The VHL/HIF oxygen-sensing pathway and its relevance to kidney disease

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Over the past decade major advances have been made in our understanding of the molecular machinery that mammalian cells use to sense and to adapt to a low-oxygen environment. A critical mediator of cellular adaptation to hypoxia is hypoxia-inducible factor (HIF), a basic helix-loop-helix transcription factor that consists of an oxygen-sensitive α-subunit, HIF-α and a constitutively expressed β-subunit, HIF-β. Under conditions of normal oxygen tension, the HIF-α subunit is hydroxylated by specific prolyl-hydroxylases and targeted for rapid proteasomal degradation by the von Hippel–Lindau (VHL) tumor suppressor, which is the substrate recognition component of an E3-ubiquitin ligase. In a hypoxic environment or in the absence of functional VHL tumor suppressor protein irrespective of oxygen concentration, HIF-α is not degraded and translocates to the nucleus, where it dimerizes with HIF-β to form transcriptionally active HIF. As a transcription factor, HIF is involved in the regulation of many biological processes that facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating genes that are involved in glucose uptake and energy metabolism, angiogenesis, erythropoiesis, cell proliferation and apoptosis, cell–cell and cell–matrix interactions, and barrier function. This review summarizes some of the most recent advances in the VHL/HIF field and discusses their relevance for pathogenesis and treatment of acute ischemic renal failure, renal fibrosis, and renal cancer.

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OVERVIEW OF REGULATION OF HIF SIGNALING

Key mediators of cellular adaptation to hypoxia are hypoxia-inducible factors (HIFs), HIF-1 and HIF-2 being the most prominent and best characterized (for recent reviews, see Wenger[1] and Pugh and Ratcliffe[2]). HIF-1 and HIF-2 (for the purpose of this review collectively referred to as HIF) are members of the PAS (per/aryl-hydrocarbon-receptor nuclear translocator (ARNT)/Sim) family of basic helix-loop-helix transcription factors. They consist of an oxygen-sensitive α-subunit and a constitutively expressed β-unit, also known as the ARNT or simply HIF-β.[1,2] As global regulators of oxygen homeostasis, these heterodimeric transcription factors facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of gene products that are involved in cellular energy metabolism (e.g. phophoglycerate kinase-1 and glucose transporter-1 (GLUT-1)), angiogenesis (e.g. vascular endothelial growth factor (VEGF)), erythropoiesis (erythropoietin (EPO)), apoptosis and proliferation (B-cell lymphoma-2 family members, cell cycle regulators p21 and p27) and other biological processes.[1,2] Direct transcriptional regulation occurs through the binding of HIF heterodimers to hpoxia-response-elements present in regulatory elements of many hypoxia-sensitive genes (Figure 1). The list of HIF target genes has grown rapidly and includes genes that have well-demonstrated relevance to the pathogenesis of acute and chronic kidney diseases. These include among others *hemeoxygenase-1*, *VEGF*, *plasminogen activator inhibitor-1*, *tissue-inhibitor of metalloproteinase-1*, and *connective tissue growth factor.*[1,3] HIF-1 and HIF-2 do not completely overlap with regard to their ability to transcriptionally regulate specific hypoxia responsive genes. For example, glycolytic genes appear to be predominantly regulated by HIF-1,[4] whereas it has been suggested by our group and others that HIF-2 is the main regulator of *VEGF* and *EPO* expression in tissues that express both HIF-1 and HIF-2.[5,6]

HIF activation is dependent upon stabilization of the oxygen-sensitive α-subunit and its subsequent translocation to the nucleus where it forms a functional complex with ARNT and transcriptional co-activators such as CBP/p300.[1,2] Normally, under conditions of adequate oxygen supply, HIF-α binds to the von Hippel-Lindau tumor suppressor protein (pVHL), which is part of an E3-ubiquitin ligase complex that

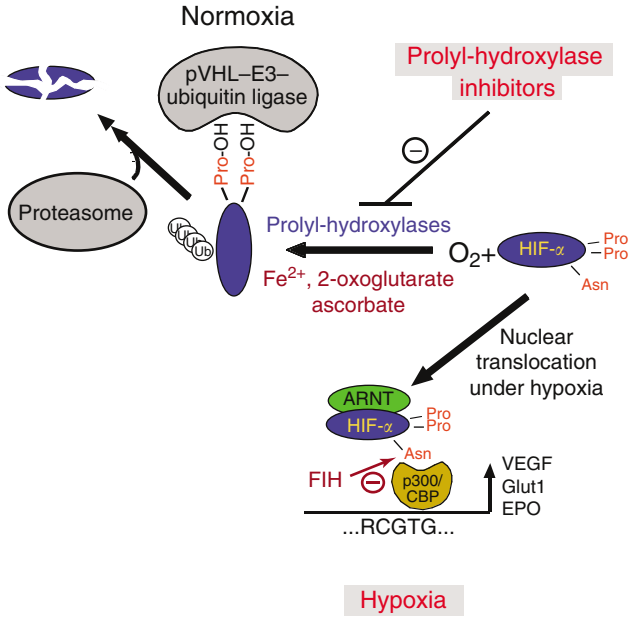

Figure 1 | The pVHL–E3-ubiquitin ligase targets hydroxylated HIF-α for proteasomal degradation. Under normoxia, hydroxylation of HIF-α subunits by prolyl-hydroxylases is required for binding to the pVHL–E3-ubiquitin ligase complex. After polyubiquitination, HIF-α is degraded by the proteasome. During hypoxia or when prolyl-hydroxylases are inhibited pharmacologically, or in the absence of functional pVHL irrespective of oxygen tension, HIFα subunits are not degraded and translocate to the nucleus where they bind to the HIF-β subunit ARNT. HIF-α/ARNT heterodimers then bind to HIF-DNA consensus-binding sites resulting in increased transcription of HIF-target genes, for example, *EPO, VEGF*, and *glucose transporter-1*. Factor-inhibiting-HIF (FIH) is an asparagine hydroxylase that modulates cofactor recruitment to the HIF transcriptional complex via asparagine hydroxylation of the HIF-α carboxy-terminal transactivation domain. Also shown is the core sequence (RCGTG) of a hypoxia-responsive element (HRE). Pro, proline; Asn, asparagine.

targets HIF-α for proteasomal degradation (Figure 1). All three known HIF-α subunits, HIF-1α, HIF-2α, and HIF-3α (the role of HIF-3α in hypoxic signaling is unclear, a HIF-3α splice variant, inhibitory PAS (IPAS), may be inhibitory[7]) have been shown to interact with pVHL. This interaction is highly conserved between species and requires iron- and oxygen-dependent hydroxylation of defined proline residues within the oxygen-dependent degradation domain of HIF-α by HIF-prolyl-hydroxylases. Prolyl-hydroxylation and binding to pVHL are absolutely required for the execution of HIF proteolysis under normoxia. During hypoxia, prolyl-hydroxylation is inhibited and HIF-α is stabilized and not degraded. Loss of pVHL function also results in HIF-α stabilization, increased HIF transcriptional activity, and upregulation of HIF target genes such as *VEGF*, *glucose transporter-1*, and *EPO*, but is independent of oxygen levels. This explains some of the clinical features that are associated with *VHL*-deficient tumors, for example, the high degree of vascularity, which is a consequence of excessive VEGF production. A second hypoxic switch operates in the carboxy-terminal transactivation domain of HIF-α with the hydroxylation of an asparagine residue; in hypoxia, asparagine hydroxylation is blocked and CBP/p300 recruitment is facilitated enabling increased levels of transcription (Figure 1). Besides hypoxic activation, a non-hypoxic increase in HIF transcriptional activity has been shown to be mediated by nitric oxide, tumor necrosis factor-α,[8] and angiotensin II,[9] and others.[1] Thus, it is easy to imagine that HIF activation is likely to occur in a variety of different renal disease settings independent of significant hypoxia.

While HIF-1, initially identified as the factor that regulates the hypoxic induction of EPO, appears to be ubiquitously expressed, HIF-2 expression is more restricted.[10] Cell types that express HIF-2α include hepatocytes, cardiomyocytes, glial cells, type-II pneumocytes, and endothelial cells.[10] In the kidney, HIF-regulated gene expression in normal, non-transformed primary renal tubular epithelial cells appears to be solely controlled by HIF-1.[3] In the adult rodent, HIF-2α was found in renal interstitial and renal endothelial cells under conditions of carbon-monoxide poisoning or renal ischemia.[10,11]

VHL AND HIF IN RENAL CANCER DEVELOPMENT

Inactivation of pVHL results in HIF-α stabilization, increased HIF transcriptional activity, and upregulation of HIF target genes. Patients with germline mutations in pVHL are predisposed to develop VHL disease, an autosomal dominant familial tumor syndrome that follows Knudson's two-hit hypothesis (loss of the remaining wild-type allele in tumors) and is characterized by the development of highly vascularized benign and malignant neoplasms at multiple organ sites, which include renal cysts and renal cell carcinomas.[12] Besides its role in familial renal cancer, VHL gene mutations are found in the majority of sporadic renal cell carcinoma of the clear cell type (CC-RCC), which is the most common form of kidney cancer. VHL associated CC-RCCs are often preceded by pre-neoplastic renal cysts, which are typically multifocal and bilateral in patients with VHL disease. Although it is widely believed that VHL associated renal tumors originate from the proximal nephron, it was recently reported that in kidneys from VHL patients, 'early' multicellular lesions appeared to be more frequently derived from Tamm–Horsfall protein-expressing distal tubular segments than from the proximal renal tubule. Interestingly, single cell foci of increased HIF-target gene expression were found more frequently in proximal tubular cells, suggesting a site-specific tumor suppressor function for pVHL in the kidney.[13] This finding raises the possibility that VHL-associated renal lesions arise from the distal nephron and should stimulate new studies into their exact histogenetic origin.

Although the highly vascular nature of *VHL*-deficient tumors is easily explained by increased HIF activity resulting in increased vascular growth factor production, VHL-associated carcinogenesis is more difficult to understand and most likely requires additional genetic events. Besides regulating the degradation of HIF-α subunits, pVHL has been shown to play a role in fibronectin extracellular matrix

assembly and matrix turnover,[14–16] microtubule stability,[17] and regulates the stability and/or activity of other proteins such as plant homeodomain protein Jade-1,[18] and atypical protein kinase C isoforms.[19–21] Other pVHL targets have been described, most notably transcription factor Sp1,[22] a kruppel-associated box (KRAB)-A domain protein, VHLak, repressing HIF transcriptional activity,[23] de-ubiquitinating enzymes,[24] the large subunit of RNA polymerase II,[25] and the RNA-binding protein hnRNP A2.[26] It is unclear however how other pVHL functions contribute to renal carcinogenesis. With regard to the contribution of HIF-1 and/or HIF-2 to VHL renal tumorigenesis, it is of interest that a substantial number of *VHL*-defective CC-RCC cell lines do not express HIF-1α, but do express HIF-2α.[27] This is in contrast to normal, non-transformed renal epithelial cells in which HIF-2α is not detectable during ischemia.[11] A bias towards HIF-2α expression was also found in clinical CC-RCC samples (94 versus 69% expressed HIF-2α in CC-RCC with confirmed *VHL* defect).[28] Thus, VHL-associated tumor development may depend on a shift in the ratio of HIF-1 versus HIF-2 levels towards an increase in HIF-2. In support of this hypothesis, VHL reconstituted 786-O CC-RCC cells transfected with a non-degradable form of HIF-2α were still able to form tumors in nude mice thereby overriding pVHL's tumor suppressor function,[29] whereas the expression of non-degradable HIF-1α in a similar experimental setting did not have a tumor promoting effect.[30] Consistent with these findings, inactivation of HIF-2α by RNA interference suppressed tumor formation in a *VHL*-deficient background.[31,32] Taken together, these reports suggest that HIF-1 and HIF-2 have diverse functions with regard to VHL renal tumorigenesis. Whether differential effects on signaling pathways critical for renal epithelial cell growth may explain the functional differences between HIF-1 and HIF-2 will have to be explored in greater detail. Important hypoxia and HIF-regulated gene products believed to play a key role in the pathogenesis and progression of CC-RCC include among others transforming growth factor-α (TGF-α), a potent renal epithelial mitogen, cell cycle regulator cyclin D1, and chemokine receptor CXCR4. Activation of the TGF-α/epidermal growth factor receptor pathway has been suggested to be the result of a predominantly HIF-2-dependent increase in TGF-α expression.[33,34] *Cyclin D1* was found to be upregulated in *VHL*-deficient renal cancer cell lines and in clinical CC-RCC specimen and has been shown to be hypoxia-regulated in a cell type-specific manner.[35–37] Cyclin D1 promotes cell cycle progression through its interaction with cyclin-dependent kinase (CDK)4/CDK6 and phosphorylation of the retinoblastoma protein thus allowing S-phase entry, and therefore is a critical target of pVHL's tumor suppressor function. Although the effects of high levels of HIF target CXCR4 on renal tumor growth itself are not clear, *CXCR4* expression levels correlate with poor survival and increased metastatic potential, suggesting that this receptor plays an important role in the clinical progression of CC-RCC.[38]

HIF SIGNALING IN RENAL ISCHEMIA REPERFUSION INJURY

During renal ischemia when HIF-α proteolysis is inhibited, HIF-1α is detected in the nucleus of renal tubular epithelial cells, where it dimerizes with HIF-1β to form transcriptionally active HIF-1. By contrast, HIF-2α is undetectable in this cell type, supporting the notion that HIF-1 is the key mediator of hypoxic HIF signaling in non-transformed renal epithelia.[10,39] As a global regulator of cellular adaptation to hypoxia, HIF-1 regulates critical biological processes important for survival of hypoxic cells such as anaerobic glycolysis (Pasteur effect), oxygen delivery through increased angiogenic growth factor production and erythropoiesis, as well as cellular proliferation and apoptosis. Nevertheless, the role of HIF-1 in the regulation of mitochondrial signaling, hypoxic cell death and recovery from ischemia–reperfusion injury is controversial and most likely context or cell-type dependent. For example, genetic studies with conditional knockout mice showed that rodent brains, which were HIF-1 deficient in neurons, were protected from cerebral ischemic injury.[40] By contrast, conventional knockout mice that have lost one copy of HIF-1α (heterozygously deficient in all cell types) did worse in a model of myocardial ischemia following ischemic preconditioning,[41] supporting the notion that a cell survival promoting effect of HIF-1 may be context-dependent. Current studies with kidney-specific HIF-1α knockout mice have not been published but are in progress.

Despite its controversial role in the execution of acute hypoxic cell death, HIF-1 is known to upregulate factors that have been shown to be 'cytoprotective' during acute renal injury including ischemia. These, for example, include VEGF,[42,43] hemeoxygenase-1,[44–47] and EPO.[48] Pretreatment of rats with a HIF-specific prolyl-hydroxylase inhibitor to increase HIF activity,[49] or cobalt chloride,[50] a chelating agent known to inhibit HIF-α proteolysis, resulted in improved renal clearance after clamping of the renal pedicle. Histologically, pretreatment with cobalt chloride was also associated with amelioration of tubulointerstitial injury with decreased macrophage infiltration, providing evidence that HIF-1 signaling is involved in ischemic pre-conditioning of the kidney, as has been proposed for other organ systems.[41,51] Although these reports suggest that HIF signaling may ameliorate acute hypoxic tissue injury, it is unclear which signaling pathways and which renal cell types mediate this effect. Nevertheless, these data suggest that pharmacological inhibition of HIF-α proteolysis with small molecule compounds that inhibit HIF prolyl-hydroxylase activity may be a useful preventative strategy to improve clinical outcome of ischemia reperfusion injuries, for example, renal ischemic injuries that are associated with procedures involving radiocontrast media, cardiothoracic surgery, or renal cadaveric transplantation. Preliminary findings furthermore suggest that certain prolyl-hydroxylase inhibitors may also have a selective effect on HIF target gene expression and HIF-controlled biological processes, as they have the potential to increase iron metabolism and erythropoiesis without affecting angiogenesis for example.[52] Although the molecular

mechanisms underlying prolyl-hydroxylase inhibitor selectivity are difficult to explain, the development of application-specific prolyl-hydroxylase inhibitors that target either erythropoiesis or angiogenesis, or HIF survival pathways alone would be of tremendous clinical importance.

A POTENTIAL ROLE FOR HIF IN THE PROGRESSION OF CHRONIC RENAL DISEASE AND TUBULOINTERSTITIAL FIBROSIS

Hypoxia has long been thought to be a major factor in the progression of chronic renal diseases irrespective of the underlying cause.[53] This is no surprise, as renal 'scarring' is associated with loss of microvasculature, leading to decreased blood flow and impaired oxygenation in areas of fibrosis. However, more recent work has suggested that discrepancies between oxygen demand and supply can even occur 'early' in diseased kidneys before visible scarring is detected.[54] The notion that hypoxia has an important role in the progression of renal fibrosis raises questions about HIF's contribution to this disease process. Potential mechanisms through which HIF signaling may contribute to renal fibrogenesis include (a) direct regulation of fibrogenic factors and synergy with TGF-β1, a potent profibrotic factor, (b) its possible role in epithelial to mesenchymal transition (EMT), and (c) HIF's role in inflammation (Figure 2).

In human renal tubular cells, hypoxia induces *collagen I*, decreases *matrix-metallopeptidase-2*, and increases *tissue-inhibitor of metalloproteinase-1* .[55] Hypoxia can also act synergistically with TGF-β1 in the regulation of certain hypoxia responsive genes such as *VEGF*,[56] *endoglin*,[57] and *EPO*.[58] Synergistic effects between hypoxia and TGF-β1 have furthermore been demonstrated with regard to the production of collagens.[59,60] These observations and the finding that several genes, which play critical roles in renal fibrogenesis are direct HIF-1 targets (e.g. *tissue-inhibitor of metalloproteinase-1*,[61] *plasminogen activator inhibitor-1*,[62] *connective tissue growth factor*[3]), suggest that increased HIF activity is likely to play an important role in the pathogenesis of tubulointerstitial fibrosis through direct transcriptional regulation of specific profibrotic genes and/or through enhancement of TGF-β1 signaling. Synergistic interaction between MAD homolog (SMAD)-3, a downstream effector of TGF-β1, and HIF-1 has been suggested by Sanchez-Elsner *et al.*[56] as a possible mechanism in the transcriptional regulation of *VEGF*. Hypoxia may also increase *SMAD3* mRNA levels or promote the thrombospondin-dependent release of latent TGF-β, as has been shown for TGF-β2.[63] Whereas direct regulation of profibrotic genes by HIF-1 can be easily investigated, the exact role of HIF-1 in TGF-β signaling may be complex and more difficult to understand.

A possible role for hypoxia and HIF-1 in the dedifferentiation and transition of renal EMT has been proposed.[64,65] This may or may not involve TGF-β1, which is a potent inducer of EMT. Transition of epithelial to mesenchymal cells is recognized as a substantial contributor to the development of renal fibrosis.[66] EMT is characterized by the disassembly of

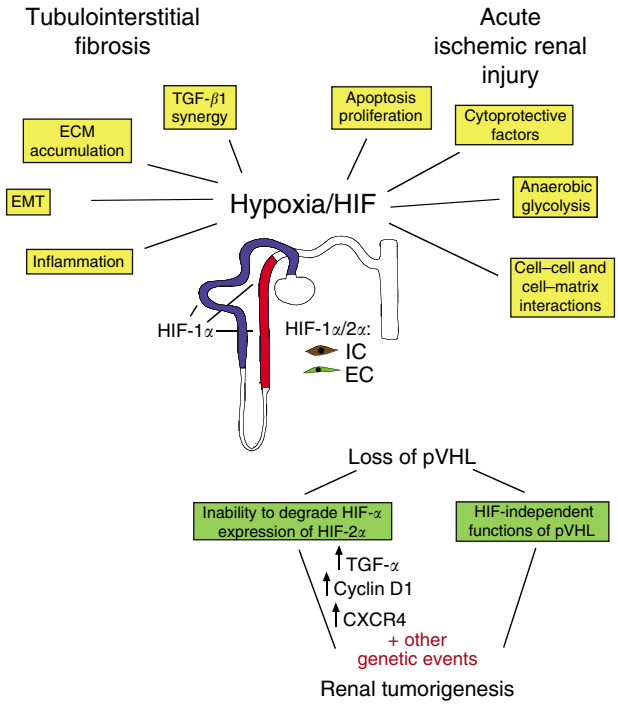

Figure 2 | The role of HIF in acute ischemic renal injury, tubulointerstitial fibrosis, and VHL-associated renal cancer. Overview of selected hypoxia/HIF-regulated biological processes that have been shown or have been proposed to play important roles in the pathogenesis of acute ischemic renal injury, renal fibrosis, and renal cancer. Shown in red (renal proximal tubule) and blue (Tamm–Horsfall protein-expressing nephron segments) are the proposed sites of origin of VHL-associated renal cancer. While HIF-1α can be found in all nephron segments, HIF-2α in the non-cancerous, normal kidney is not expressed in renal epithelial cells, but can be found in renal interstitial and renal endothelial cells. EMT, epithelial to mesenchymal transition.

intercellular contacts, such as E-cadherin adherens junctions, leading to cell–cell separations associated with an increase in motility and re-organization of the actin-cytoskeleton, which eventually results in the generation of fibroblast-like cells that express mesenchymal markers and display increased motility and invasiveness.[66] In a recent, preliminary study by our group, hypoxia increased the percentage of transitioned renal epithelial in a HIF-dependent fashion *in vitro* and *in vivo*,[65] suggesting that HIF-1 may play a direct role in EMT. The notion that hypoxia and HIF-1 influence the differentiation state of cells is supported by observations in other cell systems.[67] Although the molecular mechanisms underlying this effect need further definition, a very recent study by Gustaffsson *et al.*[68] suggested that HIF-1α through direct interaction with the intracellular domain of Notch enhances Notch signaling and in this way may directly be involved in the maintenance of an undifferentiated state. Whether this interaction will be important for EMT and the development of tubulointerstitial fibrosis remains to be investigated.

A third mechanism by which HIF may impact on the pathogenesis of tubulointerstitial disease is through its role in inflammation. It is important to realize and is not a surprise,

that micro-environmental changes, such as hypoxia, have a strong impact on inflammatory cell recruitment[69] and function.[70] Elegant studies in tissue-specific HIF knockout mice have shown that HIF-1 is essential for myeloid-cell-mediated inflammation mainly through its effects on cellular ATP generation. Absence of HIF-1 resulted in a profound impairment of myeloid cell aggregation, motility, and invasiveness, whereas forced expression of HIF-1 had the opposite effect.[69] Since inflammatory responses represent an important component in the pathogenesis of tubulointerstitial fibrosis, it is easy to imagine that HIF signaling in inflammatory cells may also have a significant role in the progression of chronic renal disease.

SUMMARY

In this review, I have summarized some of the most recent findings in VHL and HIF biology and have attempted to provide the reader with a perspective on how HIF signaling may impact on the pathogenesis and/or treatment of renal cancer, and acute and chronic kidney disease. Because of space limitations I have omitted a discussion on the potential role for HIF signaling in inflammatory and non-inflammatory glomerular disease. HIF biology in the kidney is still in its infancy and in the past has largely been dominated by investigations that examine the VHL tumor suppressor and HIF in renal carcinogenesis. Over the next 5 years, we can expect to come across a large number of very exciting genetic and pharmacological *in vivo* and cell culture studies that examine the role of the VHL/HIF oxygen-sensing pathway in all aspects of kidney pathophysiology. From a clinical point of view, small molecule compounds that specifically target HIF-specific prolyl-hydroxylases have high potential to aid in the treatment of anemia and improve iron metabolism, and also may impact on the clinical outcome of ischemic renal injuries and other renal diseases.

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