Forum Minireview

Drug Discovery for Overcoming Chronic Kidney Disease (CKD): Prolyl-Hydroxylase Inhibitors to Activate Hypoxia-Inducible Factor (HIF) as a Novel Therapeutic Approach in CKD

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Abstract. Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor composed of an oxygen-dependent α-subunit and constitutively expressed β subunit, which plays a central role in cellular adaptation to hypoxia by transcriptionally upregulating its target genes involved in angiogenesis, erythropoiesis, glycolysis, and so on. Recent studies demonstrated that hypoxia in the tubulointerstitium is involved in the pathology of progressive renal diseases and that HIF, which is activated in experimental kidney diseases, may serve to protect tubulointerstitium from the ischemic insult. The expression of HIF α-chains is post-translationally regulated and hydroxylation at one or two of the conserved proline residues by prolyl-hydroxylase domains (PHDs) is a critical step for the oxygen-dependent recruitment of the von Hippel–Lindau gene product (pVHL), a recognition component of the E3 ubiquitin ligase complex, and degradation of HIF-α. Conversely, modalities to inhibit the enzymatic activities of PHDs have been shown to activate HIF irrespective of oxygenation status and are regarded as candidate targets of pharmacological approaches against chronic kidney diseases characterized by hypoxia.

Keywords: chronic kidney disease (CKD), hypoxia-inducible factor (HIF), prolyl-hydroxylase domain (PHD)

Hypoxia and progressive renal diseases

Chronic kidney disease, be glomerular (immunological), hemodynamic or metabolic in origin, is characterized by its irreversibility of pathological process, especially when the degree of injury exceeds certain levels, with resultant development of end-stage renal disease (1). Chronic hypoxia in the tubulointerstitium, together with proteinuria, is most likely a common mediator underlying pathological progression (2). This is to be envisaged by human pathological observation that loss of peritubular capillaries correlates well with residual renal function (3). In animal experiments, we and others have provided evidence that the tubulointerstitial compartment falls into hypoxia in a number of progressive renal disease models (4–6) and modalities to retain or reconstitute peritubular capillaries contribute to preservation of residual renal function (7). Chronic hypoxia of the kidney induces pro-fibrotic changes (8) and apoptosis of resident kidney cells (9–11). Hence, oxygenation in the kidney is likely a critical determinant of its life.

Hypoxia-inducible factors (HIFs)

Hypoxia-inducible factor (HIF) is a transcription factor that plays a central role in cellular adaptation to hypoxia (12). It is a heterodimer that belongs to the basic Helix-Loop-Helix per-arnnt-sim (bHLH-PAS) family, composed of an oxygen-dependent α-subunit and constitutively expressed β-subunit (also referred to as aryl hydrocarbon receptor nuclear translocator, ARNT). The expression of the α-subunit is controlled under tight regulation of molecular oxygen, at the post-translational level (Fig. 1). In the presence of O₂, the α-subunit is hydroxylated at two conserved proline...
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The oxygen-dependent degradation domain (ODD) by members of the prolyl hydroxylase domain (PHD) family (also referred to as the EglN family) (13, 14), which recruits von Hippel–Lindau protein (pVHL), a recognition component of the E3–ubiquitin ligase complex and promotes proteasomal degradation (15). In hypoxia, the α-subunit escapes degradation, translocates into the nucleus, forms a heterodimer with the β-subunit and transactivates 100–200 of its target genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic enzymes (16). Furthermore, oxygen promotes hydroxylation by factor inhibiting HIF-1 (FIH-1) at the asparagine residue in the C-terminal transactivator domain (C-TAD), which inhibits recruitment of coactivators CBP/p300 and transactivation (17). Therefore, hypoxia regulates the operation of HIF functionally as well.

To date, three isoforms of HIF α-subunits have been reported. HIF-1α is widely expressed in virtually all organs of the body, while the expression of HIF-2α is more spatially limited, for example, to vascular endothelium and hepatocytes. Both isoforms have two transactivation domains [N-terminal transactivation domain (N-TAD) and C-TAD] and are capable of forming a heterodimer with HIF-1β, binding to the cis-element containing the core sequence −RCGTG− (hypoxia-responsive element, HRE) and transactivating HIF-target genes (18, 19). The spectrum of their target genes is partly overlapping between HIF-1 and HIF-2, but their function is largely non-redundant (20, 21). HIF-1α–knockout mice die at midgestation owing to failure of embryonic vascularization (22). Meanwhile HIF-2α–knockout mice present with multiple phenotypes, including bradycardia in association with reduced catecholamine levels (23), impaired vascular network at postvasculogenesis stages (24), and multiple organ pathology with high levels of reactive oxygen species (ROS) (25). In contrast to HIF-1α and HIF-2α, the expression and the functional role of HIF-3α is less characterized. In mice and humans, several splice variants have been reported whose expression is transcriptionally regulated. Among them, mouse Inhibitory PAS domain protein (mIPAS) (26) and human HIF-3α4 (27) may be inhibitory against other HIFs.

Expression of HIF in the kidney

Earlier immunohistochemical studies characterized renal expression of HIF-1α and HIF-2α following systemic hypoxia or subsequent to local ischemic insult (28, 29). After a number of stimuli, such as ambient hypoxia, functional anemia, and renal artery ligation, HIF-1α was expressed in tubular epithelial cells and the expression of HIF-2α was confined to the interstitial and endothelial cells. The expression of functional HIF was characterized further using transgenic rats overexpressing HRE-driven luciferase (30). In this study, tubular cells that were hypoxic and expressing functional HIF were identified in proteinuric renal disease as well as in a model of glomerular hypertension.

Functional role of HIF in the kidney

The functional role of HIF in the kidney has been explored in vivo mostly using cobalt chloride as an inhibitor of prolyl hydroxylases. Hydroxylation of one or two proline residues requires prolyl hydroxylases (discussed below) in addition to oxygen, iron, and ascorbate. Therefore, iron chelators such as cobalt and deferoxoxamine induce constitutive expression of HIF–αs. We and others have provided experimental evidence that the systemic activation of HIF protects kidneys from ischemia–reperfusion injury (31, 32).

As an alternative to up-regulate HIF in the kidney, HIF-overexpression using VP16-fused HIF-1α plasmid successfully protected the kidney against ischemia (33). The beneficial role of HIF-overexpression has been reported in other ischemic organs as well. The same expression vector of constitutively active HIF reduces infarct size and enhances neovascularization in the

Fig. 1. Role of PHD in proteasomal degradation of HIF-1α. Mechanisms are schematically illustrated to show how PHD recognizes HIF-1α and directs it toward proteasomal degradation, in the presence of oxygen. (For details, see the main text.)
coronary artery occlusion model (34). In addition, hearts from rodents exposed to intermittent hypoxia are protected against ischemia–reperfusion injury in an HIF-1–dependent manner (35). In cerebrovascular diseases, HIF has been reported to protect brain from ischemic damage (36). HIF-1α overexpression has been applied in human patients suffering from severe limb ischemia and the overall promising effect has been reported in a phase I study (37).

The net beneficial effect of HIF in the long-term application may be a bit complicated. On the one hand, 3 – 4 weeks of systemic HIF-activation protects ischemic tubulointerstitium from injury in the progressive Thy-1 nephritis model (38) as well as in the remnant kidney in the rat (39), due to inhibition of tubular cell apoptosis and/or preservation of peributular capillary networks. On the other hand, genetic ablation in tubular HIF-1α in mice resulted in the reduced occurrence of epithelial–mesenchymal transdifferentiation (EMT) in the unilateral ureteral obstruction (UUO) model (40), suggesting that fibrosis may be promoted via an HIF-dependent as well as an HIF-independent pathway. Connective tissue growth factor (CTGF) and plasminogen activator inhibitor-1 (PAI-1) are regulated by HIF and plays a crucial role in EMT. However, both of these factors are angiogenic (41, 42). Therefore, it is possible that up-regulation of these factors as a compensatory mechanism against hypoxia at the beginning may eventually lead to progression of fibrosis. Clearly the effect of HIF is multifaceted and the net effect needs to be determined in each pathological context.

**HIF hydroxylases**

The expression of HIF-α chains is mostly, if not exclusively, regulated at the post-translational level, by members of the PHD family. They belong to the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily whose activity is completely dependent on oxygen. Measurement of values for the apparent KM for oxygen (the concentration of oxygen that supports a half-maximal initial catalytic rate) using HIF-α polypeptides suggests that the PHD oxygen KM values are approximately 100 μM (43, 44). Given that oxygen concentrations in tissues are typically 10 – 30 μM and are always below the KM value for oxygen, it follows that the enzymatic activity is modulated by molecular oxygen availability over the entire physiological range. Also, hydroxylation per se is limiting for HIF-α degradation and HIF-α is limiting for the HIF-transcriptional response. In other words, HIF hydroxylation activity links changes in molecular oxygen concentration to the regulation of the HIF transcription cascade, thus operating as an oxygen sensor.

PHDs, like other 2-oxoglutarate-dependent oxygenases, employ a two-histidine–one-carboxylate facial triad to coordinate the catalytic Fe(II) center, leaving two positions for binding 2-oxoglutarates and one for molecular oxygen. During catalysis, the splitting of molecular oxygen is coupled to the hydroxylation of HIF-α and to the oxidative decarboxylation of 2-oxoglutarate to succinate and CO2. The reaction proceeds via formation of a highly reactive ferryl intermediate that oxidizes the target amino acid residue. Ascorbate is required for full catalytic activity. Changes in the availability of these cofactors potentially modulate the rate of hydroxylation irrespective of oxygen concentration. Conversely, the HIF hydroxylase activity is inhibited by metabolic intermediates such as fumarate and succinate (45, 46).

Several lines of evidence support the view that ROS also plays a role in modulating HIF-activity (47 – 49). Proposed mechanisms include direct inhibition of PHD enzymatic activity and effects on levels of ascorbate, Fe(II), or TCA cycle intermediates. In contrast, other reports showed suppression of HIF-activity by oxidative stress, especially in the kidney (50, 51). Although a detailed mechanism for this discrepancy remains to be elucidated, it may be due to a biphasic effect of oxidative stress depending on the amounts of ROS. While supraphysiological amounts of ROS are definitely cytotoxic, physiological amounts of ROS serve as a mediator of signal transduction pathways. In addition, one has to be careful about interpreting the results of mitochondrial inhibition studies when investigating the regulation of HIF. While mitochondrial inhibition suppresses production of ROS, it also results in reduction of oxygen consumption by mitochondria and an increase in oxygen tensions in the cell, leading to eventual suppression of HIF.

**PHD paralogues**

In higher metazoans, three PHD paralogues (PHD1-3) have been reported to date, and their mode of expression and its relative role in HIF-regulation is currently being explored. In expression, PHD-1 is exclusively nuclear, PHD-2 is mainly cytoplasmic, and PHD-3 is found in both the cytoplasm and nucleus (52). PHD-1 mRNA is abundant in testes, while PHD-3 mRNA is highest in the heart. PHD-2 and -3 mRNAs are increased by hypoxia. In the kidney, PHD-2 is the most abundant isoform among others, with predominant expression in the medulla (53).

In terms of regulation of HIF, PHD-2 (also referred to as EglN1) seems to play a central role in regulating
prolyl-hydroxylation, and the expression thereof, of HIF-αs under normoxic conditions (54). However, other members undoubtedly play important roles in HIF-regulation as well. For example, PHD-3 dampens the HIF-response to hypoxia in cell culture and is a more effective suppressor of HIF-2α than of HIF-1α, which might potentially determine relative abundance of HIF-2α versus HIF-1α (55). The folding and stability of PHD-3 is reportedly regulated by the Siah1α and Siah2 ubiquitin ligases and simultaneous inactivation of Siah1α/2 upregulates PHD-3, thereby downregulating EPO response as well as dampening HIF-activity in mice (56). This also suggests that the PHD-2 and PHD-3 are functionally redundant in vivo, at least in part.

In contrast to the relative target specificity of PHD-2 on HIF, it remains less clear whether other paralogues, PHD-1 and PHD-3, join signaling cascades other than HIF. In respect to this, it will be interesting to know that PHD-3 can induce apoptosis in neuronal cells following NGF withdrawal, in a manner independent of HIF-1 and HIF-2 (57). Furthermore, PHD-3–knockout mice present with impaired sympathoadrenal responses (58).

The role of PHD in the regulation of HIF has been observed in human clinical settings as well (59). A family with an inherited mutation in PHD-2 has been linked to impaired enzymatic activity and development of familial erythrocytosis, a complication of excessive RBC production following EPO overproduction. In support of this observation in humans, a recent report using heterozygous PHD-2–knockout mice again developed congestive heart failure subsequent to pressure overload, which was associated with EPO overproduction/erythrocytosis (60).

In contrast to the increase in EPO in PHD-2–knockout mice, simple knockout for PHD-3 in mice does not present with such phenotypes. However, double knock-out of PHD1 and PHD3 also develops erythrocytosis, with a different pattern of HIF-α activation and distinct sites of EPO production. In such mice, hepatic production of EPO was associated with predominant expression of HIF-2α (61).

Additionally, metabolic analysis of PHD-1–knockout mice revealed altered skeletal muscle metabolism, such as reduced glucose oxidation, enhanced glycolysis, and protection from ischemia, which appears at least partly dependent on HIF, because heterozygous inactivation of HIF-2, and to a lesser extent, of HIF-1 blunted these findings (62). These studies provided important clues about the specific interactions and roles of each isoform of HIF and PHD.

**PHD inhibitors as therapeutic targets**

As discussed above, modalities to activate HIF, if appropriately applied, are a promising way to protect kidneys from progressive injury. Inhibition of PHDs is so far one of the best candidates for genetic as well as pharmacological approaches (19).

Animal experiments have been started to address the role of PHD inhibition in a model of acute ischemic renal failure. A specific PHD-inhibitor contributes to the activation of HIF in rats in vivo and protects kidneys from the ischemia–reperfusion injury (31), an observation almost identical to that of a previous study using cobalt. In another approach from a third group as well as ours, heterozygous knockout mice for HIF-1α/2α (63) or knockdown mice of HIF-2α (64) were found to be more susceptible to acute ischemic injury in the kidney; and more importantly, it was reported that pharmacological activation of HIF again caused beneficial effects in terms of kidney protection (63). Activation of HIF by carbon monoxide also protected the transplanted kidney against prolonged cold ischemia in a rat model (65). These observations clearly offer new strategies to protect kidneys from ischemic injury.

When applying PHD-inhibition to chronic renal diseases, however, there are some hurdles that need to be overcome. First, there is a need to define a spectrum of disease in which activation of HIF works toward kidney protection (Table 1). Chronic kidney diseases are a complex of multiple factors and therefore the relative contribution of hypoxia and HIF may differ according to each pathological context. For instance, activation of HIF is beneficial in many models including a model of progressive glomerulonephritis (38), the rat remnant-kidney (39), Type 2 diabetic nephropathy (66), Habu snake venom–induced glomerulonephritis (67), and cisplatin nephropathy (68, 69). It may also be a relevant finding that the areas of tubular HIF-1α, as expressed during the pathogenesis of adriamycin nephrosis in mice, do not colocalize with those of tubular cell apoptosis (70). On the other hand, maneuvers to activate HIF may be hazardous in the unilateral ureteral obstruction model (40), as has been demonstrated using transgenic mice with knocked-out of tubular HIF-1α. Second, more insight is required about the spectrum of genes upregulated by PHD-inhibition and therefore, HIF. Inhibition of a certain PHD-paralogue does not appear to lead to the overall increase of a number of HIF-target genes, as could be envisaged in PHD-2–knockout mice presenting with polycythemia (60) or in PHD-3–knockout mice demonstrating impaired sympathoadrenal activity (58). This is even more important because most of the HIF-targets, such as EPO (71) and VEGF (7), are
obviously renoprotective, and modalities to selectively activate these protective genes, if any, might be even more effective therapeutically than others. In this regard, further research is warranted to address the renoprotective properties of individual, specific HIF-target genes.

It is still not completely understood why two of the HIF-isoforms, HIF-1 and HIF-2, transactivates their target genes in a selective way. It seems likely, at the moment, that transcriptional selectivity is not determined by selective DNA binding, but by sequences lying C terminal to the DNA-binding and dimerization domains (72). Indeed the transcriptional response of HIF-2 in cultured cell lines differs significantly from that of HIF-1. In many cell types, the transcriptional response mediated by HIF-2 is much less. Studies using mouse embryonic stem cells clarified that endogenous HIF-2 binds HREs of HIF-targets, but fails to transactivate its targets. By contrast, exogenously overexpressed HIF-2 is capable of transactivating its targets in an efficient way. One possible deduction here is that there are certain titratable inhibitors of HIF-2 (73). Further studies are needed to completely resolve this issue.

The so-called HIF-signaling goes beyond the canonical HIF–HRE axis. A growing body of evidence suggests the involvement of HIF in other transcription and signaling cascades such as Notch (74), Wnt (75), and Myc (76, 77). Interestingly, HIF-1 and HIF-2 appear to have contrasting properties on MYC and regulate cell proliferation in a negative- and positive- manner, respectively, through distinct mechanisms. Furthermore, other important signaling molecules such as NF-kB also interact with the HIF–HRE axis (78).

Nevertheless, maneuvers to activate HIF through PHD-inhibition stay as one of the most promising ways to prevent, minimize injury of, and ultimately treat, chronic renal diseases.

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