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Hypoxia. 1. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets

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Miyata T, Takizawa S, van Ypersele de Strihou C. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets. *Am J Physiol Cell Physiol* 300: C226–C231, 2011. First published October 27, 2010; doi:10.1152/ajpcell.00430.2010.—A variety of human disorders, e.g., ischemic heart disease, stroke, kidney disease, eventually share the deleterious consequences of a common, hypoxic and oxidative stress pathway. In this review, we utilize recent information on the cellular defense mechanisms against hypoxia and oxidative stress with the hope to propose new therapeutic tools. The hypoxia-inducible factor (HIF) is a key player as it activates a broad range of genes protecting cells against hypoxia. Its level is determined by its degradation rate by intracellular oxygen sensors prolyl hydroxylases (PHDs). There are three different PHD isoforms (PHD1–3). Small molecule PHD inhibitors improve hypoxic injury in experimental animals but, unfortunately, may induce adverse effects associated with PHD2 inhibition, e.g., angiogenesis. As yet, no inhibitor specific for a distinct PHD isoform is currently available. Still, the specific disruption of the PHD1 gene is known to induce hypoxic tolerance, without angiogenesis and erythrocytosis, by reprogramming basal oxygen metabolism with an attendant decreased oxidative stress in hypoxic mitochondria. A specific PHD1 inhibitor might therefore offer a novel therapy against hypoxia. The nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) regulates the basal and inducible expression of numerous antioxidant stress genes. Disruption of its gene exacerbates oxidative tissue injury. Nrf2 activity is modulated by Kelch-like ECH-associated protein 1 (Keap1), an intracellular sensor for oxidative stress. Inhibitors of Keap 1 may prove therapeutic against oxidative tissue injury.

oxygen sensor; hypoxia; prolylhydroxylase; hypoxia-inducible factor; nuclear factor-erythroid 2 p45-related factor 2; Kelch-like ECH-associated protein 1

Hypoxia and Oxidative Stress

ALL MAMALIAN ORGANS require a sufficient and consistent supply of oxygen to fuel various biometabolic processes, including oxidative phosphorylation during mitochondrial respiration. A decreased oxygen supply, i.e., hypoxia, induces not only acute disorders like ischemic heart disease but also chronic disorders, such as renal fibrosis (38). Ries et al. (46) demonstrated tissue hypoxia in the kidneys of streptozotocin-induced diabetic rats by blood oxygen level-dependent imaging. This finding was confirmed later by pimonidazole staining (a probe to detect hypoxia) and by the levels of hypoxia-inducible factor (HIF) (47). Tissue hypoxia was also documented in a hypertensive, type 2 diabetic rat model (21). Interestingly, the localization of tissue hypoxia may differ in the same organ, according to the type of disease. Tanaka et al. (58) have taken advantage of an hypoxia-responsive reporter vector, carrying hypoxia-responsive elements (HRE) of the rat vascular endothelial growth factor (VEGF), to generate a novel hypoxia-sensing transgenic rat, the first in vivo biosensor system that allows quantitative evaluation of local hypoxic status at a cell-to-cell resolution (58). The hypoxia-responsive transgene works optimally under the oxygen concentration of 1 to 5% (7–40 mmHg). With this model, they identified in the kidney a "diffuse cortical" hypoxia pattern in the puromycin aminonucleoside-induced nephrotic syndrome and a "focal and segmental" hypoxia pattern in the remnant kidney model.

Oxidative stress during hypoxia may sound paradoxical. Yet, it may be induced not only by a rise but also by a fall in oxygen tension. The hypoxic cell relies on anaerobic glycolysis to generate ATP, whereas its residual low oxygen supply supports some level of oxidative production of ATP through the tricarboxylic acid cycle and electron transport chain (ETC). Electrons leaking from the mitochondrial ETC generate an excess of reactive oxygen species (ROS), i.e., oxidative stress. Reoxygenation or high oxygen levels following severe hypoxia further exaggerate ROS generation. This concept is validated by the clinical benefits accruing from the use of agents able to scavenge ROS or preventing their formation in hypoxic lesions (27).

Cellular Defense Mechanisms

Hypoxia. Defense against hypoxia hinges upon the HIF (33, 51), which activates a broad range of genes that stimulate erythrocytosis, angiogenesis, glucose metabolism, or cell proliferation/survival and eventually protect hypoxic tissues.

HIF-α is constitutively transcribed and translated in cultured cells. In vivo, hypoxia or ischemia induces HIF-1α mRNA expression (3, 7, 29, 64, 67). Its oxygen-dependent degradation rate determines its level (Fig. 1, *top*). In the presence of oxygen, HIF-α undergoes enzymatic hydroxylation by prolyl hydroxylases (PHDs) (13, 49). Hydroxylated HIF-α is then recognized by the Hippel-Lindau tumor suppressor protein (pVHL) (17, 20), acting as an E3 ubiquitin ligase, and is rapidly degraded by the proteasome (34, 42). During hypoxia, the nonhydroxylated HIF-α escapes interaction with pVHL and is thus stabilized. After binding to its heterodimeric partner HIF-1β mainly in the nucleus (12), it transactivates genes involved in the adaptation to hypoxic-ischemic stress.

Three isoforms of the HIF-α subunit have been identified (22) (i.e., HIF-1α, HIF-2α, and HIF-3α). HIF-1α and HIF-2α are structurally and functionally similar. In contrast, HIF-3α lacks the structures for transactivation present in the COOH-

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termini of HIF-1α and HIF-2α and might play an alternative role as a negative regulator of hypoxia-inducible gene expression (32).

Recent studies in mice, utilizing gene disruption of either HIF-1α or HIF-2α, disclosed that HIF-2α acts as a physiological regulator of erythropoietin (56). The HIF-2α gene is responsible for familial erythrocytosis in humans (45) and for comparatively high hemoglobin concentrations in polycystic kidney disease (pericystic hypoxia leading to HIF-2 induction) (5). In addition, it plays a crucial role in the defense against oxidative stress (1, 26).

PHDs belong to the Fe(II) and 2-oxoglutarate-dependent dioxygenase superfamily (22), which incorporates two atoms of molecular oxygen into their substrates: the first, used in the oxidative decarboxylation of 2-oxoglutarate, yields succinate and carbon dioxide, whereas the second is incorporated directly into the proline residue of HIF-α. They are called "oxygen sensors" as their activity rigorously depends on oxygen tension (15).

Iron is essential for PHD activity. As a consequence, transition metal chelators should inhibit PHD activity. Cobalt chloride inhibits PHD activity through an intracellular depletion of ascorbate necessary for iron (reduced) activity (48). The erythropoietic effect of cobalt is known in humans since the 1940s (4, 54) and has been utilized in the 1970s to treat the anemia associated with chronic renal failure (11). Unfortunately, cobalt chloride proved too toxic for further clinical use.

Three different PHD isoforms have been identified (22) (i.e., PHD1, PHD2, PHD3), each of which has its own tissue and subcellular distribution (36). PHD1 is exclusively nuclear, PHD2 is mainly cytoplasmic [but shuttles between nucleus and cytoplasm (55)], and PHD3 is present in both cytoplasm and nucleus. PHD2 acts as a decisive oxygen sensor in the HIF degradation pathway (2). Although hypoxia decreases overall PHD activity, upregulation of HIF-1α induces the expression of PHD2 and PHD3 (14). This HIF-induced PHD expression ensures rapid removal of HIF-α after reoxygenation. Feedback loops may thus exist duiring hypoxia signaling.

Hypoxia (recreased availability of oxygen) and nitric oxide (NO) decrease PHD activity (2, 60). Wang et al. (62) recently demonstrated that under normal conditions, individual mitochondria undergo spontaneous transient bursts of quantal superoxide generation, termed "superoxide flashes." Superoxide flashes are observed in all cell types investigated to date and are triggered by a surprising functional coupling between the mitochondrial permeability transition pore activation and ETC-dependent superoxide production. Importantly, reoxgenation following hypoxia leads to uncontrolled superoxide flash gen-

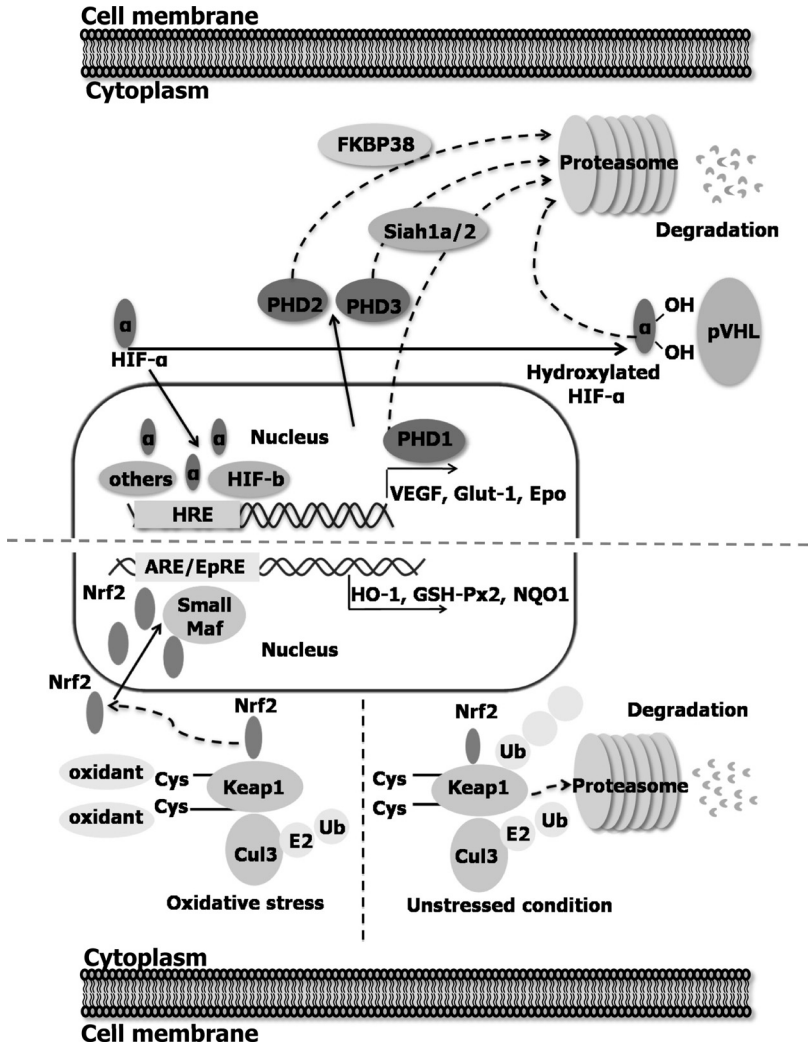

Fig. 1. Cellular defense mechanisms against hypoxia and oxidative stress. *Top*: hypoxia-inducible factor-prolyl-1 hydroxylase (HIF-PHD) pathway under hypoxia. HIF-α is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation. Oxygen determines its stability through its enzymatic hydroxylation by prolylhydroxylases (PHDs). The hydroxylated HIF-α is recognized by Hippel-Lindau tumor suppressor protein (pVHL), an E3 ubiquitin ligase, and is rapidly degraded by the proteasome. Nonhydroxylated HIF-α cannot interact with pVHL and is thus stabilized. It binds to its heterodimeric partner HIF-β mainly in the nucleus and transactivates genes involved in the adaptation to hypoxic-ischemic stress. Expression of PHDs (PHD2 and PHD3) is regulated by HIF. PHDs interact with Siah1a/2 (PHD1 and PHD3) or FKBP38 (PHD2) and are subject to proteasomal degradation. PHD activity is inhibited under hypoxia or by nitric oxide, reactive oxygen species (ROS), transition metal chelators, cobalt chloride, 2-oxoglutarate analogs, or TM6008/TM6089. *Bottom*: nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway under oxidative stress. Nrf2 is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation through the Keap1-Cullin3 (Cul3) system. Nrf2 is ubiquitinated continuously and degraded within the proteasome. Under oxidative stress, reactive cysteines within the Keap1 moiety undergo conformational changes, eventually leading to detachment of Nrf2 from Keap1 and to inhibition of its ubiquitination. Oxidative stress thus inhibits the degradation of Nrf2 and facilitates nuclear translocation of Nrf2. Nrf2 then heterodimerizes with a small Maf protein, binds to the antioxidant/electrophile responsive element (ARE/EpRE), and transactivates a variety of antioxidant genes.

esis and contributes to increased oxidative stress during hypoxic injury. ROS affects PHD activity and stabilizes HIF-1α activity by chelating and oxidizing PHD-bound Fe(II) to Fe(III) or through the activation of mitchondrial-dependent ROS signal (8, 44, 50).

Oxidative stress. Hypoxia is intimately related to oxidative stress. It is of interest that the genetic disruption of the PHD1 gene in hypoxic mice lowers oxygen consumption in the mitochondria of skeletal muscle, reduces oxidative stress, and eventually enhances cellular survival (1). In agreement with this observation, the activation of HIF-1α reduces, whereas its inhibition worsens ROS generation (6, 24).

Concurrently, oxidative stress exacerbates the status of hypoxia. In vitro studies in rat proximal tubular cells or in vivo studies in streptozotocin-induced diabetic rats show that high glucose blunts the activation of HIF, an effect fully reversed by treatment with antioxidants, such as α-tocopherol or tempol (23, 47). NADPH oxidase activation also aggravates renal hypoxia (65). Altogether, hypoxia and oxidative stress are closely linked in the kidney.

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a basic leucine zipper redox-sensitive transcriptional factor that regulates the expression of several cellular antioxidant and cytoprotective genes. Upon exposure to oxidative stress and/or electrophiles, Nrf2 translocates into nuclei, heterodimerizes with a small Maf protein, eventually binds to the antioxidant/electrophile responsive element (ARE/EpRE), and activates the transcription of antioxidant genes, including heme oxygenase-1, glutathione peroxidase-2, NAD(P)H-quinone oxidoreductase 1, and glutathione *S*-transferase (25, 41). Nrf2 causes thus a broad and coordinated set of downstream reactions against oxidative stress.

Nrf2-mediated transcriptional responses are protective in a variety of experimental animals models including oxidative lung injury and fibrosis, asthma, and brain ischemia reperfusion injury (9, 10, 52). For example, induction of renal ischemia followed by reperfusion in wild-type mice elevate Nrf2 levels and activate their downstream target genes in the kidneys (30). By contrast, Nrf2 deficiency enhances susceptibility of mice to both ischemic and nephrotoxic acute kidney injury (31). Treatment of Nrf2 knockout mice with the antioxidants *N*-acetyl-cysteine or glutathione improves renal function. Furthermore, Nrf2 knockout mice with streptozotocin-induced diabetes progressively increase their urinary excretion of NO metabolites (an indirect evidence of oxidative stress) and develop renal injury (66). Upregulation of Nrf2 might thus be a potential therapeutic target to mitigate oxidative stress-induced tissue injury.

The regulation of Nrf2 has been recently elucidated (Fig. 1, *bottom*). Nrf2 is constitutively transcribed and translated. It is ubiquitinated continuously through the Kelch-like ECH-associated protein 1 (Keap1)-cullin3 (Cul3) system and degraded within the proteasome (18, 61). Its level depends on its rate of destruction. Keap1 is a sensor of oxidative stress and acts as a negative regulator of Nrf2. Under oxidative stress, reactive cysteines within the Keap1 moiety undergo conformational changes, eventually leading to the detachment of Nrf2 from Keap1 and the inhibition of its ubiquitination. Oxidative stress thus inhibits the degradation of Nrf2, facilitating its nuclear translocation.

In Keap1 knockdown mice, Nrf2-regulated gene expression significantly increases and ameliorates oxidative liver injuries in the obstructive cholestasis (43). Inhibition of Keap1 could thus afford tissue protection against ischemia through an increased nuclear translocation of Nrf2 and the subsequent activation of antioxidant genes.

Therapeutic Perspectives

Hypoxia. The degradation of HIF-α through the oxygen-dependent hydroxylation of specific proline residues by PHDs is amenable to inhibition. Small molecular inhibitors of PHDs have thus been investigated (15). The binding of the substrate 2-oxoglutarate to the catalytic domain of PHDs appears essential for the enzymatic PHD activity. Chemical compounds whose structure mimick 2-oxoglutarate [e.g., *N*-oxalylglycine (dimethyloxalylglycine) (19, 39), *N*-oxalyl-D-phenylalanine (35), L-minosine (16)] are therefore able to inhibit PHD activity.

Relying on a different strategy including docking simulation based on the three-dimensional protein structure of human PHD2, we synthetized two novel inhibitors of PHDs (TM6008 and TM6089) (40). Both compounds bind to the active site within the PHD2 molecule where HIF binds (Fig. 2). As anticipated, given orally, they stimulate HIF activity in various organs of transgenic rats expressing a hypoxia-responsive re-

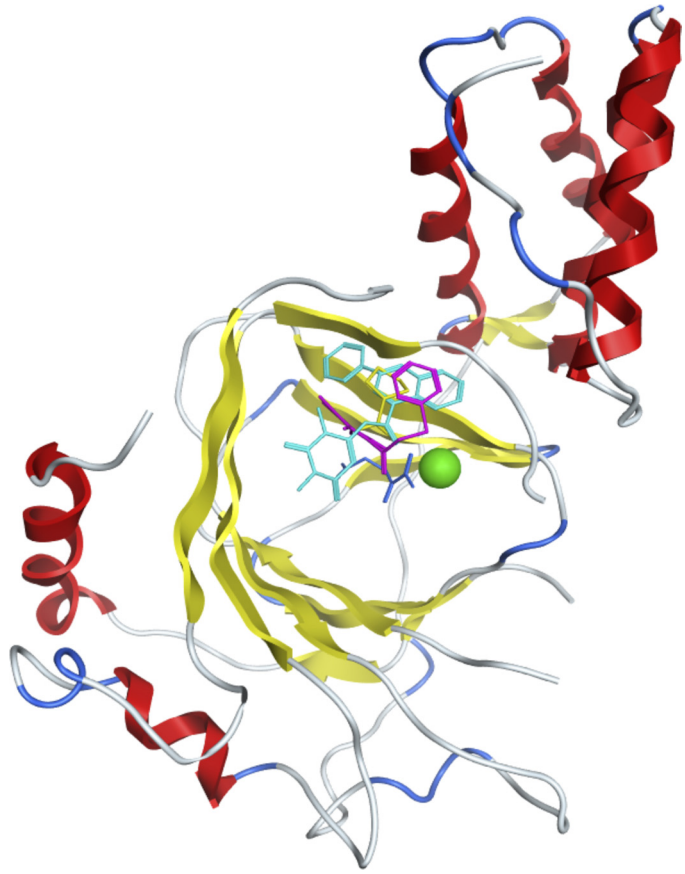

Fig. 2. Predicted binding modes of PHD inhibitors. TM6008, TM6089, HIF proline, and 2-oxoglutarate are shown in light blue, magenta, yellow, blue, respectively. Fe(II) is shown by a green sphere. Figure is drawn by the software Discovery Studio Visualizer 2.0 (Accelrys Software, San Diego, CA).

porter vector. Given locally, they induce angiogenesis in a mouse sponge assay (40).

Unfortunately, nonspecific inhibition of HIF-α degradation also augments VEGF and erythropoietin production, both of which have proven detrimental for proliferative diabetic retinopathy in humans by multivariate logistic-regression analyses of VEGF and erythropoietin levels in the vitreous fluid (63). Dissociation of the benefits of HIF activation from its noxious effects on VEGF and erythropoietin is therefore needed.

The role of each PHD isoform has been recently delineated by the specific disruption of each PHD gene. Broad-spectrum conditional knockout of PHD2 induces VEGF and an hyperactive angiogenesis, with the formation of mature and perfused blood vessels. In agreement with these observations, TM6008, a compound potentially binding human PHD2 in docking simulation studies, induces angiogenesis in mice (40). PHD3 is also involved in angiogenesis: in mice with hindlimb ischemia, therapeutic revascularization is better after PHD3 than after PHD2 gene silencing (55).

Both PHD1 and PHD3 gene knockout in mice has no apparent effect on erythropoiesis (56) but double PHD1 and PHD3 knockout induces the accumulation of HIF-2α in the liver with a moderate erythrocytosis. Adult PHD2-deficient mice develop a severe erythrocytosis with a dramatic increase in the levels of serum erythropoietin and erythropoietin mRNA in kidney (56). These results are taken to indicate that PHD1/3 double deficiency leads to erythrocytosis partly through the activation of the hepatic HIF-2α/erythropoietin pathway, whereas PHD2 deficiency leads to erythrocytosis by activating the renal pathway.

Dissociation between the benefits of HIF activation and its effects on angiogenesis and erythropoiesis has been recently illustrated by the group of Carmeliet (1). The specific disruption of PHD1 unexpectedly induces hypoxic tolerance in muscle cells, without angiogenesis and erythrocytosis, at least in part through the activation of HIF-2α. Basal oxygen metabolism is reprogrammed and oxidative stress generation is decreased in hypoxic mitochondria. Inhibition of PHD1 likely stimulates various protective mechanisms, such as ATP production through enhanced glycolysis and a restriction of substrate for oxidative phosphorylation through the induction of pyruvate dehydrogenase kinase, with the eventual attenuation of electron entry into ETC. As a consequence energy is conserved, oxidative damage is reduced, and cells are protected from hypoxic damage. A similar sequence of events has been proposed to explain why hibernating or hypoxia tolerant animals are more resistant to ischemic insults (68, 69).

Unfortunately, none of the present PHD inhibitors is specific for a distinct PHD subtype. A specific PHD1 inhibitor should protect hypoxic tissues through a reduced oxidative stress devoid of the adverse effects associated with PHD2 inhibition [e.g., polycythemia (28, 37, 56), congestive heart failure (53), and placental defects during pregnancy (57)].

Oxidative stress. Unfortunately, in contrast with the HIF-PHD pathway, synthetic small molecule compounds able to interfere with the Nrf2-Keap1 system are rare. Bardoxolone methyl, a potent inducer of Nrf2, is currently being tested in a Phase II clinical study of diabetic nephropathy (http://clinicaltrials.gov/). No effective Keap1 inhibitor is currently available. Recently, the X-ray crystal structure of Keap1 and the molecular mechanism of interaction between Nrf2 and Keap1 have been elucidated (59). A compound binding the active site of Keap1 and inhibiting the interaction between Nrf2 and Keap1 could be theoretically searched by computer-based virtual screening based upon the three-dimensional structure of Keap1. If its benefits are confirmed, a specific Keap1 inhibitor may offer an alternative approach to blunt oxidative stress injury.

Conclusion. Hypoxia and oxidative stress are a final, common pathway in a wide variety of disorders. Advances in the unraveling of the molecular events delineated in the present review, especially those targeting sensor molecules for oxygen and oxidative stress, should herald new concepts in the management of a broad spectrum of chronic illnesses sharing an impaired oxygen metabolism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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