hydrocarbon receptor), CLOCK, HIF-1α, HIF-2α (EPAS1/HLF/HRF/ MOP2), HIF-3α, NPAS1 (MOP5), NPAS2 (MOP4), SIM1, or SIM2 (19,20).

HIF-1α and HIF-2α have a high degree of amino acid sequence identity within the bHLH-PAS and transactivation domains and, as a result, their ability to dimerize with ARNT or ARNT2 and bind to DNA, and to activate transcription are similar when tested *in vitro* or in cell transfection assays, respectively (21). A cysteine residue present in the basic domain of HIF-2α but not HIF-1α might provide a mechanism for specific redox regulation of DNA binding by HIF-2α-ARNT heterodimers (22). It is not clear whether HIF-2α functions to augment HIF-1α expression in certain cell types or whether heterodimers containing HIF-2α activate target genes distinct from those regulated by HIF-1 as no examples of the latter have been described.

The HIF family members show expression patterns that are more cell type-specific than HIF-1α, although the patterns do partially overlap (23,24). HIF-2α has many biochemical properties similar to HIF-1α, most significantly the ability to bind HIF-1β and induce hypoxic gene expression (24). The HIF-3α locus has recently been shown to produce several splice variants (25), one of which encodes an inhibitory PAS domain protein that may act as a negative regulator of hypoxia-inducible gene expression through competitive inhibition of HIF-1α by binding to HIF-1β (26).

The presence of a HIF-1-binding site is necessary but not sufficient to direct gene expression in response to hypoxia (27), indicating that HIF-1 must interact with other transcription factors bound at adjacent sites (28). Interactions between HIF-1α and hepatocyte nuclear factor 4 (HNF4), which binds at an adjacent site in the hypoxia response element of the human erythropoietin gene, have been demonstrated (29).

Interestingly, HIF-1α is not only responsive to hypoxia. A number of studies indicate that HIF-1β is responsive to hormones such as insulin (30), growth factor such as PDGF, EGF, FGE, TGF- and IGF (1,31), coagulation factors such as thrombin (32), vasoactive peptides such as angiotensin II (33), cytokines (34), or carbachol which activates muscarininic acetylcholine receptors (35) already under normoxia. Moreover, HIF-1α has also been activated by metal ions such as cobalt, calcium (36), chromium (37) and arsenite (38) as well as by mechanical stress (39). The molecular basis enabling these non-hypoxic stimuli to induce HIF-1α is not completely known but may involve reactive oxygen species (ROS) generated either at the endoplasmic reticulum or within mitochondrial and various kinases (40) (Fig. 1).

Oxygen-dependent regulation of HIF-1α subunit

Hypoxia enhances HIF-1α protein levels by inhibiting its degradation, thereby allowing it to accumulate, to dimerize with HIF-1β, and to bind to the HRE in the promoter or enhancer regions of various genes. Thus, the functional HIF-1α-HIF-1β complex is primarily regulated by the abundance of the HIF-1α subunit.

The mechanism by which HIF-1 activation is initiated during hypoxia remains unclear. Both HIF-1α and HIF-1β mRNAs are constitutively expressed, indicat-

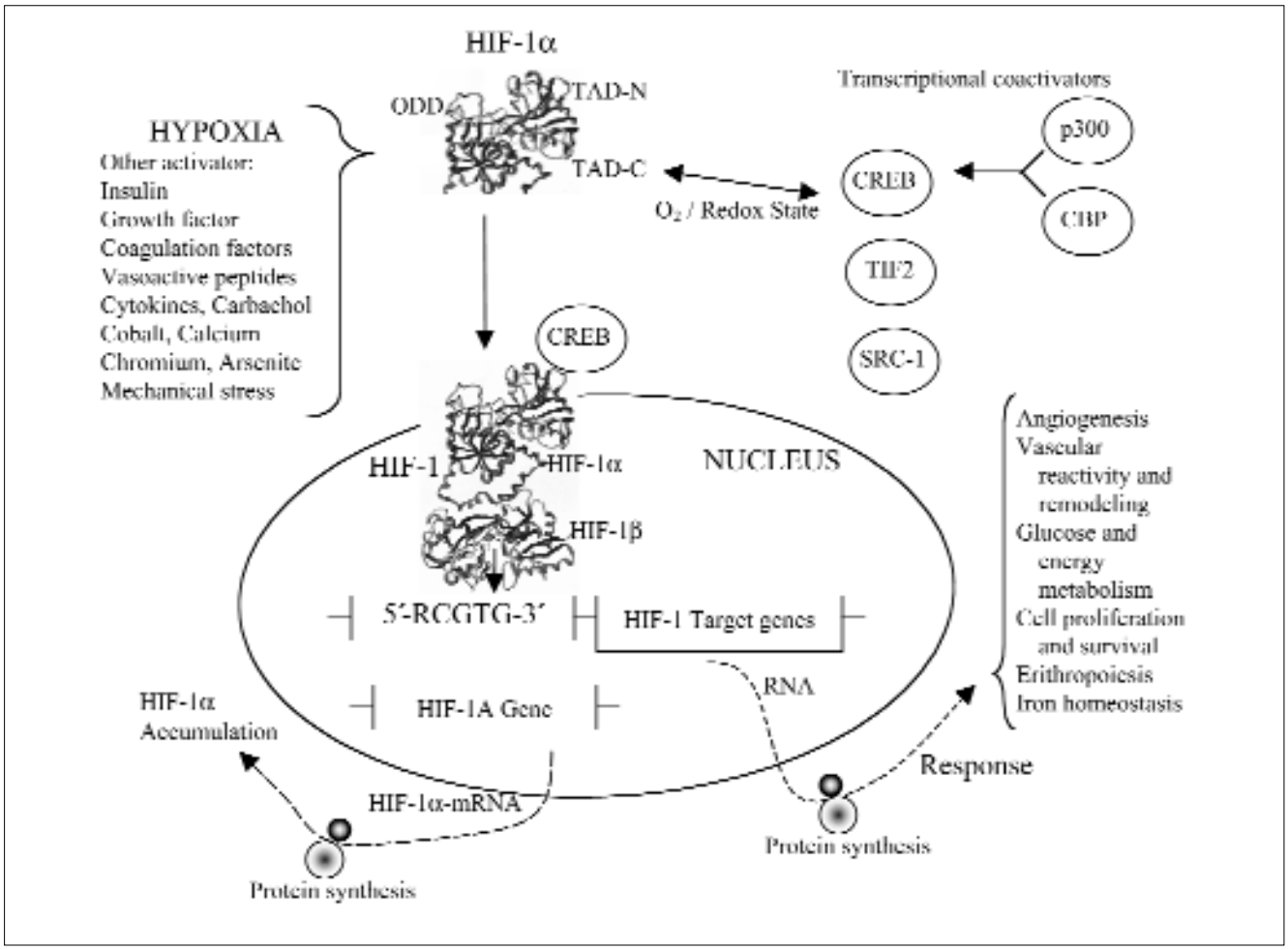

Fig. 1 - Regulation of hypoxia-inducible gene expression by hypoxia inducible factor (HIF-1). HIF-1α interacts with coactivator such as CBP, p300, SRC-1 and TIF2 and this association is regulated by both oxygen concentration and redox state. The ODD (oxygen-dependent degradation domain) is a critical component of HIF-1α and is involved in HIF-1α protein stability. Under hypoxic conditions, HIF-1α is couple with HIF-1β, and binding to the core DNA recognition sequence 5´-RCGTG-3´ which is contained within hypoxia response elements of target genes. The binding of HIF-1 activates increased transcription of these genes and ultimately results in the synthesis of proteins that mediate (patho)physiological responses to hypoxia.

ing that functional activity of the HIF-1α-HIF-1β complex is regulated by post-transcriptional events; although evidence exists that it can also be regulated at the translational levels (41,42). HIF-1β levels are not significantly affected by [O_2], whereas HIF-1α protein is rapidly degradated under normoxic conditions by the ubiquitin-proteasome system. Normoxia desestabilized HIF-1α subunit by O_2-dependent hydroxylation of at least two proline residues (P402, P564) within the oxygen-dependent degradation domain (ODD) (43). This is a prerequisite for the binding of the von Hippel-Lindau tumour suppression protein (pVHL) (44). In association with a multiprotein complex containing elongins B/C, Cullin-2 and Rbx1, pVHL forms a E3 ubiquitin ligase complex which then initiates ubiquitynation and proteosomal degradation (45). Cells lacking functional pVHL express a high level of HIF-1α even under normoxia, which induces expression of many HIF-1-dependent genes (25). Moreover, hereditary pVHL disease is known to be associated with highly vascularized tumours in retinal and central nervous system hemangioblastomas, in part driven by inhibition of HIF-1α degradation (46).

The hydroxylation reactions are carried out by at least four HIF-α prolylhydroxylase domain containing protein (PHD), distinct from those involved in collagen stabilization, although the experimental support for the alter one is limited. Another hydroxylase named factor

inhibiting HIF (FIH) prevents the recruitment of the coactivator CREB binding protein CBP/p300 by hydroxylating an asparaginyl residues (N803) in the C-terminal transactivation domain (TAD-C). In addition, TAD-C can be modified by redox factor 1 (Ref-1) and interact with steroid receptor coactivator-1 (SRC-1) and transcription intermediary factor 2 (TIF-2) (1) in a redox dependent manner (47), thus suggesting the role for ROS in modulating HIF-1 activity. Prolylhydroxylase are mixed-function oxygenases that required, in addition to substrate, molecular oxygen, Fe^{2+} and α-ketoglutarate. During reaction, molecular oxygen is incorporated into both succinate and proline (Fig. 2).

In addition, ascorbate is necessary to achieve full activity, presumably to maintain iron in the Fe^{2+} state indicating that the catalytic hydroxylation process exerted by these enzymes requires a radical cycling system (48) and supporting the important role of ROS in regulating HIF-1α. Thus, changes in the cellular levels of any of these substrates, products or cofactors would have the potential to influence hydroxylase activity. Indeed, treatment of vascular smooth muscle cells with ascorbate has been shown to mediate HIF-1α degradation (49). This appeared to be due to increased PHD activity (50) since under physiological conditions the ascorbate levels are in the range of 25-50 μM which is far below the *K*m for ascorbate of about 140-170 μM (51). It is thus assumed that changes in the ascorbate levels can significantly alter hydroxylase activities. The mechanism by means of which ascorbate regulates hydroxylase activity is not completely solved, yet. On the one hand, ascorbate is thought to reduce Fe^{3+} to Fe^{2+} within the enzyme active site, thus rendering the enzyme active after it became inactive due to spontaneous Fe^{2+} to Fe^{3+} self-oxidation. On the other hand, ascorbate might enhance the provision of Fe^{2+} from an intracellular pool such as ferritin by conversion of Fe^{3+} into Fe^{2+}. This may explain why the iron chelator desferrioxamine enhances HIF-1α levels whereas addition of ascorbate antagonized this effect (50). Moreover, addition of iron to cultured human prostate adenocarcinoma PC3 cells stimulated HIF-1α degradation (50) possibly by rendering HIF-1α susceptible to further modifications exerted by hydroxyl radicals (OH). These radicals could be generated in a Fenton reaction and would be able to reconvert ferric iron into ferrous iron or to facilitate the recycling of dehydroascorbate into ascorbate. Both processes would increase PHD activity leading to the degradation of HIF-1α.

ROS and HIF-1α in the hypoxic signaling process

Since the formation of ROS requires molecular oxygen, it has been suggested that ROS may be involved in the response to hypoxia. Indeed, there has been a long-lasting debate about the role of ROS in oxygen sensing stimulated by the ideas that: (a) a heme protein functioning as an oxidase generates ROS as

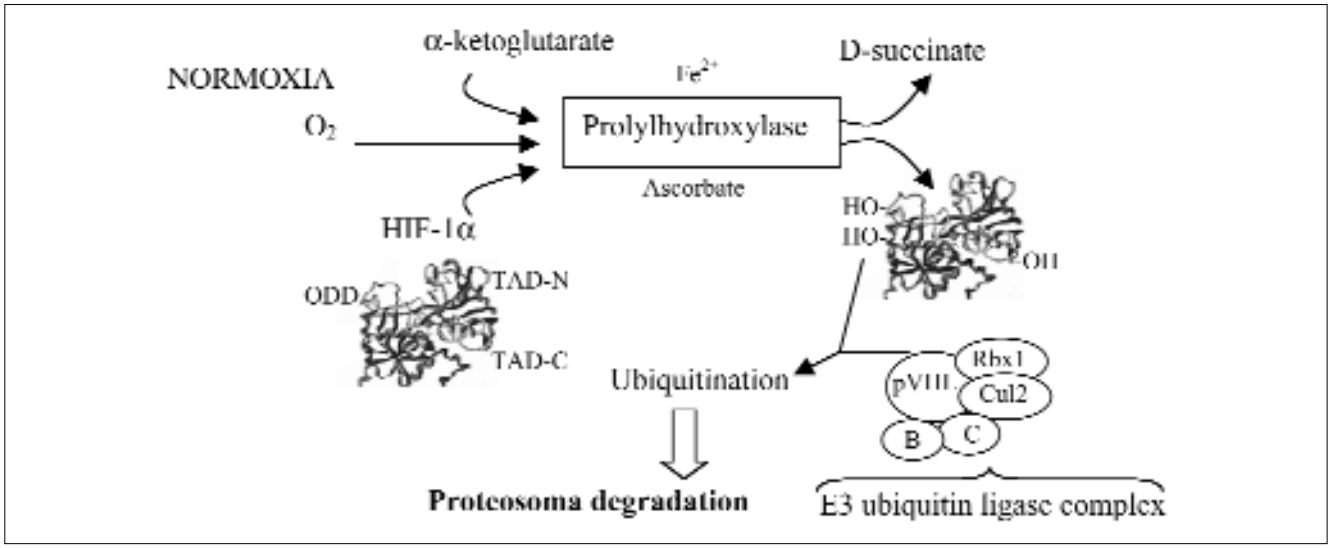

Fig. 2 - Hypoxia inducible factor alpha (HIF-1α) destabilization during normoxia. Under non-hypoxic conditions, the HIF-1α subunit is bound by the von Hippel-Lindau tumour suppressor protein (pVHL), which is the recognition component of a E3 ubiquitin–protein ligase complex that also contains the proteins elongin B, elongin C, Rbx1 and cullin 2. Ubiquitination of HIF-1α targets it for degradation by the 26S proteasome.

signalling molecules; (b) ROS derived from the mitochondrial respiratory chain could contribute to oxygen signalling; or (c) ROS production may be linked to the activity of signalling components upstream of HIF-1α such as hydroxylases or kinases.

Heme-containing oxidases modulate ROS levels and HIF-1α under hypoxia

Spectrophotometrical analyses of carotid body preparations and HepG2 cells demonstrated the presence of a non-mitochondrial, b-type cytochrome similar to the cytochrome b558 of the NADPH oxidase from neutrophils. It was thus suggested that a heme-containing oxidase similar to the NADPH oxidase is able to convert O_2 to radical anion superoxide ($O_2^{\cdot-}$) and subsequently to H_2O_2 and that these ROS act as signalling molecules mediating the response to hypoxia. In addition, a pO_2 and NADH regulated non-mitochondrial oxidase activity was found in microsomes containing a cytochrome b558. Moreover, a cytosolic cytochrome b-type NADPH oxidoreductase was proposed to act as a candidate oxygen sensor (40). Accordingly, inhibition of heme synthesis and addition of CO to the atmosphere prevented oxygen-dependent expression of the EPO and phosphoenolpyruvate carboxykinase (PCK) gene. In addition, application of H_2O_2 repressed the hypoxia-induced EPO production in HepG2 cells and decreased the upregulation of aldolase A, glucokinase and tyrosine hydroxylase by hypoxia in hepatocytes or PC12 cells. Reciprocally, addition of H_2O_2 prevented hypoxic downregulation of the glucagon-dependent PCK expression in primary hepatocytes (40).

The sensitivity of HIF-1 target genes towards application of exogenous oxidants under hypoxia confirmed that also HIF-1 itself is redox-sensitive as seen in experiments in which treatment of purified HIF-1 with H_2O_2 and the oxidative agent diamide abolished HIF-1 DNA-binding activity. In addition, alkylation of free sulfhydryl groups with *N*-ethyl-maleimide led to a loss of DNA-binding activity. This effect could be circumvented by prior addition of dithiothreitol suggesting that HIF-1 DNA-binding activity requires reducing conditions. Indeed, Ref-1, a dual function DNA repair endonuclease and redox regulatory protein and its regulator thioredoxin (Trx) have enhanced HIF-1 activity and protein levels under normoxia and hypoxia, respectively. In contrast, a redox-inactive Trx (C32S/C35S) markedly decreased HIF-1α protein levels (52). Subsequently, Ref-1was found to bind to n-terminal transactivation domain (TAD-N) and TAD-C with a predominant effect on TAD-C (46). Within this domain, the oxidation/reduction states of cysteine 800 in HIF-1α and cysteine 848 in HIF-2α are critical for transactivation and recruitment of CBP/p300, SRC-1 and TIF-2 (46). Mutation of cysteine 800 prevented the decrease in HIF-1α TAD-C activity in response to OH formed in a Fenton reaction at the endoplasmic reticulum (53), further indicating that reducing conditions may promote HIF-1α activity under hypoxic conditions.

Additional evidence that H_2O_2 and other ROS play an important role in oxygen sensing and HIF regulation came from experiments where addition of H_2O_2 to cells grown under hypoxia resulted in destabilization of the HIF-1α protein in Hep3B cells and of the HIF-2α protein in HeLa cells. Consistently, increased $O_2^{\cdot-}$ production by the xanthine/xanthine oxidase system decreased hypoxia-induced HIF-1α protein levels in renal medullar interstitial cells (54).

Whereas H_2O_2 treatment prevented hypoxic accumulation of HIF-1α and its target genes, increased levels of HIF-1α protein under normoxia have been detected in H_2O_2-treated aortic smooth muscle cells (49) and Hep3B cells (55). Moreover, a threshold concentration of H_2O_2 may exist since low concentrations of H_2O_2 (10-50 μM) increased HIF-1α levels, whereas high concentrations of H_2O_2 prevented HIF-1α accumulation (49). Thus, only slight changes in the redox state may be required to activate the HIF pathway. However, the sensitivity of the HIF system towards H_2O_2 may also depend on the cell type since H_2O_2 did not activate HIF-1α in urinary bladder carcinoma ECV304 cells (49). Moreover, the redox-sensitive NFκB pathway may be involved in the regulation of HIF-1α by TNF-α (56), thus suggesting a crosstalk between different redox-sensitive cascades in normoxic cells.

Mitochondria modulate ROS levels and HIF-1α under hypoxia

Mitochondria have long been implicated to play a role in the hypoxic response since inhibitors of the mitochondrial respiratory chain have been shown to mimic hypoxia in the carotid body (57). Two different concepts have been proposed to explain how the response to hypoxia can be related to mitochondrial function. First, *decreased* mitochondrial ROS production would mediate the hypoxic response or, second, *increased* ROS generation would allow the hypoxic response (40). The first concept is based on the assumption that under O_2 limiting conditions ROS formation is

diminished. Indeed, during alveolar hypoxia pulmonary ROS production was reduced. This has been supported by findings in pulmonary arteries where hypoxia and the proximal respiratory chain inhibitors rotenone and antimycin A decreased generation of ROS as measured by three different techniques (lucigenin-based chemiluminescence, the AmplexRed H_2O_2 assay kit and 2',7'-dihydrochlorofluorescin (DCFH) fluorescence) (58). Consistently, many other laboratories reported decreased ROS formation under hypoxia in the lung, hepatocytes and cardiac myocytes (49,50).

Although somehow counterintuitive, the concept that hypoxia increases ROS production was based on findings that ROS levels were elevated under hypoxia in pulmonary myocytes, cardiac myocytes, Hep3B cells, HeLa cells and adipocytes (40,60). Since in hepatocytes pO_2 levels below 2-3 mmHg decrease cytochrome c oxidase activity, it was proposed that hypoxia is sensed by cytochrome c oxidase which then reduces its *V*max, thereby enhancing the half-life of reduced electron carriers upstream of cytochrome aa3 such as ubisemiquinone anions. The ubisemiquinone anion may then transfer an electron to molecular O_2 yielding $O_2^{\cdot-}$ which is then dismutated by Mn-SOD to the signal molecule H_2O_2 (61). Thus, inhibitors which block the respiratory chain upstream of ubisemiquinone such as rotenone and myxothiazol would prevent ROS formation whereas downstream inhibitors such as cyanide or azide would increase ROS levels. The H_2O_2 so produced could diffuse into the cytosol or could be degraded by the mitochondrial antioxidant system. Superoxide generated at Complex III could alternatively enter the cytosol via anion channels in the mitochondrial membranes (62). Higher concentration of $O_2^{\cdot-}$ blocked PHD activity, although molecular concepts to explain this effect remain multifarious it is assumed that H_2O_2 will promote oxidation of iron, thereby blocking PHD activity (63). In addition, it was reported that inhibition of cell respiration by NO accounts for destruction of HIF-1α under hypoxia (64). This model implies that binding of NO to cytochrome c oxidase ceased motochondrial respiration with increased availability of non-respiratory oxygen in cell. Oxygen which is no longer reduced to water in the respiratory chain is redistributed and become available to PHD to restore their activity under hypoxia (65) (Fig. 3).

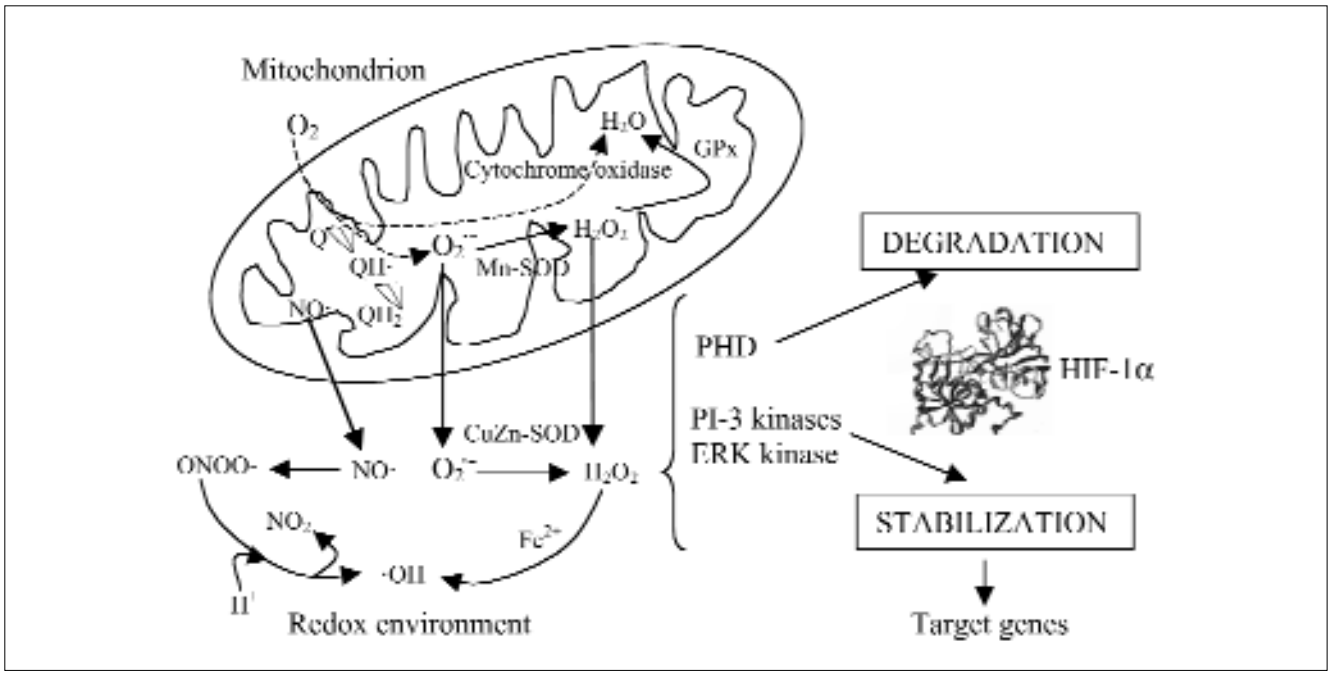

Fig. 3 - Involvement of mitochondria and reactive oxygen species (ROS) in Hypoxia inducible factor alpha (HIF-1α) regulation. Hypoxia stimulates radical anion superoxide ($O2^{\cdot-}$) generation at Complex III. Superoxide enters the cytosol via anion channels and is converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 activates phosphatidylinositol 3-kinase (PI-3 kinases), ERK kinase and triggering HIF-1a stabilization. Cross-talks between different signaling cascades activated by high or low ROS levels may also influence the stability and / or activity of HIF-1a, this allowing to fine tune HIF-1-dependent target gene expression in a cell-type specific manner. GPx, glutathione peroxidase; NO, nitric oxide; NO_2, nitrogen dioxide; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite anion; PHD, Prolyl hydroxylase domain-containing protein; Q, ubiquinone; QH·, ubisemiquinone; QH_2, ubiquinol.

Interestingly, most of the studies showing increased ROS production under hypoxia were performed by exposing cells for several hours to the non-fluorescent dye DCFH which is converted by ROS into fluorescent dichlorofluorescein (DCF) (40). However, ROS measurements by DCF are debatable since it has been shown that DCFH can autooxidize and that deacetylation of DCFH-DA can produce H_2O_2 (66). Thus, although DCFH is among the most frequently used fluorophores for ROS measurements; it should be considered that the sensitivity of this assay could be influenced by various conditions not directly related to intracellular ROS generation.

A role for mitochondrial ROS production in the regulation of HIF was also suggested from studies, where rotenone and myxothiazol decreased DCF fluorescence and prevented HIF-1 accumulation and activation of its target genes by hypoxia (67). By contrast, in cells lacking mitochondrial DNA (Hep3Bρ0) hypoxia did not enhance DCF fluorescence and HIF-1α levels (67,68). These data suggested that inhibition of mitochondrial ROS production would prevent the hypoxic response. However, antimycin A which enhances mitochondrial ROS production did not mimic the hypoxic response in Hep3B (61,67) and HEK293 cells (69). The relevance of the mitochondrial respiratory chain as an ubiquitous oxygen sensor was further questioned since mitochondrial production of ROS was not involved in O_2 sensing in the airway chemoreceptor cell line H146 (70). Similarly, H_2O_2 levels decreased under hypoxia in Hep3Bρ0 as well as in control cells suggesting that the source of H_2O_2 was not located in the mitochondria (71). Consistently, hypoxia-dependent induction of HIF-1 was neither affected by rotenone or a lack of mitochondria in Hep3B and HeLa cells (71,72) nor by defects in genes encoding different components of the electron transport chain in the Chinese hamster ovary cell lines Gal32 and CCLI 6B-2 (71,72). Further, hypoxia-mediated upregulation of GLUT1 was mediated by binding of HIF-1 to the HRE, whereas the induction of GLUT1 by antimycin A or rotenone involved a serum response element. Moreover, inhibition of mitochondria-derived ROS did not prevent HIF-1α stabilization in gastric cancer cells infected with *Helicobacter pylori* (73).

In summary, these data support the assumption that modulation of ROS levels has an important impact on the hypoxic response mediated by HIF-1α. However, the sources of ROS generation, the exact kinetics and conditions of ROS production and their specific relevance to HIF-1α activation are not completely resolved, yet.

Activation of HIF-1 in Tumour Progression

Microenvironmental hypoxia is a hallmark of tumour progression. This is attributable to a decrease in capillary density and functional disorganization of the vasculature surrounding proliferating tumour cells. Although hypoxia alone has negative effects on cellular proliferation (74), the acclimation to decreased oxygen concentration transcriptionally activates many genes involved in angiogenesis, anaerobic metabolism and the cell cycle, through the activation of HIF-1 (11,16). Recent immunohistochemical analyses using an antibody specific for the HIF-1α subunit revealed high-level expression of the protein in various types of human cancers, including mammary gland, prostate, lung, colon, and brain, suggesting critical roles of HIF-1 in tumour formation (23). In addition, clinical studies have shown that HIF-1α immunostaining is pronounced in aggressive tumours (Tab. I) (75-77). In addition to the effects of intratumoural hypoxia, genetic alterations in a number of tumour suppressor genes and oncogenes have also been implicated in the normoxic activation of HIF-1 pathways in the multistep process of tumour development.

Table I - Association HIF-1α overexpression-tumour progression[a]

BRAIN TUMOUR	**HIF-1α Score**[b]
Low grade astrocytoma or mixed glioma	2.0
Anaplastic astrocytoma or oligodendroglioma	2.9
Glioblastoma multiforme	3.6
BREAST TISSUE	**%(+)Cells HIF-1α**[c]
Normal	0.0
Hyperplasia	0.0
Ductal carcinoma well differentiated	3.5
Breast cancer well differentiated	14.9

Notes:
a Data from Refs 75-77
b All brain tumours that were analyzed over-expressed HIF-1α relative to surrounding normal brain tissue. Scoring: 1, nuclear staining in <1% of cells; 2, 1–10%; 3, 10–50%; 4, >50%. Mean score within each group is shown.
c Mean percentage of cells analyzed per biopsy (normal and hyperplasia n=10, cancer n=20) with detectable HIF-1α expression.

Effected molecules include pVHL, PTEN (phosphatase and tensin homologue deleted on chromosome 10), Ha-*ras*, v-*Src*, and p53, and comprise a diverse and complex array of pathways that involve not only protein stabilization, but also phosphorylation, nuclear translocation, and transcriptional cofactor recruitment of HIF-1α (2,16,47), Regardless of the stimulus for HIF-1α activation, tumour progression appears to favour sustained HIF-1α expression, inasmuch as HIF-1 acts as a major regulator of angiogenesis and anaerobic energy metabolism under certain conditions. Among over two dozen HIF-1 target genes, vascular endothelial growth factor (VEGF) is the most prominent and plays an important role in tumour neovascularization, which supplies sufficient amounts of oxygen and nutrients to tumour cells to allow tumour propagation and metastatization (78). Furthermore, induction of anaerobic glycolytic enzymes and genes involved in glucose uptake allows the tumour to maintain energy production regardless of oxidative phosphorylation and survive with a limited oxygen supply. Indeed, tumour cells, along with other highly proliferative cells, have been shown to use glycolytic pathways, rather than oxidative phosphorylation for ATP production. This might be due to constitutive HIF-1 expression even under normoxia (10). Further support for the view that HIF-1 is a positive regulator of tumour progression comes from studies of experimental tumours using mouse hepatoma cells either wild-type or null for HIF-1β (78). Tumours lacking HIF-1β grow more slowly and form a defective vascular network relative to wild-type HIF-1β cells when injected into mice, although both cell types are able to proliferate on tissue culture dishes under either normoxia or hypoxia. These results indicate that loss of HIF-1 activity suppresses tumour angiogenesis, but not tumour proliferation, due to reduced production of angiogenic factors such as VEGF. Another recent study used peptides to block the interaction between HIF-1α and p300. These cells displayed tumour growth retardation, as well as decreased angiogenesis (12). Some results are consistent with the concept of HIF-1 as a positive modulator of tumourigenesis in which tumours derived from HIF-1α null mouse embryonic stem cells showed a significant reduction in size. However, alterations in vascular density within tumours were not observed in HIF-1α null tumours (13,79). Collectively, these data suggest HIF-1 activation has different effects on tumour progression with a variable net outcome under different conditions.

Future directions

The benefits of exploiting tumour hypoxia have yet to be fully realized. Despite this, the positive clinical results with the combination of the hypoxic cytotoxin tirapazamine with cisplatin to treat advanced non small-cell lung cancer and with chemoradiotherapy to treat advanced head and neck cancer demonstrate the potential of this approach (6,80). Pharmacological research is often limited by bioassays that only monitor a narrow set of HIF-1 regulatory pathways. It may be necessary to incorporate both environmental and genetic factors into the *in vitro* model(s) when developing bioassays to screen chemical libraries for HIF-1 inhibitors. Since hypoxic tissues are intrinsically undervascularized, drug delivery will remain a critical factor that can hinder or contribute to the clinical success of HIF-1 inhibitors. We now have tools for quantifying the ability of prodrugs, and their activated metabolites, to diffuse in tumour issue, so designing second-generation prodrugs with properly optimized micropharmacokinetic properties is a clear possibility for the future.

In general, the rationale of molecular-targeted drug research is to identify new therapeutically useful agents that selectively target a disease-specific molecular mechanism(s) or pathway(s). In oncology, the goal is to discover new chemotherapeutic agents that are directed only at tumour cells and would not cause general cytotoxicity-related side effects (81). The discovery of new natural product-based inhibitors of HIF-1 holds such promise. Therefore, it is surprising to see that so much effort in the discovery of HIF-1 inhibitors is currently aimed at characterizing molecules that inhibit HIF-1 at cytotoxic concentrations (summarized in Tab. II). Efforts to discover therapeutically useful HIF-1 inhibitors, including natural product-based inhibitors, should include an early stage deselection criteria that exclude cytotoxic compounds. Molecular-targeted drug discovery programs that fail to consider the cytotoxic properties of the active compounds identified are destined to continue producing relatively non-selective cytotoxic antitumour agents. On the other hand, programs that combine well designed bioassays and unique sources of chemical diversity, such as natural products, will have great potential to discover tumour-specific chemotherapeutic agents.

Other strategies as: -hypoxia activated gene therapy, targeting HIF-1 and the use of recombinant clostridia ; - are certainly promising and have demonstrated antitumour efficacy in preclinical studies. What is now needed is the development of the optimum drug or vector combination for each strategy and their clin-

Table II - Examples of inhibitors of hypoxia-inducible factor-1 alpha (HIF-1α) by different mechanisms

Final effect	Mechanism	Active compound
Decrease HIF-1α synthesis[a]	Inhibit transcription	Actinomycin D
	Degrade HIF-1α mRNA	Picroside-I
	Decrease translation	Topotecan
Increase HIF-1α degradation		
Inhibit hsp90b		
Geldamycin		
	Block the binding of HIF-1α to hypoxia response elements	
Radicicol		
	Disrupting the interaction between HIF-1α and hsp90	Apigenin
	Facilitate proteasome-mediated HIF-1α degradation	Resveratrol
	Over-expression of HIF prolyl hydroxylase	Cyclosporine
	Preventing oxygen-dependent degradation domain	Cyclosporine
	Mitochondrial inhibitors	Rotenone
	Increase expression of pVHL	R-trichostatin
	Enhance interaction HIF-1α / pVHL	Berberine
	Promoting proteosoma mediated degradation	Pseudolaric acid
	Blocking the induction of HIF-1α	Genistein

Notes:
a Cycloheximide inhibited general eukaryotic protein synthesis and block hypoxia-induced HIF-1α protein accumulation and HIF-1 activation.
b hps90 (heat shock protein 90) binds to the HIF-1α PAS domain and stabilizes HIF-1α protein
Legend: pVHL, Von Hippel–Lindau protein

ical testing. Relevant to the latter is the appropriate selection of patients with whom to use hypoxia-directed treatments. As with any targeted anticancer strategy, hypoxia-directed therapy can only be effective on those tumours expressing the target or, in this case, with sufficient levels of hypoxia. Performing clinical trials on unselected patients whith hypoxic and better-oxygenated tumours runs the clear risk of rejecting a treatment that could be of significant benefit to a subset of patients. The most appropriate means of assessing tumour hypoxia to perform such a selection is under active investigation (82). Clearly, there is much to be done to exploit the unique features of hypoxia, HIF-1α, other molecular targets upregulated under hypoxia and necrosis in human solid tumours, but the future is promising.

Conclusion

Hypoxia plays an important role in various types of pathophysiological conditions, such as cancer, as well as physiological and developmental processes. HIF-1 is a central regulator of cellular hypoxic response and controls expression of critical hypoxia-responsive genes, allowing cells to acclimate to different oxygen concentrations. Recent studies of HIF-1 regulatory systems provide new clues as to how organisms sense alterations in oxygen concentration and transmit this information to downstream effectors. It is still to be clarified how HIF-1 role in the maintenance of cell and organ integrity, how loss of HIF-1 function alters cellular mechanisms, and how other transcription factors, including other HIFs, cooperate to control gene expression. As new information is collected, we will gain a more complete picture of the effect of oxygen on biological processes.

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Abbreviations

·OH, Hydroxyl Radical
AHR, aryl hydrocarbon receptor
ARNT, aryl hydrocarbon receptor nuclear translocator
bHLH, basic helix-loop-helix
CBP, cAMP-response element-binding protein
CREB, (cAMP-response element-binding protein)-binding protein (E1A-binding protein, 300 kD)
DCF, 2',7' dichlorofluorescein
DCFH, 2',7' dihydrochlorofluorescein
DFO, Desferoxamine
EPO; erythropoietin
FIH-1, factor-inhibiting hypoxia-inducible factor-1
HIF-1, hypoxia-inducible factor-1
HRE, hypoxia response element
$O_2^{\cdot-}$, Radical anion superoxide
ODD oxygen-dependent degradation domain (residues 401-603)
p300, E1A-binding protein, 300 kD
PAS, Per/ARNT/Sim
PCK, phosphoenolpyruvate carboxykinase
PHD, Prolyl hydroxylase domain-containig protein
PTEN, phosphatase and tensin homologue deleted on chromosome 10
pVHL,Von Hippel-Lindau
Ref-1, Redox factor 1
ROS, Reactive Oxygen Species
SRC-1, Steroid Receptor Coactivator-1
TAD-C, C-terminal transactivation domain (residues 786-826)
TAD-N, N-terminal transactivation domain (residues 531-575)
TIF-2, Transcription intermediary factor 2
Trx, thioredoxin
VEGF, vascular endothelial growth factor

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